



Department of Animal and Plant Sciences
The University of Sheffield
Western Bank
Sheffield S10 2TN
England

**Endocrine and Haemodynamic Investigations
of
Normal and Laminitic Horses**

A thesis submitted for the degree of Doctor of Philosophy.

Karen Ann Hinckley

**Department of Animal and Plant Sciences
University of Sheffield.
Sheffield**

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This thesis is dedicated to the mares

Pride of Armagh, Solar Star, Biscuit, Cracker and Rose Marie

whose courage and brave endurance
provided motivation and inspiration throughout the investigations of
laminitis.

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Endocrine and Haemodynamic Investigations of Normal and Laminitic Horses

Summary

The historical and evolutionary perspectives of equine laminitis were placed in a contemporary context of hippology. A survey revealed 3% of the equine population in the UK to be affected by laminitis. Physiological aspects affecting pedal blood flow, namely endocrine and haemodynamic relationships, were considered under controlled management.

Pedal haemodynamics, investigated non-invasively using Near Infrared Spectroscopy, detected changes in concentrations of oxyhaemoglobin, deoxyhaemoglobin and cytochrome oxidase, indicating tissue utilisation of oxygen and hence perfusion. Responses of chronic laminitics suggested attenuated blood flow and local hypoxia. Haemostasis was shown to occur in acute laminitis

The role of nitric oxide was investigated by assessment of the effects of concentrations of substrate, synthetic nitric oxide donors, and endogenous and exogenous inhibitors of nitric oxide synthesis. Reperfusion of ischaemic laminal tissue during acute laminitis was initiated by iv administration of l-arginine, a substrate of nitric oxide (NO) synthesis; this also increased blood flow in laminal tissues of a normal horse, but had little effect on blood pressure. Grass induced acute laminitis was successfully treated with transdermal application of glyceryl trinitrate paste to the pasterns. A competitive inhibitor of l-arginine synthesis increased blood pressure of normal horses but did not induce acute laminitis. Plasma l-arginine increased when normal and chronically laminitic horses went to grass. Plasma asymmetric dimethyl-l-arginine (ADMA) was lower in chronic laminitics than normal ponies; ADMA decreased when at grass from 1.2 $\mu\text{mol/L}$ to 0.7 $\mu\text{mol/L}$ in normal horses and from 0.8 $\mu\text{mol/L}$ to 0.47 $\mu\text{mol/L}$ in chronically laminitic ponies.

Radioimmunoassays for angiotensin II, atrial natriuretic peptide and endothelin were validated and basal values for the horse established as 24 ± 2 pg/ml, 34 ± 2 pg/ml and 1.78 ± 0.2 pg/ml respectively. Seasonal differences in AII and ANP were observed. Endocrine changes, observed during acute laminitis, were neither large nor sustained.

Blood pressures and heart rate were significantly raised at the acute stage but otherwise unremarkable. Although transient hypertension occurs during acute laminitis, chronic laminitics are not hypertensive. Moderate hypertension accompanies refractory laminitis but this does not appear to be mediated by angiotensin II or endothelin. Electrolyte concentrations were largely unaffected but slight seasonal differences were seen alongside sodium retention in refractory and some chronic cases. Digestive disturbances of acute laminitis were reflected in decreased urinary contents of hippuric acid.

Seasonal changes in ingested electrolytes and water soluble carbohydrates are part of the complex pathogenesis of acute laminitis. An hypothesis outlines potential and actual relationships between dietary factors, endotoxaemia and the vasoactive hormones whose interplay may adversely influence pedal haemodynamics in developmental and acute equine laminitis.

Endocrine and Haemodynamic Investigations of Normal and Laminitic Horses

Contents

	Page
Summary	IV
List of Figures	VI
List of Tables	X
List of Abbreviations	XII
Prologue	XIV
Chapter 1. Introduction	1
Chapter 2. Epidemiology of equine laminitis	54
Chapter 3. Blood pressure and laminitis	86
Chapter 4. Haemodynamics of the equine hoof	134
Chapter 5. Nitric oxide and equine laminitis	183
Chapter 6. Radioimmunoassay of plasma angiotensin in normal and laminitic horses	267
Chapter 7. Atrial natriuretic peptide	306
Chapter 8. Endothelin	356
Chapter 9. Serum electrolytes and observations on selected urinary metabolites in normal and laminitic horses	375
Chapter 10 Discussion - Speculation, Conclusions and a Field Theory of the aetiology of laminitis.	418
References	452
Appendix I Blood pressure data	486
Appendix II Experimental and clinical blood pressures	503
Appendix III Electrolyte analysis	522
Appendix IV Treatment schedules	539
Publications List of publications and presentations	543
Addendum Reprints of published papers and abstracts	

List of Figures

	Chapter 1	Page
Figure		
1.1a	Evolution of the horse	3
1.1b	Evolutionary changes in body mass	4
1.2	Evolutionary development of the equine digit and dentition	6
1.3	Changes in weight bearing structures with evolution	8
1.4	Laminitic horse depicted in mediaeval painting	9
1.5	Founder described in early veterinary books	10
1.6	Skeletal anatomy of the equine forelimb	15
1.7	Parasagittal section of the equine digit	16
1.8.	Hoof capsule removed to show laminal tissue	17
1.9.	Epidermal and dermal laminae	18
1.10..	Photomicrograph of epidermal and dermal laminae	18
1.11	Microcirculation of the laminae	20
1.12	Electron microscopy of the arteriovenous anastomoses (AVA)	20
1.13	Microvascular function of AVA	21
1.14	Arterial circulation of the equine limb	23
1.15	Angiography of the equine digit	24
1.16	Severe clinical case of laminitis	26
1.17	Laminitic stance	26
1.18	Abnormal hoof growth in chronic laminitis	33
1.19a	Morphology and radiography of a normal hoof	34
1.19b	Morphology and radiography of a chronic laminitic hoof	35
1.20	Sagittal section of a hoof showing 'sinking' and solar prolapse	36
1.21a	Radiography of normal hoof with markers	37
1.21b	Radiography of 'sinking' and solar prolapse with markers	37
1.22	Diagrammatic representation of ischaemia in the laminae	44
	 Chapter 2	
Figure		
2.1	Questionnaire sent to BEVA members	59
2.2	Questionnaire sent to IDHS(GB) members	61
2.3	Aetiology of equine laminitis in the UK	70
2.4	Seasonality of equine laminitis in the UK	72
2.5	Breed differences in the incidence of equine laminitis in the UK	73
	 Chapter 3	
Figure		
3.1	First measurement of blood pressure in 1733	91
3.2	Relative levels of measurement sites	93
3.4	Non-invasive measurement of blood pressure in field conditions	99
3.5	Changes in blood pressure in Week 1 of <i>post partum</i> laminitis	113
3.6	Changes in blood pressure in Weeks 1 -5 of <i>post partum</i> laminitis	114
3.7	Radiography of the hoof of the <i>post partum</i> laminitis case	115

Chapter 3 (cont.)

Figure		
3.8	Blood pressure of normal and chronic laminitic ponies	120
3.9	Blood pressure of normal and chronic laminitic horses	121
3.10	Radiography of the front hooves of two chronic laminitis cases	122
3.11	Chronic laminitic blood pressures compared with another study	130

Chapter 4

Figure		
4.1	Thermal image of an equine digit	140
4.2	Thermal images and spot temperatures of equine digits	141
4.3	Thermal images and temperature gradients of equine digits	142
4.4a	Thermal images / temperature gradient of a normal hoof	143
4.4b	Thermal images /temperature gradient of a chronic laminitic hoof	144
4.5	Coronary band circulation of normal and laminitic animals	147
4.6	NIRS of ischaemia and reperfusion of a human forearm	155
4.7a	NIRS sensors in place on the equine hoof	158
4.7b	Relationship of NIRS sensors to internal pedal structures	159
4.8	NIRS of pedal haemodynamics of unsedated normal horses	164
4.9	NIRS of ischaemia and reperfusion of a normal equine hoof	165
4.10	NIRS of pedal haemodynamics - abnormal response	166
4.11	NIRS of pedal haemodynamics of unsedated horse - 30 mins	168
4.12	NIRS of pedal haemodynamics of sedated normal horse - 30 mins	169
4.13	NIRS of pedal haemodynamics of sedated normal horse - saline iv	170
4.14	NIRS of pedal haemodynamics of sedated normal horse during saline iv and manoeuvres to alter blood flow	171
4.15	NIRS of pedal haemodynamics of a case of acute laminitis showing haemostasis	173

Chapter 5

Figure		
5.1	Complex interactions of hormones	187
5.2	Molecular structure of l-arginine	190
5.3	Biosynthesis of l-arginine	192
5.4	Nitric oxide mediated relaxation of vascular smooth muscle	193
5.5	Molecular structure of glyceryl trinitrate	195
5.6	NIRS of pedal haemodynamics of sedated normal horse - 30 mins	207
5.7	NIRS of pedal haemodynamics of sedated normal horse - saline iv	210
5.8	NIRS of pedal haemodynamics of sedated normal horse during saline iv and manoeuvres to alter blood flow	211
5.9	NIRS of pedal haemodynamics of sedated normal horse during l-arginine iv	212
5.10	NIRS of pedal haemodynamics of a sedated case of acute laminitis showing haemostasis	214
5.11	NIRS of pedal haemodynamics of a sedated case of acute laminitis during l-arginine iv showing reperfusion	215

Chapter 5 (cont.)

Figure		
5.12	Glyceryl trinitrate patches in place on a pony's pasterns	218
5.13	Blood pressures of acute laminitis cases treated with glyceryl trinitrate	219
5.14	Blood pressures of mild untreated cases of laminitis	220
5.15	Case of acute laminitis 2 days after treatment	221
5.16	Renal biosynthesis of l-arginine from l-citrulline	239
5.17	Molecular structure of ADMA	240
5.18	HPLC peaks of l-arginine and ADMA	243
5.19	Seasonal variations in plasma l-arginine of normal and chronically laminitic horses and ponies	246
5.20	Seasonal variations in plasma ADMA of normal and chronically laminitic horses and ponies	247
5.21	Vasodilatory action of glyceryl trinitrate	263

Chapter 6

Figure		
6.1	Endocrine regulation of vascular tone	270
6.2	Synthesis of angiotensin II	271
6.3	Serial dilution of equine angiotensin II	284
6.4	Seasonal variations in plasma angiotensin II of normal horses and ponies	287
6.5	Seasonal variations in plasma angiotensin II of chronically laminitic horses and ponies	290
6.6	Comparison of seasonal changes in plasma angiotensin II of normal and chronically laminitic horses and ponies	292

Chapter 7

Figure		
7.1	Molecular structures of ANP and BNP	309
7.2	Comparison of human, equine and rat sequences of ANP	310
7.3	ANP receptors and their affinities	312
7.4	Radioimmunoassay standard curve	325
7.5	Serial dilutions of equine ANP	326
7.6	Plasma ANP in normal horses and ponies	332
7.7	Plasma ANP in chronically laminitic horses and ponies	333
7.8	Seasonal variation in mean plasma ANP in normal animals	343
7.9	Seasonal variation in mean plasma ANP in chronically laminitic animals	344
7.10	Seasonal variation in mean plasma AII for comparison with Figures 7.8 and 7.9	345
7.11	Regression analysis of plasma AII and ANP of normal animals	348
7.12	Regression analysis of plasma AII and ANP of chronically laminitic animals	349

Chapter 8

Figure

8.1	Amino acid sequences of the endothelin family	358
8.2	Biosynthesis of endothelin	359
8.3	Vascular interaction of nitric oxide and endothelin	361

Chapter 9

Figure

9.1	Relationship between plasma ANP, AII and sodium	388
9.2 -	GCMS analysis of urinary organic acids of normal horses	395 -
9.8	GCMS analysis of urinary organic acids of normal horses	401
9.9	GCMS analysis of urinary organic acids of acutely laminitic horses	402
9.10	GCMS analysis of urinary organic acids of acutely laminitic horses	403
9.11 -	GCMS analysis of urinary organic acids of a horse during	405 - 412
9.18	administration of antibiotics	

Chapter 10

Figure

10.1	Hypothesis of the aetiology of developmental and acute equine laminitis	429
10.2	Chart to aid clinical diagnosis of acute laminitis	439
10.3	Amputation of forelimb and fitting of prosthesis in the USA	440
10.4	Forced recumbency as adjunct therapy for acute laminitis in the USA	441

List of Tables

	Chapter 1	Page
Table		
1.1	Endocrine changes in equine laminitis	50
1.2	In vitro studies of equine vessels to vasoactive agents	51
Table	Chapter 2	
2.1	Geographical division of areas surveyed	58
2.2	Percentage of equine population affected by laminitis	63
2.3	Outcome of acute cases (BEVA)	65
2.4	Outcome of acute cases (IDHS)	65
2.5	Incidence and outcome of acute equine laminitis in the UK	66
2.6	Treatment of equine laminitis	67
2.7	Financial impact of equine laminitis in the UK	68
2.8	Geographical distribution of equine laminitis in the UK	75
2.9	Fate of cases of acute equine laminitis	80
	Chapter 3	
Table		
3.1	Published values of equine blood pressure	95
3.2	Description of animals used in this study of blood pressure	97
3.3	Basal blood pressure and heart rate of normal ponies	102
3.4	Blood pressure and heart rate of normal ponies (winter)	103
3.5	Blood pressure and heart rate of normal ponies (spring)	103
3.6	Blood pressure and heart rate of normal ponies (summer)	103
3.7	Basal blood pressure and heart rate of normal horses	104
3.8	Blood pressure and heart rate of normal horses (winter)	104
3.9	Blood pressure and heart rate of normal horses (summer)	105
3.10	Blood pressure and heart rate of pregnant and non-pregnant horses	106
3.11	Blood pressure and heart rate of normal horses of various ages	107
3.12	Blood pressure of acute laminitis before treatment	109
3.13	Comparison of blood pressures of normal and acutely laminitic ponies	110
3.14	Blood pressures of individual cases of acute laminitis	110
3.15	Blood pressure of an atypical case of mild acute laminitis	111
3.16	Blood pressure of a case of <i>post partum</i> acute laminitis	112
3.17	Blood pressure of Days 1-2 of <i>post partum</i> acute laminitis compared with normal	116
3.18	Blood pressures of chronic laminitic horses (winter)	117
3.19	Blood pressures of chronic laminitic horses (summer)	117
3.20	Blood pressures of chronic laminitic ponies (winter)	118
3.21	Blood pressures of chronic laminitic ponies (summer)	118
	Chapter 4	
Table		
4.1	Infrared thermography of equine hoof temperatures	145
4.2	Factors causing signal changes in NIRS	154
4.3	Summary of NIRS responses to altered pedal haemodynamics	162

Chapter 5 (cont.)		Page
Table		
5.1	Description of animals used in the the study	199
5.2	Doses of transdermal glyceryl trinitrate	202
5.3	Blood pressures and heart rates of acute laminitic ponies	207
5.4	Summary of NIRS of normal and acutely laminitic ponies	216
5.5	Blood pressure of a <i>post partum</i> case of acute laminitis given transdermal glyceryl trinitrate as an adjunct therapy	222
5.6a	Effects of l-arginine on the blood pressure of a normal horse	225
5.6b	Effects of l-arginine on the blood pressure of an acutely laminitic pony	226
5.7	Short term effects of glyceryl trinitrate on grass induced laminitis	227
5.8	Short term effects of glyceryl trinitrate on <i>post partum</i> laminitis	228
5.9	Urinary and serum nitrates after administration of l-arginine and glyceryl trinitrate	229
5.10	Plasma l-arginine and ADMA in normal and chronic laminitic ponies	245
5.11	Changes in plasma l-arginine and ADMA over the course of acute grass induced laminitis	248
5.12	Changes in blood pressure during an infusion of L-NAME	258
 Chapter 6		
Table		
6.1	Cross reactivity of Nichols Institute antibody	279
6.2	AII immunoreactivity of clinical cases of acute laminitis	281
6.3	AII immunoreactivity of dialyse equine plasma	283
6.4	Suitability of various antisera for radioimmunoassay of equine AII	283
6.5	Summary of suitable antisera and extraction methods for equine AII	283
6.6	Plasma angiotensin II of normal horses and ponies	286
6.7	Plasma angiotensin II of chronically laminitic horses and ponies	288
6.8	Comparison of summer and winter plasma AII in normal and chronically laminitic horses and ponies	291
6.9ab	Comparison of plasma AII in normal and chronically laminitic horses and ponies	291
6.10	Comparison of plasma AII in normal ponies with values during acute laminitis	293
6.11	Plasma AII over the course of acute grass induced laminitis (Beau)	294
6.12	Plasma AII over the course of acute grass induced laminitis (Misty)	295
6.13	Plasma AII over the course of acute <i>post partum</i> laminitis	296
6.14	Electrolyte composition of grass and fodder	301

	Page
Chapter 7	
Table	
7.1	311
7.2	319
7.3	327
7.4	327
7.5	329
7.6	332
7.7	335
7.8	336
7.9	337
7.10	338
7.11	339
7.12	340
Chapter 8	
Table	
8.1	362
8.2	365
8.3	366
8.4	369
8.5	371
Chapter 9	
Table	
9.1	381
9.2	382
9.3	383
9.4	384
9.5	385

List of Abbreviations

AB	Antibiotics
ACP	Acepromazine
AII	Angiotensin II
ANP	Atrial Natriuretic Peptide
AVP	Arginine Vasopressin
B	Bound
B0	No binding
BEVA	British Equine Veterinary Association
BP	Blood Pressure
CGRP	Calcitonin Gene Related Peptide
CL	Chronic Laminitis
cytaa3	Cytochrome oxidase
D	Daltons
DDFT	Deep Digital Flexor Tendon
DWR	Dorsal Wall Resection
EDRF	Endothelium Derived Relaxing Factor
ET	Endothelin
FX	Flunixin Megulamine
GTN	Glyceryl Trinitrate
HHb	Deoxyhaemoglobin
HR	Heart Rate
IDHS	Irish Draught Horse Society (GB)
K	Potassium
L	Laminitic
M-L	Medial-Lateral
Mg	Magnesium
mg	milligram
N	Normal
n	number
Na	sodium
NIRS	Near Infrared Spectroscopy
NO	Nitric Oxide
NSAIDS	Non-Steroidal Anti-inflammatory Drugs
NSB	Non Specific Binding
O₂Hb	Oxyhaemoglobin
P3	Third (distal) phalynx
PBZ	Phenylbutazone
PCV	Packed cell volume
PG	Prostaglandins
Ph	Phosphorous
PTHrP	Parathyroid Hormone Related Peptide
RAAS	Renin Angiotensin Aldosterone System
RAS	Renin Angiotensin System
RIA	Radioimmunoassay
S.N.	Sedated Normal
TC	Total Counts
tHb	Total Haemoglobin
TNF	Tumour Necrosis Factor
TOF	Time of Flight
TRIS	tris [hydroxy-methyl] methylamine
U.N.	Unsedated Normal
VIC	Vasoactive Intestinal Contractor

Endocrine and Haemodynamic Investigations of Normal and Laminitic Horses

Prologue

The central objective of this thesis was to gain insight into, and further understanding of, the complex haemodynamic and endocrine processes involved in the pathogenesis of equine laminitis, a disease that has afflicted horses probably since they were befriended by mankind. The treatise has been arranged sequentially to give as complete an analysis as possible. First, the evolution of horses gives a backcloth to the historical aspects of equine laminitis. An epidemiological investigation into the incidence of the disease in the U.K. is described. A novel non-invasive method of investigating pedal haemodynamics is applied to assess differences between normal and diseased equids. Seasonal changes in endocrine profiles of normal animals and those suffering spontaneously induced laminitis of different aetiologies is described. Endocrine investigations relate specifically to measurement of systemic and paracrine hormones that have not been established for the horse. The results are used as a basis for a scheme to integrate the complex physiological relationships involved in the disease. The thesis begins and ends with the horse as a whole animal; between, equine vascular physiology and its hormonal control are examined and a therapy for a disease of domestication developed; finally an hypothesis is presented that, if correct, will improve the welfare of this most sympathetic animal.

Chapter 1 Introduction

	The horse	Page
1.1	Evolutionary aspects	2
1.2	History and definitions of equine laminitis	9
1.3	Anatomy	14
1.4	Clinical signs of equine laminitis	25
1.5	Aetiology and pathogenesis	41
1.6	Equine laminitis in the context of modern endocrinology	45
1.7	Specific research aims and objectives	53

*"Think, when we talk of horses, that you see them
Printing their proud hoofs i' the receiving earth"*

**King Henry V, Prologue
William Shakespeare**

1.1 Evolutionary aspects

Equids have been associated with man for between 35,000 and 100,000 years (MacFadden, 1992). Horses, ponies and donkeys have been crucial to the development of civilisations, playing a vital role in agriculture, transport and warfare (Hartley-Edwards, 1987; McMiken, 1990; Clutton-Brock, 1992). Although workhorses still exist in some countries, the more usual role of the modern horse is for recreational or sporting activities. Intelligent, with gentle and understanding temperaments, equids enjoy a special relationship with man as useful, interesting and sympathetic commensals.

The evolution of the horse over the last 50 million years is well documented; indeed, more is known about the evolutionary history of the horse than about any other mammal (Young, 1981; MacFadden, 1992). Figure 1.1(a) shows the evolutionary progress of equids over the last 50 million years. Classification of the horse into the Order *Perissodactyla* ('uneven toes') is based on the evolutionary development of the equine digital skeleton and hooves. All other members of the Order are herbivores and include rhinoceroses and tapirs, which, with slightly different pedal structures, belong to other suborders of the *Equidae*. All ungulates of the family *Equidae* share digital architecture and are members of the suborder *Hippomorpha* (horse-like). Extant equine species, namely horses, asses and zebras belong to the genus *Equus*. Contemporary horses and ponies (*Equus caballus*) are usually classified as different breeds or types.

Figure 1.1 (a) Evolution of the horse (from Young, 1981, based on Stirton). The evolution of the horse is seen in the centre and changes in body size and shape described. On the left, concurrent skeletal changes in the equine forelimb and foot are shown. Changes in dentition during evolution are shown on the right.

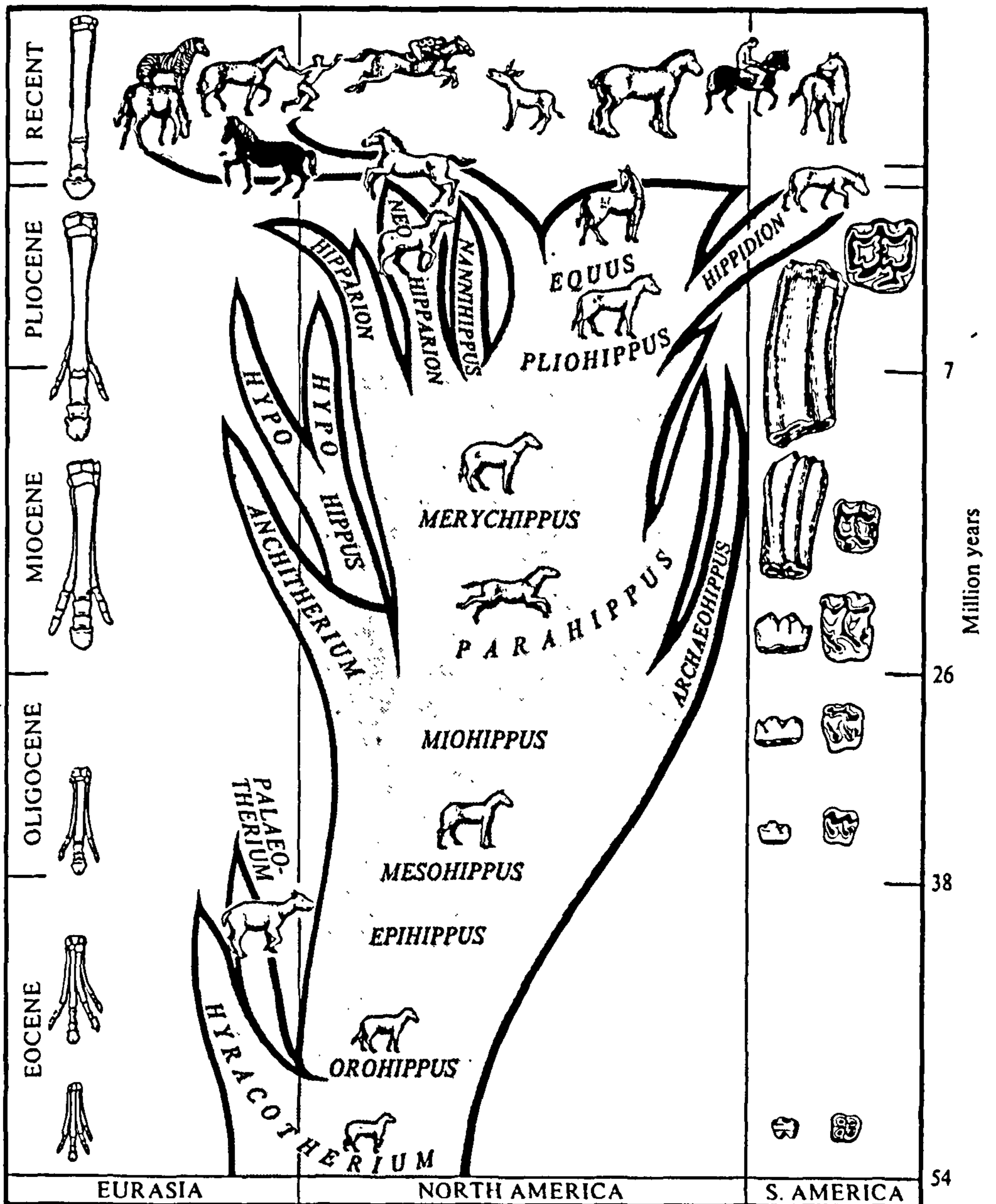
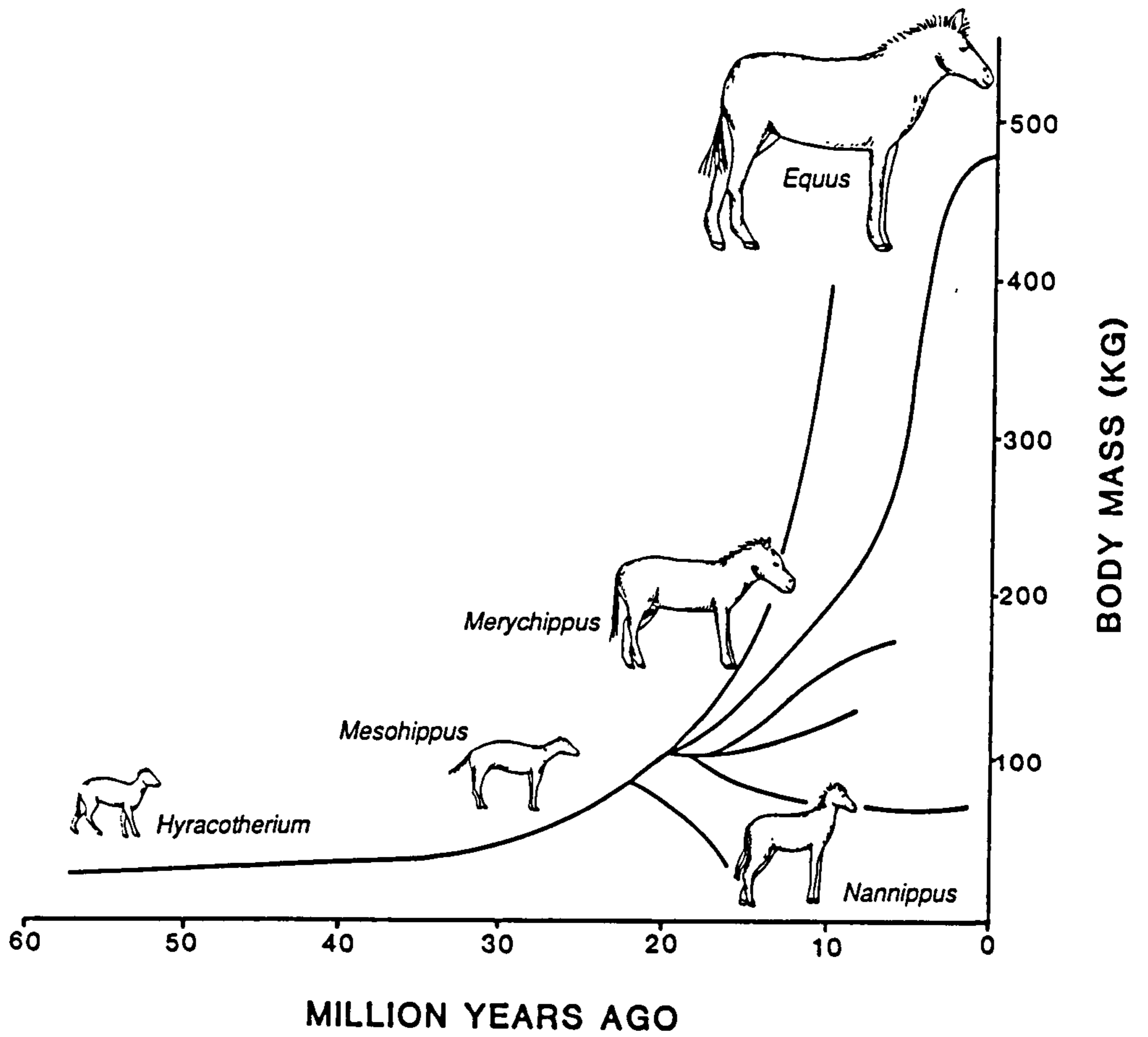


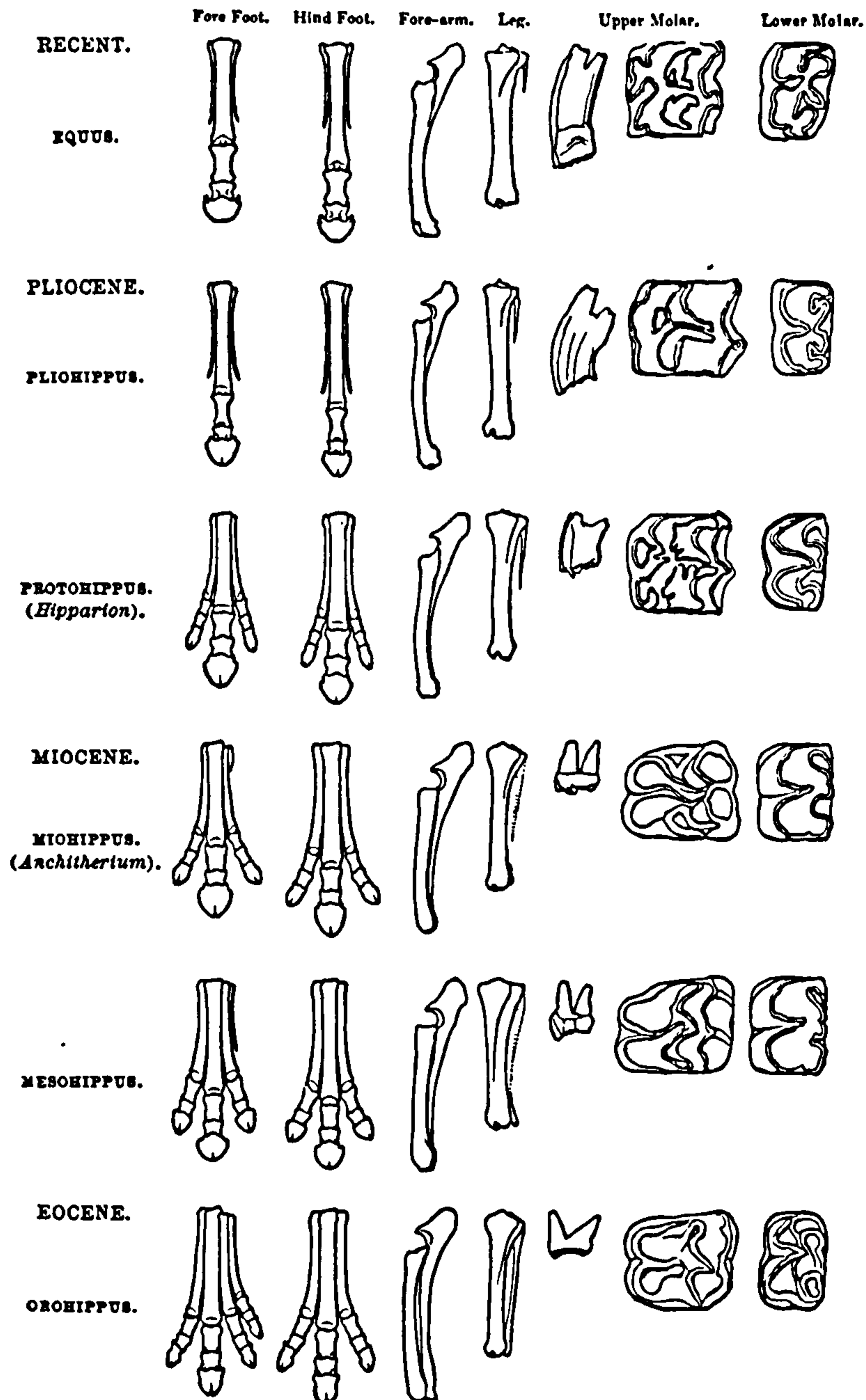
Figure 1.1 (b) Changes in equine body mass with evolution (MacFadden, 1992)



Of these, some breeds live under conditions which have changed little over the last thousand years; examples are native pony breeds that still live nearly wild on moors or mountains. Others, like the Thoroughbred or Irish Draught Horse, have been selectively bred, in more recent times, to fulfil particular purposes.

The evolution of the general dental, cranial and skeletal architecture of equines is extremely well detailed. Anatomical changes are identified in fossils of equines since the Eocene period 50 million years ago (Young, 1981; MacFadden, 1992). Although skeletal changes are the most obvious, there were concurrent increases in body size (Figure 1.1b) and from these, the size and development of the brain and other soft tissues were deduced. Exact changes in brain morphology and function are difficult to assess but there were significant effects on behaviour. An increased body size, alongside altered dentition, suggest changes in digestion and/or feeding habits. Alterations of early equine anatomy reflect adaptation to environmental changes of the period. The changing climatic conditions, alongside the appearance of more species of plant, and presumably geological characteristics, saw the horse adapt to optimize grazing ability and swift movement as the forests turned to temperate grass plains. A coarse textured grass diet was most efficiently digested by hard molar mastication and subsequent hind gut digestion of cellulose by symbiotic micro-organisms, especially in the caecum. Speed was essential to avoid predators (a horse would be clearly more visible in open grasslands), and for the long migratory journeys undertaken to take advantage of seasonal availability of food (MacFadden, 1992). Over many generations, equines have developed longer distal portions of their limbs and a reduction in accessory digits (Figure 1.2).

Figure 1.2 Evolutionary development of the equine digit - the equine digit changes from a four toed foot to a single toed foot; other digits have become vestigial. Concurrent changes in dentition occur; the molars in recent periods have become larger, open rooted and flat topped (Macfadden, 1992).



The entire weight of the horse is borne through the bony column of the third digit, or phalanx (Figure 1.2), which is equivalent to the second finger of the human hand. The other digits, laterally and medially, are vestigial in the modern equine and are present as splint bones (Figure 1.2).

Accessory digits of early equids prevented over extension and were anti-concussive as the distal portions of each digit bore pads. However, during the Miocene and Pliocene periods, the distal tip of the weight bearing digit gradually evolved to accrue a discreet hoof capsule, instead of a pad (Figure 1.3). Weight was carried through the bony column to the hoof which bore concussive forces through its suspensory structure; over extension was prevented by development of ligaments and tendons (MacFadden, 1992). This anatomical arrangement donated greater speed. The vestigial remnants of the equivalent of the human thumb are seen as horny growths (known as 'chestnuts') on the medial aspects of the limbs of all equids, proximal to the carpal joints. Chestnuts are also found proximal to the tarsal joints of horses and zebras, but not other equines. Sometimes horses are born with extra accessory digits whose significance is obscure; they are usually removed surgically (C.M.Colles, personal communication) .

Advantages bestowed by these anatomical changes have ensured the prosperity of the species over thousands of years, even during early domestication, when swiftness in hunting or battle and efficient feed conversion meant survival. Evolutionary efficiency turned to disadvantage when environmental conditions became less demanding. As soon as horses were fully domesticated, that is they were housed, fed a high energy diet of grain rather than low carbohydrate roughage, and subjected to artificial exercise regimes, they were liable to "diseases of domestication". One such disease is equine laminitis. Laminitis occurs in all breeds, indeed in all hoofed animals, but anecdotally, laminitis has a higher incidence in those equine breeds that have been domesticated most recently. The complicated structure of their limbs, together with a highly specific digestive system and metabolism, were no longer ideal for the imposed environment. Unable to adapt, selection pressure turned against them. The affliction of equine laminitis probably has a greater incidence now than ever before. Laminitis, like colic, is a disease of domestication.

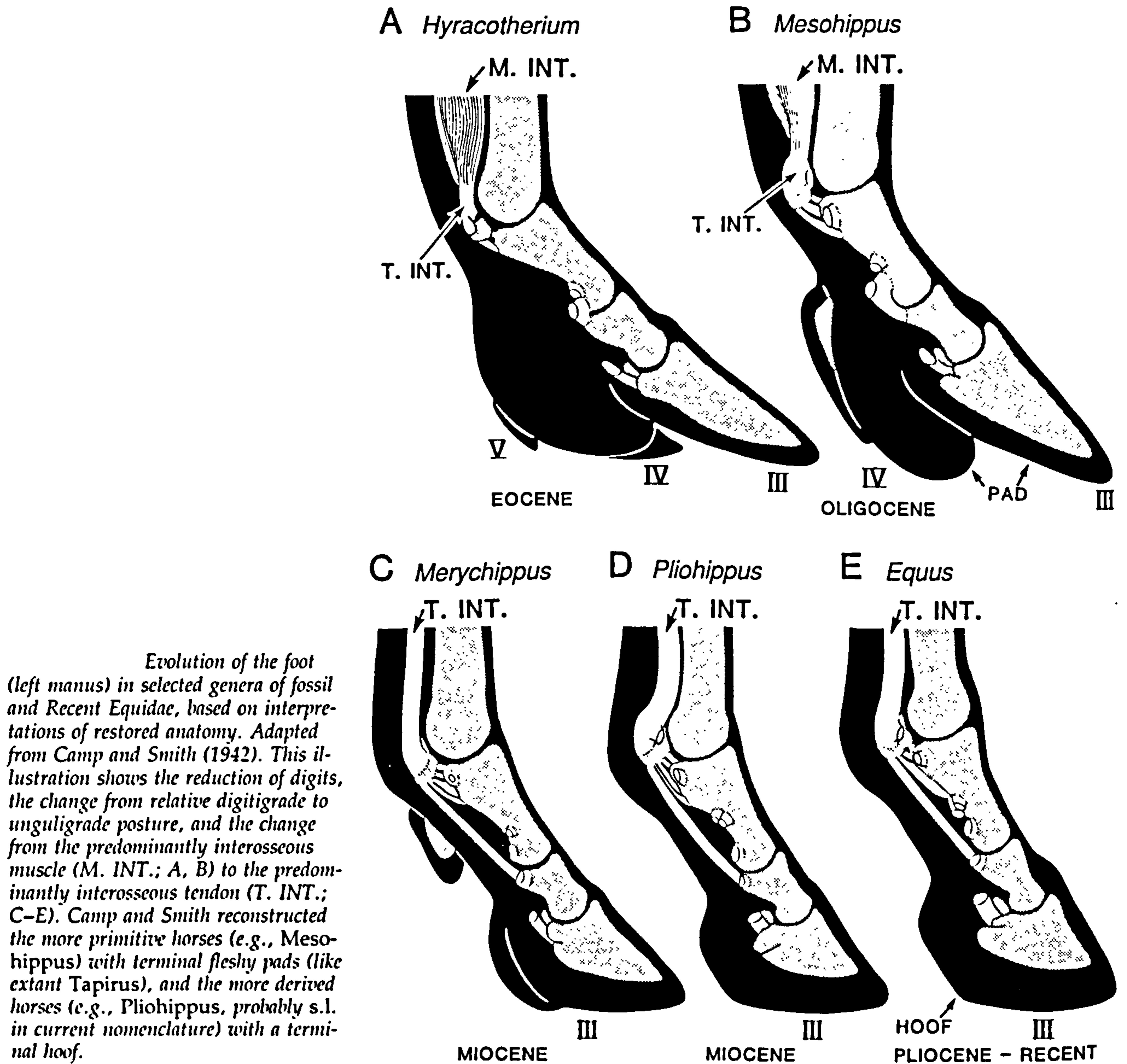


Figure 1.3 Comparison of digital weight bearing structures during evolution of the horse (MacFadden, 1992).

1.2 History and definition of equine laminitis

Although laminitis is probably a recent disease in the evolutionary timescale, it is certainly not a new disease in recorded history. Laminitis is seen in early paintings (Figure 1.4) and is mentioned in early veterinary and farriery books where it is described as 'founder' (Hodson *et al.*, 1662; Markham, 1662; Halfpenny, 1670), (Figure 1.5).



Figure 1.4 Early picture of a horse showing a laminitic stance with weight borne on the heels (Uccello, 1397 - 1445).

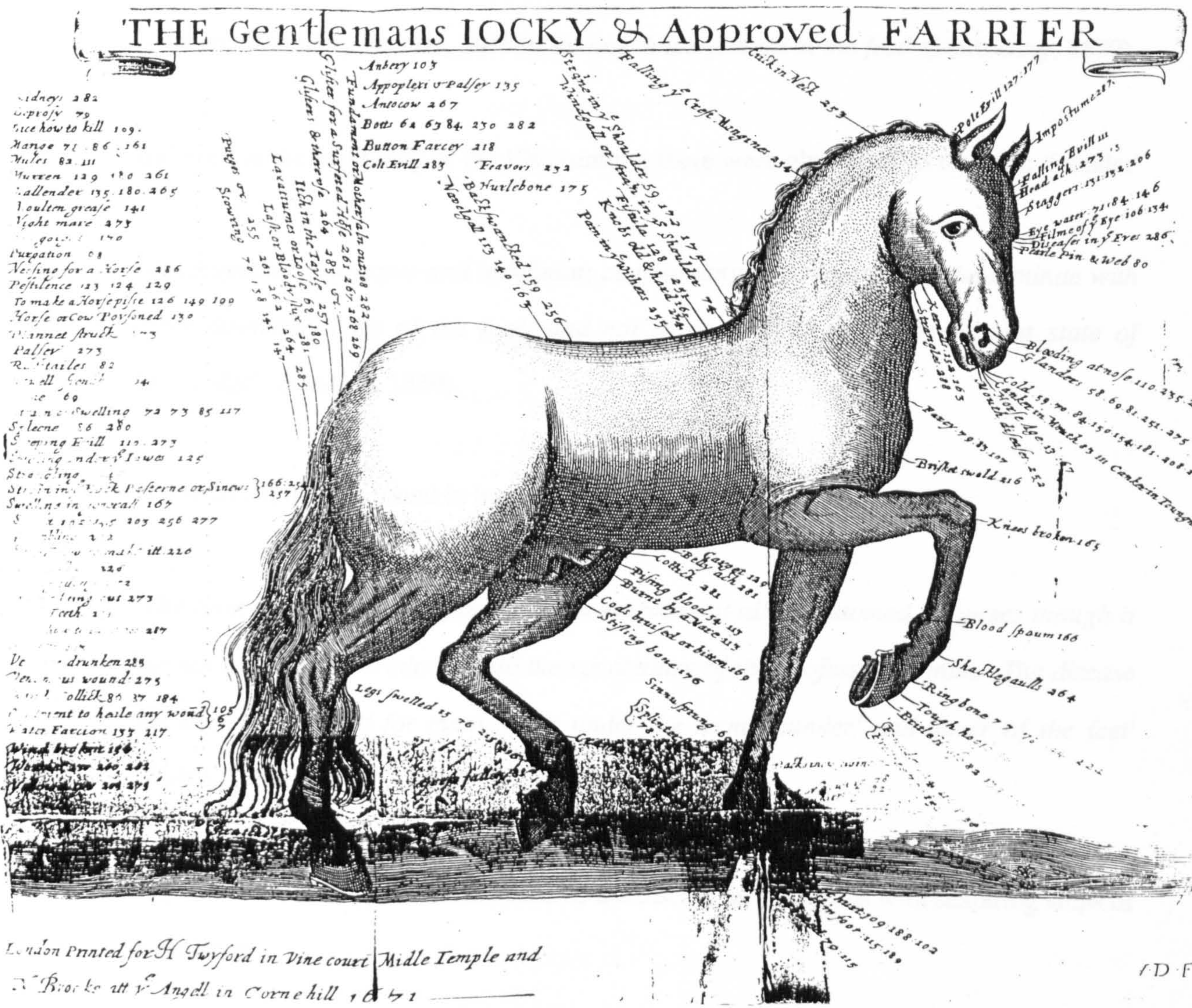


Figure 1.5 Early illustration from a veterinary textbook of 1672 showing ‘founder’ as a known disease of the horse (Hodson *et al.*, 1672).

Some early quotations may be noted:

"For any Founder, Frettize, Surbait, or any imperfection in the feet" (Halfpenny, 1670)

But even in the beginning of the 19th century, there were objections to the term 'founder'

"as altogether too vague and indefinite; confounding inflammation of the laminae with other diseased states of the foot; and not in accordance with our present state of knowledge" (Castley, 1830).

The term laminitis is found in books of the 19th century

"The term laminitis is now familiar with everyone at all accustomed to horses though it has not long been introduced into the vocabulary of the professional man. The disease has been recognised for many years under the term 'founder' and 'fever of the feet' (Walsh, 1888).

Such terms do convey the seriousness of the disease as analogous with seafaring ships of those times

"..the animal is a cripple for life. To this state of the foot I have no objection that the term Founder be applied. Here, indeed, I think it to be very expressive of the lost and sinking state of the animal machine." (Walsh, 1888).

Some veterinary clinicians describe the biomechanical movements of the distal phalanx following the original acute disease by the archaic names of 'founder' and 'sinking' (Eustace, 1992). Nonetheless, the term 'laminitis' was adopted and as texts on equine management increased during the 19th century, so did references to equine laminitis. Laminitis was a major problem in war horses and an interesting account is given of laminitis in cavalry chargers after travelling by sea to Corunna, Galicia, between 1808 - 1809. Half of the 400 horses belonging to the 7th Regiment that had assembled at Villa-Franca were left behind because they had contracted laminitis (Walsh, 1888). Magner's Stock Book published in the U.S.A has an entire chapter on the disease (Magner, 1901). Cavalry manuals (1908) describe laminitis. Laminitis in pit ponies has been described in the 1000 or so ponies that lived below ground in the Mansfield area in the first half of this century. The disease had a high incidence because of the practice of each miner feeding a huge feed to his pony before leaving his shift. This practice of overfeeding led to laminitis, especially on rest days (J. Mason, personal communication). References abound both in horse owners books (Hayes 1976; Hayes 1990; Eustace, 1992) and in veterinary texts (Blood and Radostits, 1989; Robinson, 1987; Stashak, 1987; Wyn-Jones, 1988; Evans *et al.*, 1990; Rose and Hodgson, 1993; Powell and Jackson, 1992; Boden, 1993; Kobluk *et al.*, 1995).

The noun 'lamina', defined in the Oxford English Dictionary as " Thin plate, scale, layer or flake, of metal, bone, membrane, stratified rock, vegetable tissue, etc." (Latin); is a very apt, as will be seen later, description of the inside of the horse's hoof which contains numerous layers of tissue. These tissues are known as dermal and epidermal laminae or laminar corium. A suffix of 'itis', added to a word, describes inflammation of the area. Therefore, laminitis is thus inflammation of the dermal laminae (Fessler *et al.*, 1982) This strict definition is, however, an oversimplification of a very complex disease and, although inflammatory mediators are involved, laminitis is not primarily an inflammatory disease.

Laminitis is now considered to be, not simply a disease of the feet, but a physiological disturbance of the whole animal (Yelle, 1986, Hood and Stephans, 1981; Overton, 1995). The systemic disorder is varyingly manifested in the feet as laminitis. Laminitis is therefore just one symptom of a systemic dysfunction such as endotoxaemia for example. The disease may require redefinition and hence be more appropriately named. It has been suggested that the disease be renamed "acute laminar degeneration" (Baxter, 1992). This title describes more accurately the processes within the hoof but does not include the systemic malfunction that precedes the secondary degeneration.

1.3 Anatomy

A knowledge of the anatomy, and mechanics of weightbearing, within the horse's hoof is essential for an understanding of the sequelae of acute laminitis. A brief outline is given and veterinary textbooks give finer details (Ashdown and Done, 1992; Stashak, 1987). Two thirds of the horse's weight is borne on the front limbs (Figure 1.6). The hoof, at the distal part of the limb, has several mechanisms to dissipate concussive forces. Towards the rear of the hoof there is a cartilaginous pad, the digital cushion, within the hoof itself and, on the exterior, a triangular shaped pad on the sole of the hoof capsule, the frog. The hoof capsule has a convex sole and the weight is taken on the outside surface, through the hoof wall. Only in fast paces does the frog come into contact with the ground (Colles 1989). In essence, the weight of the horse (in the bony column) is suspended inside the hoof capsule between the deep digital flexor tendon (DDFT) and the attachment of the distal phalanx or pedal (coffin) joint to the dorsal interior of the hoof capsule. The structures within the hoof capsule are shown in Figure 1.7. The equine foot is composed of a hard horny exterior, the hoof capsule and the distal or third phalanx (P3) within the hoof capsule. The horn and bone are joined by soft tissue - the dermal and epidermal laminae.

The structure of the equine hoof is remarkable not only for its mechanical weightbearing structure but also for the fact that soft tissue that binds two hard and relatively inelastic surfaces - the pedal bone and the hoof capsule - together. The thin layer between bone and keratinous exterior of the hoof is at the most 2cm wide but its integrity maintains the weightbearing capacity and is literally vital to the well being of the horse. The thin layer of laminar tissue, the interdigitating of dermal and epidermal laminae are shown in Figures 1.8 and 1.9. The weight bearing structures within the hoof are complex cantilever and suspensory arrangements. The deep digital flexor tendon (DDFT) is attached to P3 and the direction of force of the horse's body weight is through the bony column and then into a cantilever structure composed of P3, DDFT and hoof capsule (Figure 1.7).

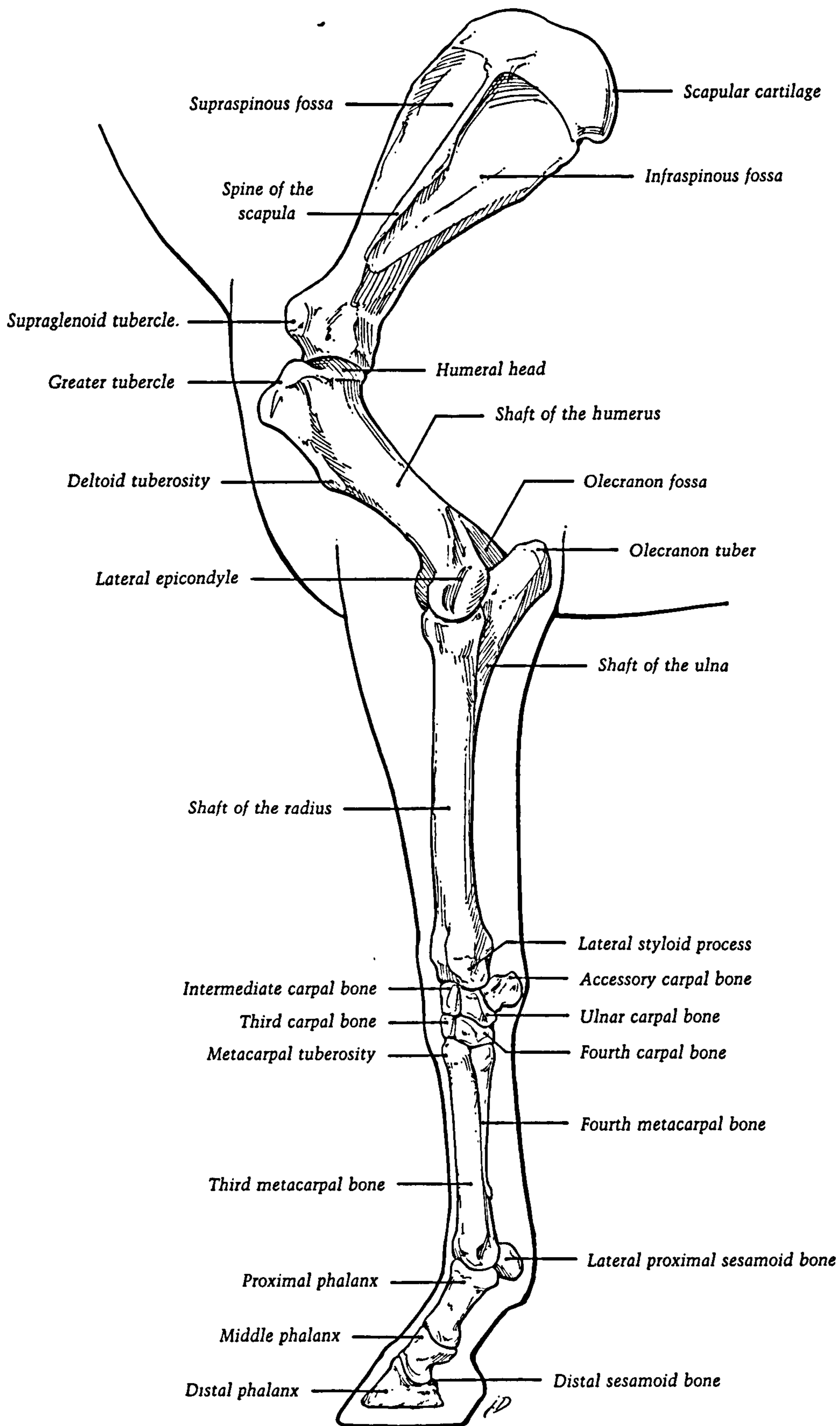


Figure 1.6 Anatomy of the skeleton of the equine forelimb (Stashak, 1987)

Figure 1.7 Parasagittal section of the equine digit and pastern (Pollitt, 1995)

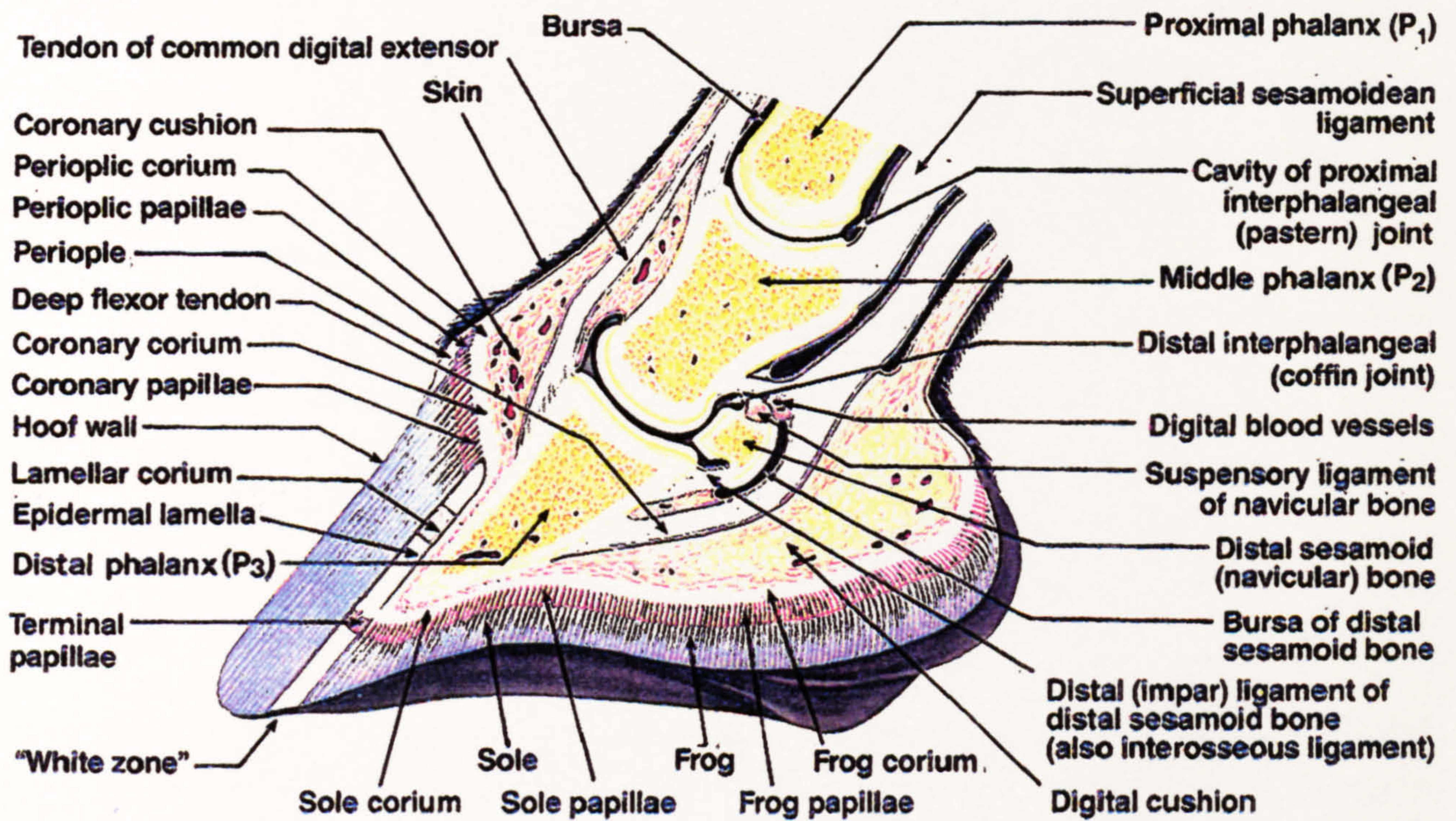
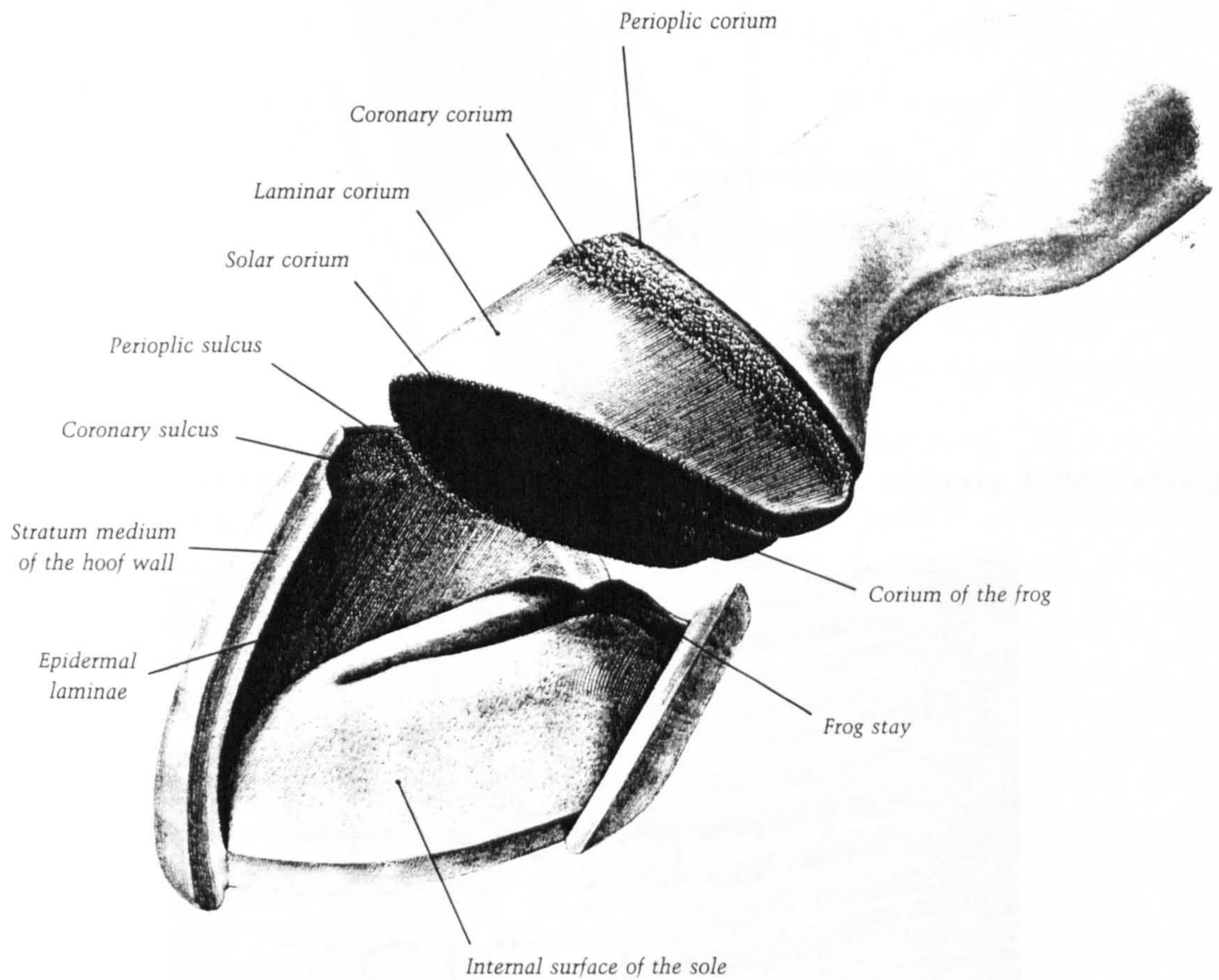


Figure 1.8 Hoof capsule removed from equine digit showing laminar tissue (Stashak, 1987).



Dissected view of the relationships of the hoof to the underlying regions of the corium.

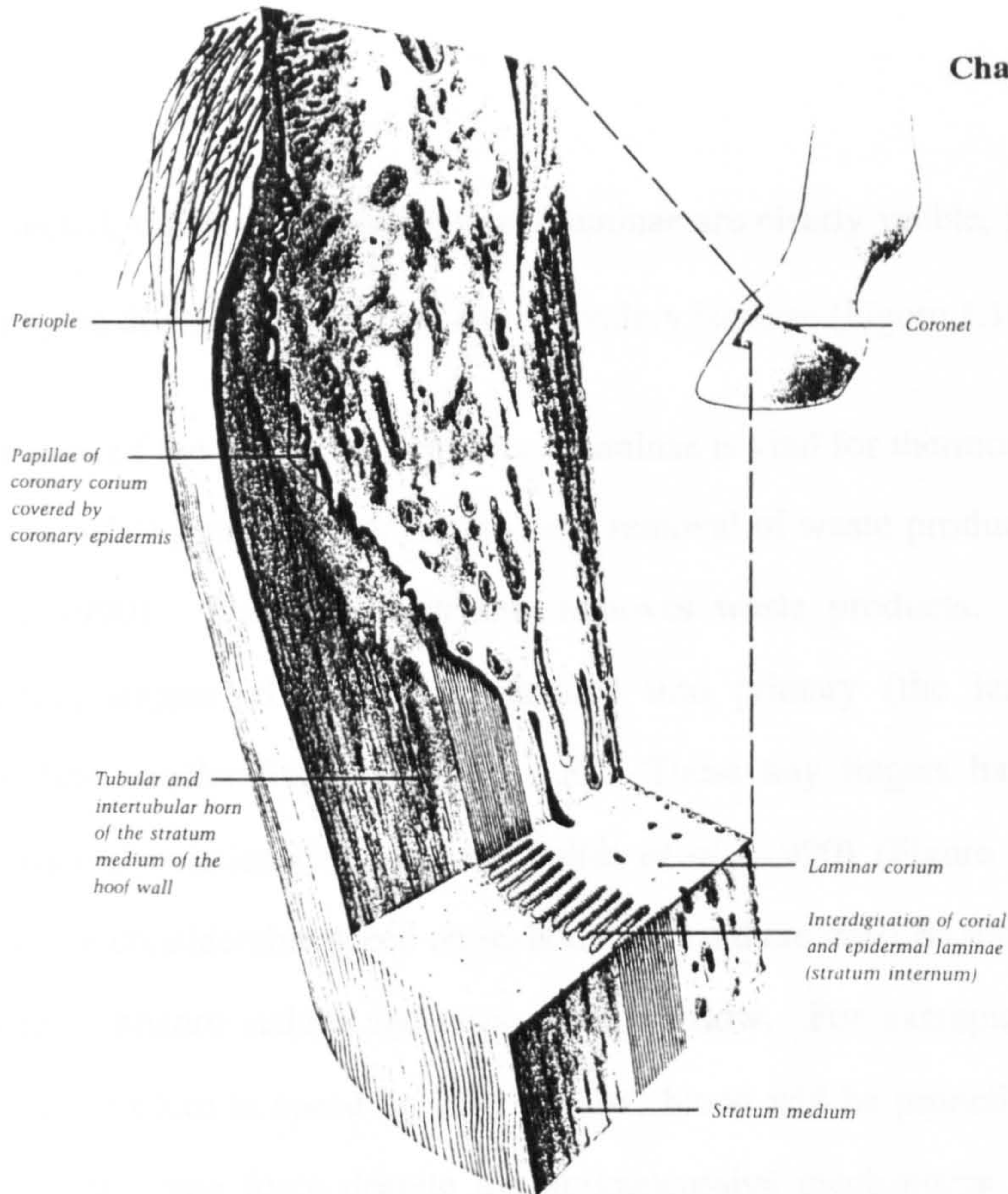
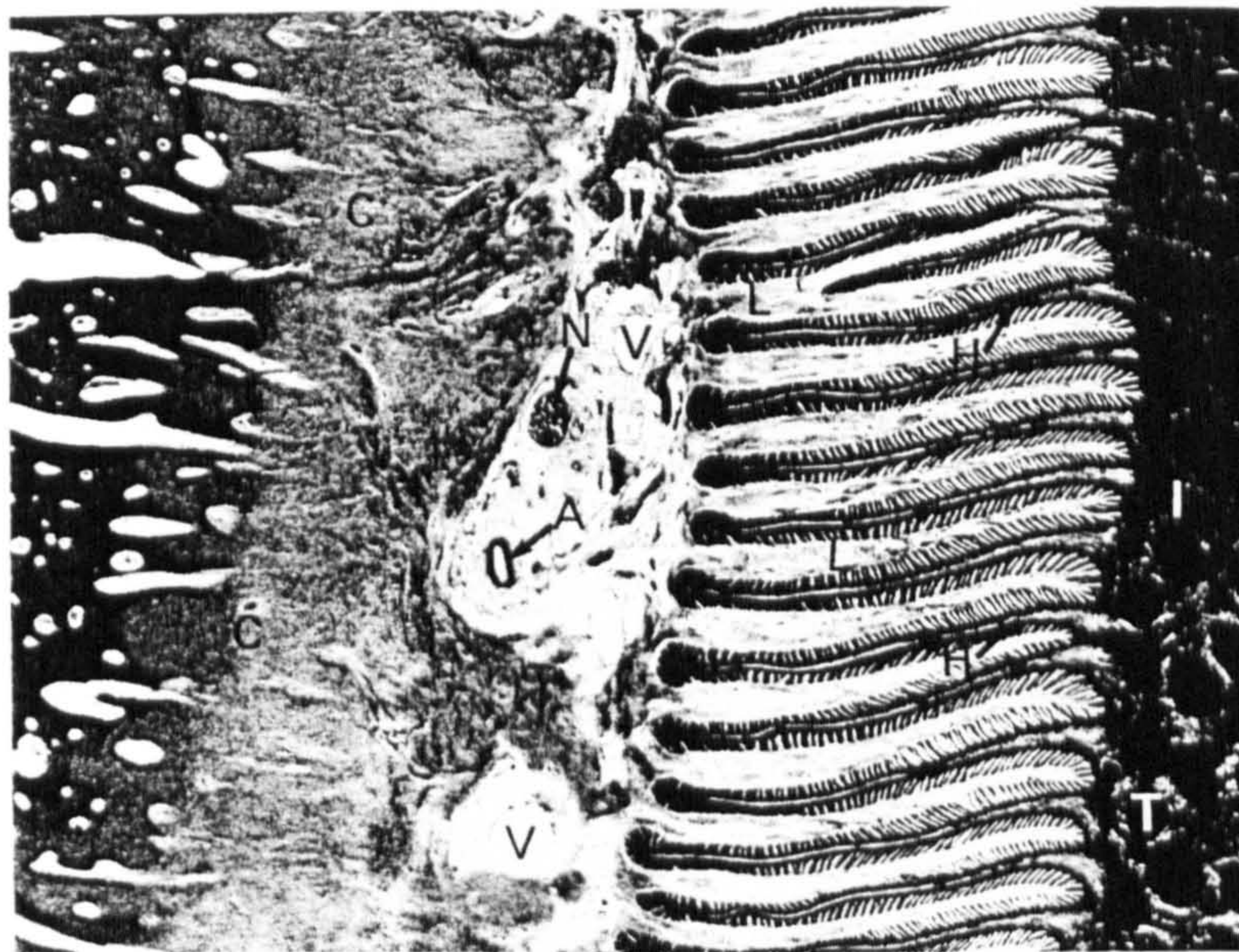


Figure 1.9 Diagram of a dissection of the hoof wall and coronary band showing epidermal and dermal laminae (Stashak, 1987)



This is a transverse sectional view through the hoof wall as seen through the microscope. The pedal bone (P) is on the left and the outside of the hoof is on the right. The pedal bone is covered by the connective tissue (C) of the periosteum which contains arteries (A), veins (V) and nerves (N) and merges with the dermal laminae (L). The two sets of laminae, dermal (L) and epidermal (H) slot together like interlocking fingers. The tubular horn (T) from the coronary papillae and the intertubular horn (I) from the pits between the papillae are clearly visible making up the bulk of the hoof wall.

Figure 1.10 Photomicrograph of dermal and epidermal laminae (Eustace, 1992).

When dissected, the dermal and epidermal laminae are clearly visible; in finer detail, the laminae may be divided into primary and secondary laminae (Figure 1.10).

The circulation of the dermal and epidermal laminae is vital for thermoregulation and for the provision of oxygen and nutrients to, and removal of waste products from, the hoof (Robinson, 1990). The circulation also removes waste products. The laminae are interdigitating fingers of soft tissue divided into primary (the larger fingers) and secondary (the smaller fingers) (Figure 1.8). These tiny fingers have a profuse and complex microcirculation described by Pollitt *et al.*, (1990) (Figure 1.11). Since the horse moves at considerable speed on its hooves then there must be some capacity within the laminae to absorb sudden increases in blood flow. For example, when the horse lands on one forefoot at speed on hard ground, blood will be propelled into the digital circulation with some force despite the anticoncussive mechanisms of the foot (see Chapter 4). To guard against injury the microcirculation has numerous arteriovenous anastomoses (AVA) (Figure 1.11 and Figure 1.12).

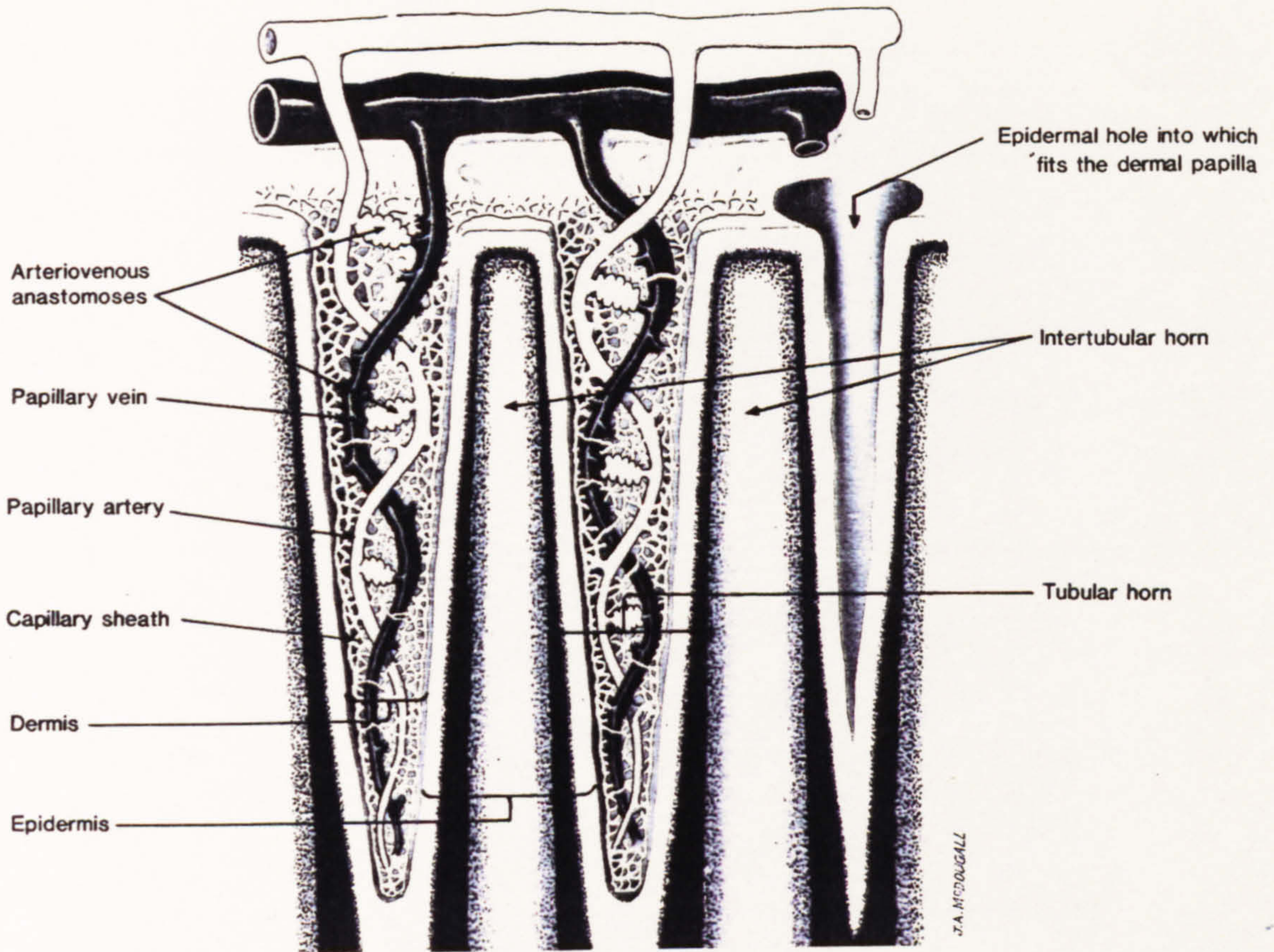


Figure 1.11 Microcirculation of the digital dermal papillae; numerous AVAs connect the central artery and vein of each papilla. The largest AVAs are found nearest to the origin of the central artery and vein (Pollitt, 1995).

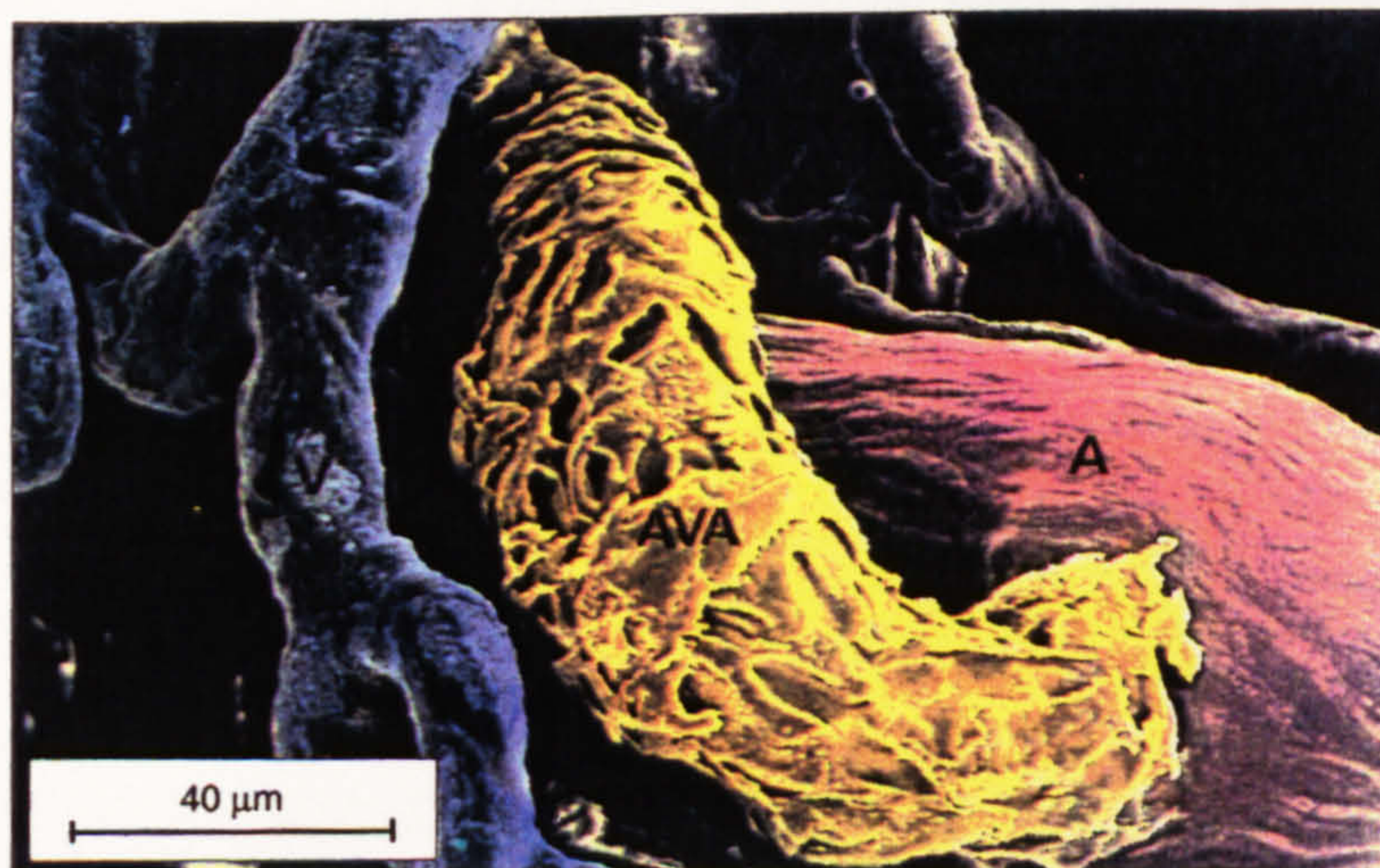


Figure 1.12 Electron microscopy of AVA within dermal and epidermal tissue (Pollitt, 1995).

Arteriovenous anastomoses (AVA), first noted by Hyrtle (1864), have been described by Rooney (1984), Pollitt and Molyneux (1990). AVA have a density of 500/cm³ and a lumen diameter of 15 μ m (Robinson, 1990; Trout, 1990). AVA are involved in thermoregulation - closing to allow increased blood supply to the hoof to increase heat loss and opening to redirect blood away from the periphery to conserve heat. AVA are under sympathetic control but are likely to be governed by non-adrenergic mechanisms particularly vasoactive peptides (Molyneux *et al.*, 1994). Clinical symptoms of laminitis include a warm hoof and a bounding digital pulse which together suggest that the primary pedal supply, and/or drainage, is involved in the disease. The micro circulation to the epidermal and dermal laminae was described by Pollitt *et al.*, (1990) (Figure 1.13)

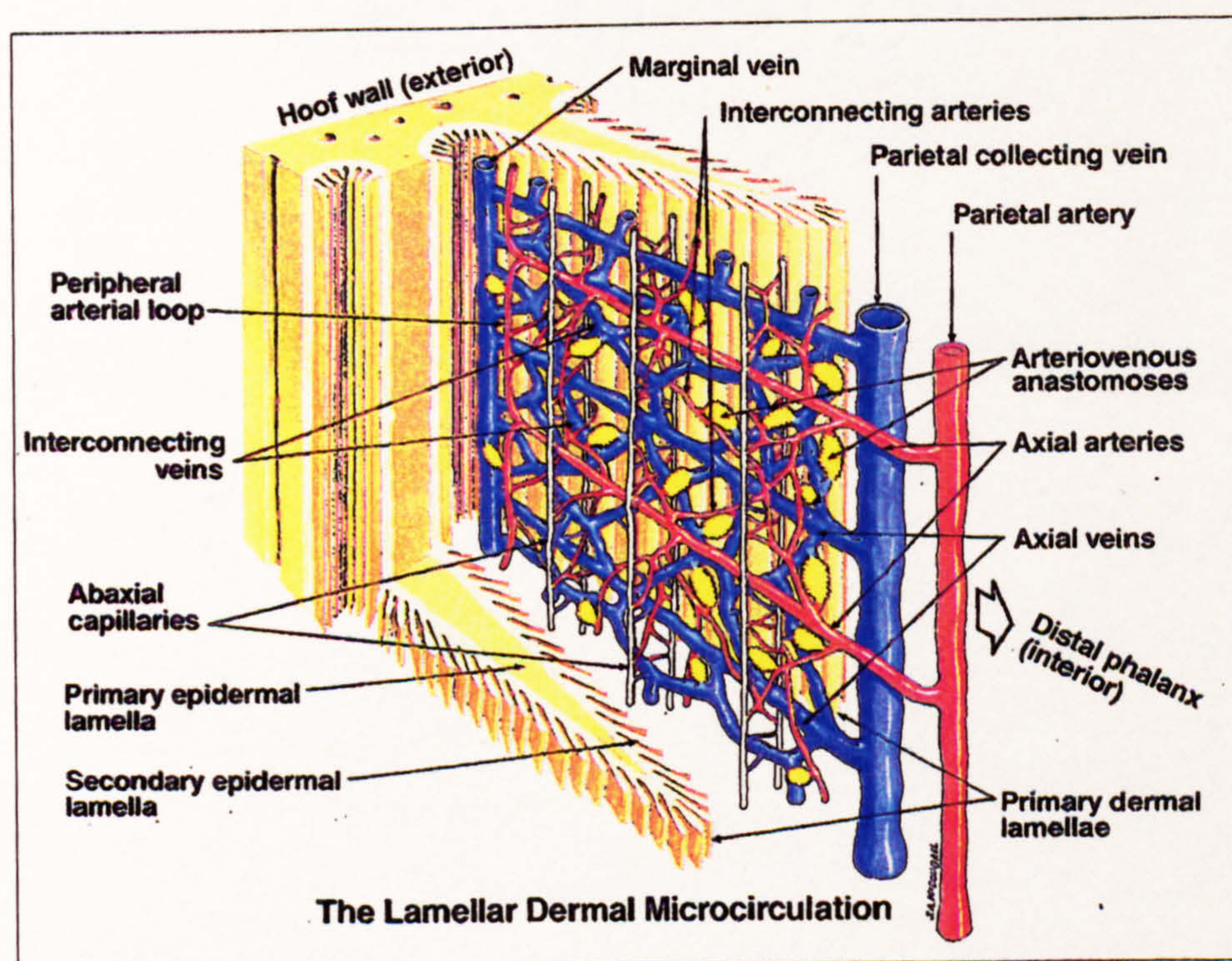


Figure 1. 13 The micro circulation of the epidermal and dermal laminae (Pollitt *et al.*, 1990).

Vascular supplies to these areas are obviously crucial. The gross circulation of the equine limb is shown in Figure 1. 14. The arterial supply to the hoof is from the median and lateral digital arteries which branch to supply the coronary band, then further divide into the circumflex artery and the terminal arch. The circumflex artery runs near the sole just within the hoof wall and the terminal arch supplies the laminar corium through the foramen in P3.

Venous drainage capillaries are profuse : plexuses of large veins are present near the sole, frog and coronary band with smaller veins forming plexuses near the hoof walls (Mishra and Leach, 1983 b; Robinson, 1990). In this latter area, capillary tufts with anatomically unique diverticula and enlargements are found (Mishra and Leach, 1983 a). All venous vessels in the hoof are thick walled to resist pressure within the hoof capsule (Korthuis *et al.*, 1983; Hunt, 1991) and may therefore participate in the potential venoconstriction that may be involved in laminitis (Hunt, 1991). Latex angiographic casts and angiographic scans using radiolabelled media have been used to describe the gross anatomy, and electron microscopic techniques to detail the microcirculation (Figure 1.15).

Figure 1.14 Arterial supply to the equine digit (Stashak, 1987).

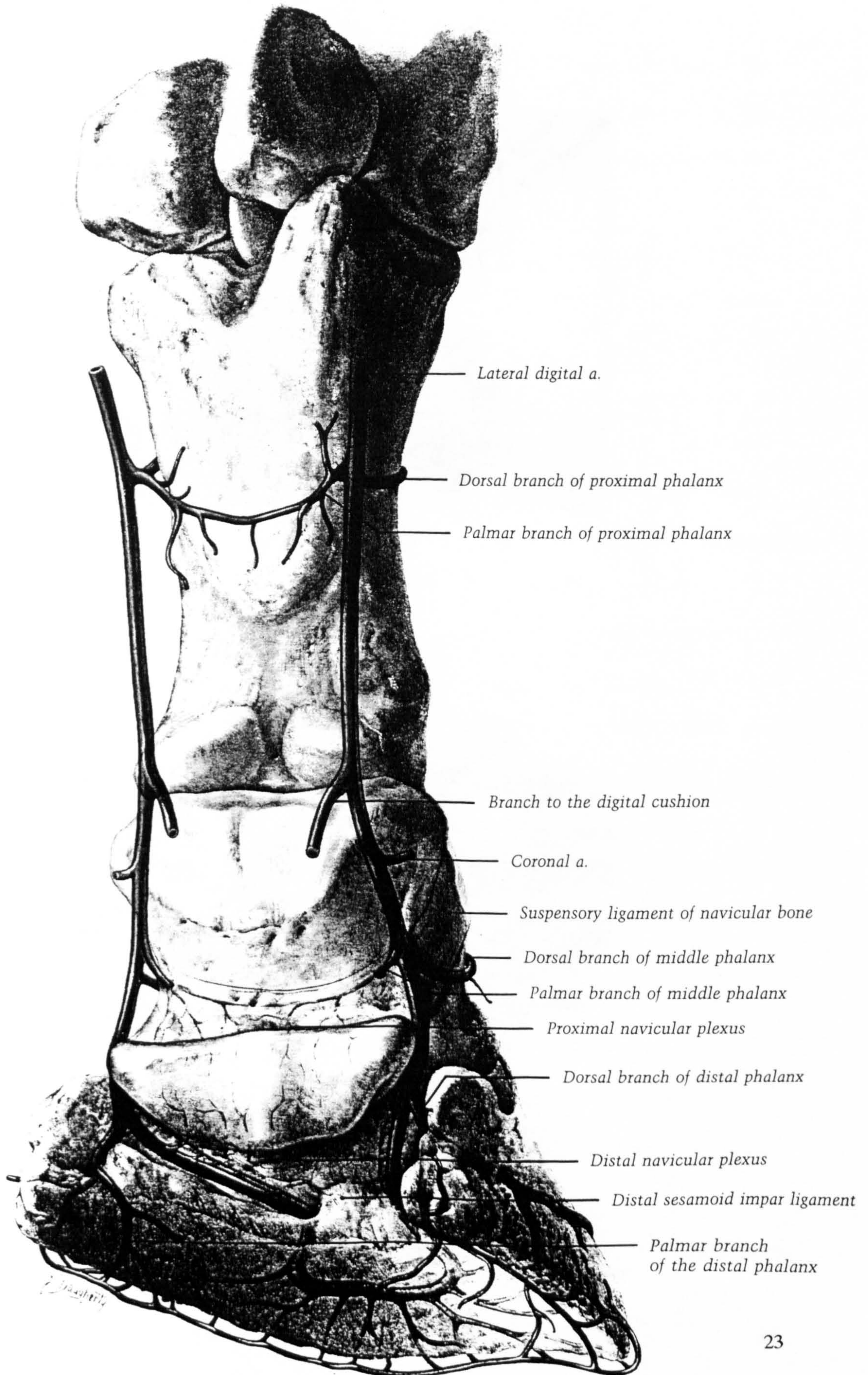
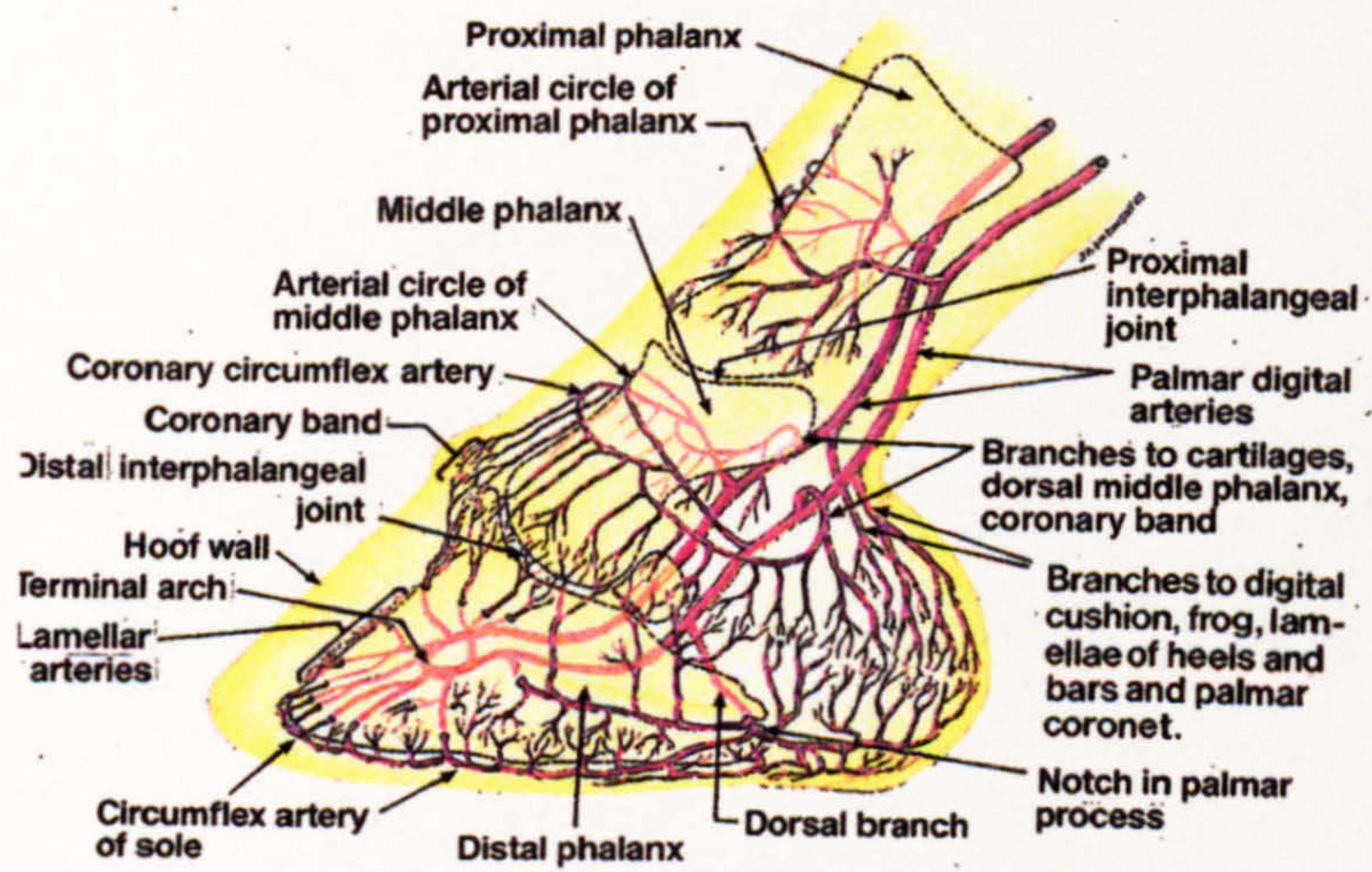


Figure 1.15 Latex angiograph showing gross circulation of the hoof (Pollitt, 1995).



1.4 Clinical signs of laminitis

Definition of phases. Laminitis may be broadly classified into different, although really rather temporally indistinct sequential phases - developmental, acute and chronic (Hood, 1979; Hood *et al.*, 1993). During the developmental phase, pathological changes are probably underway but there is no overt lameness (Hood *et al.*, 1993). Hood *et al.*, (1993) defined developmental laminitis as "the period between initiation of mechanisms that result in the disease and the appearance of acute lameness". The developmental stage, in experimental laminitis, is usually around 56 hours but under field conditions it may be as long as weeks or months. Acute laminitis "begins with the onset of characteristic lameness and ends with the mechanical collapse of the digit" and occurs over about 72 hours (Hood *et al.*, 1993). Clinically, classifications are acute, sub-acute, refractory and chronic (Colles and Jeffcott, 1979; Stick, 1992; Baxter, 1992). In using these definitions, sub acute is equivalent to the developmental phase and refractory is the stage, following an acute attack, during which the animal's condition neither improves or worsens; that is to say it is static but inherently unstable. Hood (1995) classified acute laminitis cases into either progressive or static states and further divided these into compensated or uncompensated categories. Compensation is when the horse experiences physiological changes but is seemingly able to adapt and the changes are not prohibitive to health. Uncompensated situations occur when the pain and mechanical changes are prohibitive and aggressive therapeutic approaches are essential (Hood, 1995).

Clinical signs of acute laminitis

Acute laminitis, clinically obvious in all but the mildest and most severe recumbent cases, is characterised by lameness and obvious pain of varying degrees. During a moderate to severe acute attack, the horse has an increased heart rate, sweats and presents signs of pain. A 'bounding' pulse is apparent in the digital arteries. Immobility and recumbency are extreme, but not uncommon, symptoms (Fig 1.16).



Figure 1.16 A horse with severe pain and distress shortly before destruction; laminitis was diagnosed by a veterinary surgeon. (K.A. Hinckley, 1993).

In less severe cases, animals adopt a particular stance as the hind limbs are brought forward under the animals to take a greater proportion of the bodyweight (Figure 1.17).

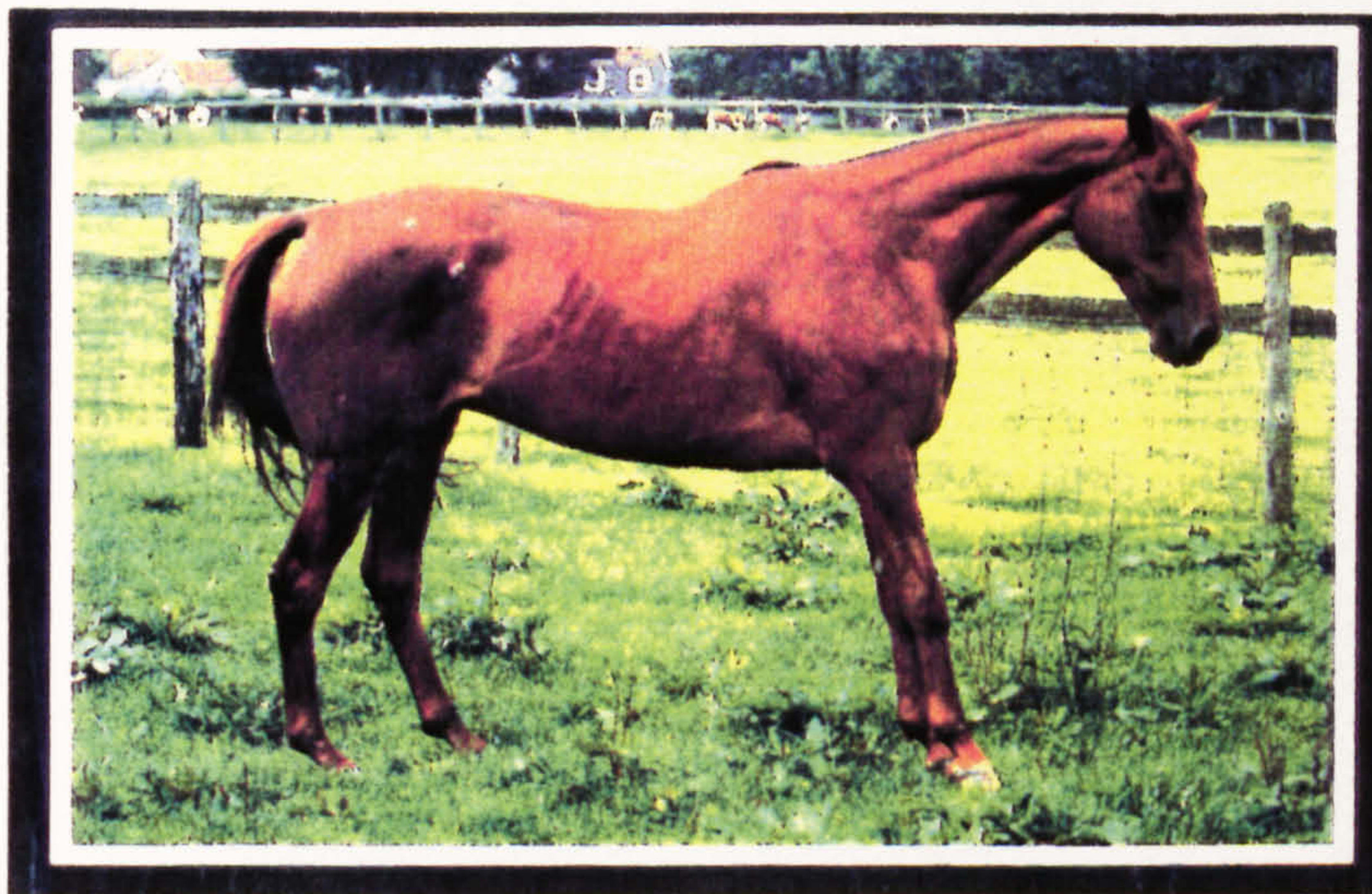


Figure 1.17 A horse with acute laminitis showing the typical stance (Eustace, 1992).

Usually all four feet are affected but there are cases where only the front feet are affected, or most often after surgery, when only the support limb shows laminitis. As the horse's bodyweight is carried on the heels of the feet, animals have a characteristic gait where the heels are placed on the ground before the toes. The characteristic lameness was classified into Grades I - IV by Obel (1948) and these grades are still widely used (Table 1).

Table 1.
Grades of Lameness during Laminitis (Obel, 1948)

Grade I	horse lifts feet incessantly, no lameness evident in walk but trot is a shortened or stilted gait.
Grade II	horse is willing to move but with characteristic gait.
Grade III	horse reluctant to move and will not lift forefeet.
Grade IV	horse moves only when forced

The Obel Grading, although useful is inherently imprecise and some authors (Owens *et al.*, 1995), including the present, have subdivided the scale into 0.5, 1, 1.5 and so on to describe the slight changes in lameness existing between the grades. Other methods of assessment include percentage 'standing time' (recorded by 24 hour video cameras) and a novel weight bearing balance which records the amount of weight borne by each foot and the number of times that the weight is shifted - a characteristic sign of the disease (Hood, 1995). Clearly, the clinical definition of lameness in relation to laminitis requires detailed assessment possibly using novel methods; this subject is addressed in Chapter 4. Anatomical changes may occur with time following an acute attack; these may be both severe and rapid. Refractory laminitis is the static stage between acute and chronic phases.

Chronic laminitis is the stage after an episode of acute laminitis in which secondary mechanical changes take place within the hoof following ischaemia and subsequent necrosis of the tissues within the laminar corium. The chronic phase may last weeks, months or years; in severe cases, a horse can be a chronic laminitic for the remainder of its life. Chronic laminitis has been classified by a clinical scoring (CS) system (Hood, 1995):

CS 1 is a horse that has returned to full athletic function after acute laminitis;

CS 2 able to perform limited athletic work but not to its previous standard;

CS 3 not able to partake in ridden work but is able to be used for breeding purposes;

CS 4 requiring constant analgesics to maintain ordinary functions;

CS 5 euthanised.

Although moderate laminitis is an easy diagnosis to make, mild and severe cases are less straightforward. Mild laminitis may be differentially diagnosed as pedal osteitis, infection, trauma to the sole, 'corns' or other lameness proximal to the hoof. The bounding digital pulse may also be present in some of these conditions. Severe laminitis can be an equally difficult and more demanding condition as severe laminitis is both dramatic and pitiful. There are few clinical signs to differentiate a horse that lies recumbent, sweating and groaning from laminitis from some types of colic. Many horses are referred to veterinary hospitals for surgical intervention of strangulating colic only to be rediagnosed as laminitis (J.G. Lane, personal communication).

Clearly, from both theoretical and diagnostic points of view a scheme for differential diagnosis of laminitis is desirable. Firstly, a chart of symptoms would assist those investigations of clinicians, especially those not specialising in equine work. Secondly, the non-invasive measurement of blood pressure under field conditions could help identify mild cases correctly and assess treatment. Thirdly, as Hunt (1991) points out "there are currently no predictive indices available to determine whether or not a horse will develop laminitis after a particular insult". Ideally, a laboratory diagnostic test would indicate whether a horse is developing the disease at a stage where good management could prevent the onset of the acute stage or confirm the diagnosis of laminitis; such a test is not available at present .

This thesis in part addresses some of these problems. A diagnostic chart is designed. The non-invasive measurement of blood pressures of normal and laminitic horses is investigated in Chapter 3. Possible laboratory tests as predictive indices for assessing predisposition to the disease are outlined in Chapters 9 and 10.

Acute laminitis must be treated as an emergency both to stabilise the condition as well as for humane reasons (Yelle, 1986; Colles, 1977). Unfortunately, most treatments are palliative at best and at worst quite empirical and ineffective. A thorough assessment of many clinical cases reveals that no therapy is capable of reversing the course of the disease, or even stabilising it. To quote Dr. D. Byars "When you look in a trunk of a veterinarian's car and there are 10 different products for one disease, you know no one product works that well" (Biles 1990). In fact, many of the therapeutic approaches currently used by veterinary clinicians are similar to those 70 -100 years ago but, as one clinician notes, "not because of their success " (Colles, 1991a). Recommendations of latter day veterinarians include : reduction of blood pressure by bleeding, slowing the heart rate by administration of aconite, together with surgery to the hoof, special shoes, laxatives, and hot or cold fomentations. Although many pharmacological agents are to hand, in principle very little has changed.

The aims of treatment are to:

correct primary illness and/ or remove causative agent(s);

block pain;

improve digital blood flow;

prevent rotation;

promote hoof growth (Baxter, 1992).

Treatment of acute laminitis thus encompasses pharmacological, surgical and management approaches.

Pharmacological approaches to the treatment of laminitis have been described at length (Colles and Jeffcot, 1977; Stashak, 1987; Baxter, 1992 ; Stick, 1987). Therapeutics commonly used by veterinary surgeons are listed in Chapter 2. The strategies concentrate on the relief of pain, principally by non-steroidal anti-inflammatory drugs (NSAIDS) such as phenylbutazone (PBZ). There are several agents in this category notably meclofenamic acid and flunixin meglumine. The latter is also a cyclo-oxygenase inhibitor and in quantities of 1.1mg/kg produces an 'anti-endotoxic effect' (Templeton *et al.*, 1985) to reduce plasma concentrations of thromboxane, PGF 1α , prostacyclin and other prostanoids synthesised by cyclo-oxygenase from arachidonic acid. Other more recent effective analgesics include carprofen (Owens *et al.*, 1995) but the use of opiate derivatives is unusual mostly because of their short duration of action and because other remedies produce more effective analgesia for this particular type of pain. Butorphanol is rarely used for the same reasons. Microthombi have been implicated in the disease (Weiss *et al.*, 1994) and large doses of aspirin (5 -20 mg/kg) are suggested for their analgesic actions, anti-platelet aggregation actions and their ability to reduce thromboxane synthesis.

Other remedies are aimed at sedation and/or reduction of blood pressure. The reduction of blood pressure by bleeding was described by MacGillivray (1883) and has only been discontinued recently (Colles, 1991a). One very commonly used drug is acepromazine,

an α adrenergic blocker, which is an effective sedative agent and lowers blood pressure by inhibiting sympathetic vasoconstriction (Purohit *et al.*, 1981). Anecdotally, Isoxsuprine, which enhances vasodilatation has also been used effectively especially in those horses that are profoundly sedated by even the smallest amount of acepromazine; the effect is however short-lived and if the drug is discontinued the effect is rapidly reversed. The mode of action is uncertain and is not reproducible *in vitro* on isolated digital veins and arteries (Elliot and Soydon, 1995). Heparin has been used in veterinary hospitals with success (Belknap and Moore, 1989). In an evaluation of heparin for prophylaxis in equine laminitis, a reduction in the incidence of acute laminitis after surgery of 71 cases (1980 - 1986) was noted but may be difficult to use in field conditions because some animals potentially may go into shock. DMSO (0.1g/kg) may also be useful because of its anti inflammatory action, free radical scavenging properties and vasodilatory properties (Baxter, 1992) but its efficacy is uncertain and proprietary brands usually contain dexamethasone.

Today, the use of exogenous corticosteroids and antihistamines are contraindicated because of adverse effects and the precipitation of laminitis (Stashak, 1987; Eustace, 1992). Other pharmacological approaches include the use of β -adrenergic blockade (propranolol) to lower heart rate (Hood, 1979) but this is not used in practice and the overall effect was less effective than PBZ treatment. Topical nerve 'blocks' to the digital nerves lowers blood pressure and heart rate (Hood, 1979). The total abolition of pain and lameness is contraindicated since increased mobility and weight bearing damages the structures within the feet. Antibiotics have been recommended (Colles and Jeffcott, 1977) but there seems to be no real evidence to support this recommendation, except in laminitis of post parturient aetiology; even here a more effective treatment is iv oxytocin and intra-uterine irrigation / antibiotics to remove the source of endotoxins.

Surgical approaches range from amputation of the limb and fitting with prostheses (R. Redden, B. Payne, personal communication) to corrective trimming of the horn to reshape and balance the foot. Surgical approaches aim to relieve fluid exudate and pressure within the hoof capsule by drilling holes in the dorsal hoof wall; some practitioners remove part or all of the dorsal wall, known as a dorsal wall resection (DWR) (Eustace, 1992; Peremans *et al.*, 1991). Surgery can be performed on the tendons likely to cause rotation. Tenotomy of the deep digital flexor tendon (DDFT) and check ligaments is performed as a last resort (Eustace, 1992; Baxter, 1992). Amputation of the front limbs of acute cases and the fitting of artificial limbs has been attempted in Thoroughbreds in the USA by Ric Redden (personal communication) but with equivocal results (B. Payne, personal communication). In this country, amputation, without the provision of artificial limbs, resulted in the clinician concerned being prosecuted for cruelty. The practise of 'grooving' (cutting of channels from coronary band to sole so the sensitive laminae are visualised) had a similar result (and did not benefit the horse). It has been suggested that forced recumbency is beneficial; this is done by putting acutely laminitic animals in a box with a very low ceiling so that they are unable to stand upright (Wattle *et al.*, 1995) but it is unlikely that many horses would succumb with any alacrity; this bizarre 'treatment' published in 1995 surely exemplifies the state of the art (or rather lack of) in contemporary therapies !

Special shoeing has returned to 'fashion'; originally described by Dollar and Wheatley (1897) and more recently by Chapman and Platt (1984), it was reassessed by Eustace and Caldwell (1989). Special shoeing during the acute stage is controversial (Colles and Ware, 1995) but is usually beneficial in chronic stages where support of P3 is advised because of secondary anatomical changes resulting from the acute attack. Secondary changes usually characterise the chronic stage but may occur in the acute stage if the episode is very severe.

Physiological changes associated with morphological alterations to the hooves during and after laminitis are described in early farriery and veterinary textbooks (Dollar and Wheatley, 1897; Magner, 1902) and are an obvious feature of the disease. In particular, the chronic stage of the disease which continues for months, or years, after the original episode, is described in old and modern texts alike (Magner, 1902; Stashak, 1987; Pollitt, 1995).

Gross morphological changes occur when the integrity of the laminar tissues including oedema, necrosis and / or sepsis; mechanical forces tear the laminar tissue as the distal phalanx, now unopposed, is pulled (rotated) in a palmar direction by the deep digital flexor tendon (DDFT) (Eustace, 1992). There is then rotation during the acute phase, when it is more common (but less obvious on examination) than in the chronic stage. The hoof is then deformed with unusual growth. In chronic stages, mechanical forces distort the horn tubules and alter the direction of horn growth (Eustace, 1992); this usually occurs in the dorsal aspect of the hoof at the coronary band. If left untrimmed this abnormal growth leads to bizarre hoof growth (Figure 1.18).

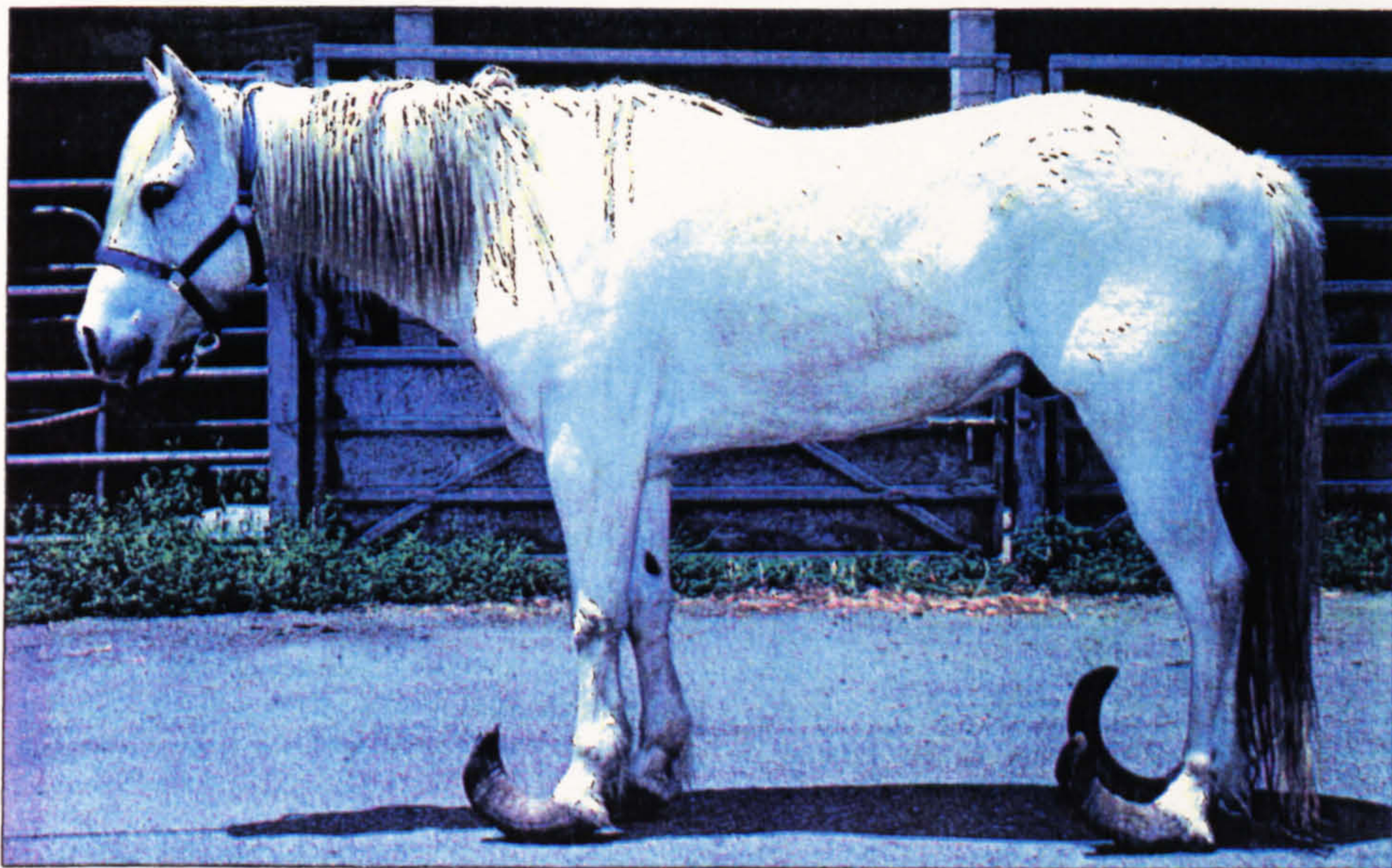


Figure 1.18 Severe chronic laminitis left untreated for 18 months showing gross morphological changes in hoof growth (Pollitt, 1995).

Since the circulation of the hoof is physically restricted by rotation of the distal phalanx (P3), the hoof grows less quickly at the toe than at the heel. In addition, abnormal hoof growth creates pressure on the coronary band which restricts growth still further. In chronic cases; divergent rings - wider at the heels and narrow at the toes are seen (Eustace, 1992). This abnormal arrangement of growth and alignment continues until the hooves are grossly deformed and it is extremely difficult without considerable experience and/or radiographs, to predict the alignment of P3 and so commence remedial trimming or farriery. As a general guide, the dorsal face of P3 is aligned with new horn growth at the coronary band on the dorsal aspect of the hoof wall. However, corrective trimming or remedial shoeing is best done with the aid of recent reference radiography. Figure 1. 19 compares the gross morphology of a normal foot with those cases of chronic laminitis.

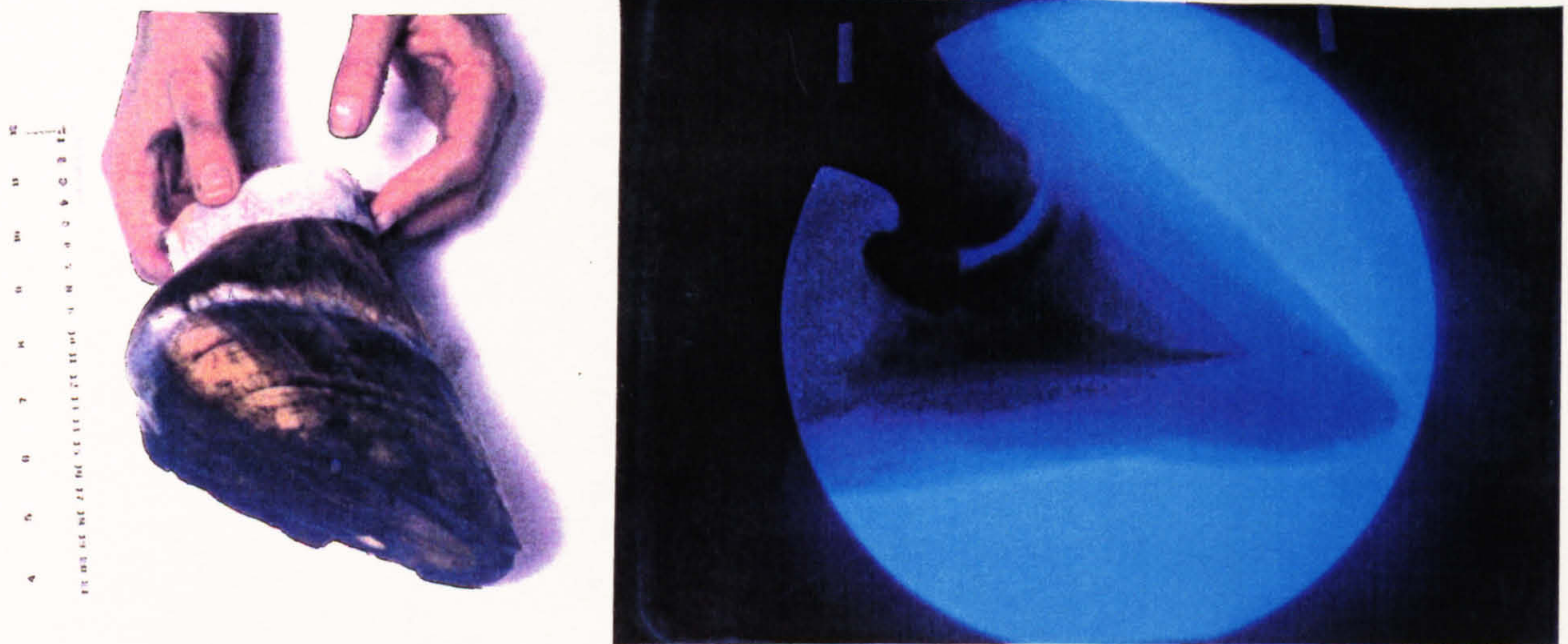


Figure 1.19 (a) Comparison of the gross morphology of a normal equine hoof with a medial - lateral radiograph; the dorsal wall of P3 is aligned with the hoof wall.

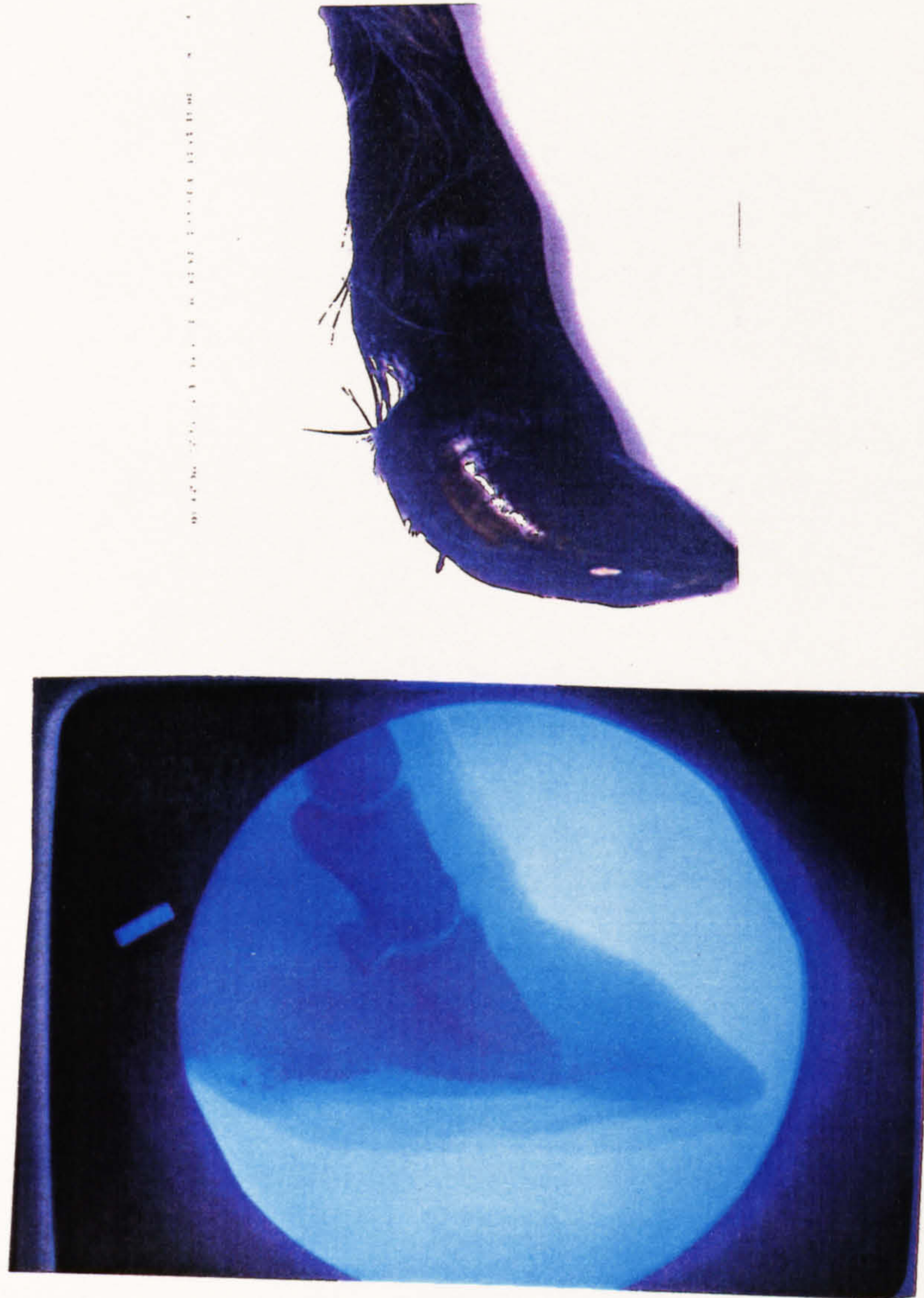


Figure 1.19 (b) Comparison of the gross morphology of a chronically laminitic equine hoof with a medial - lateral radiograph; the dorsal wall of P3 is not aligned with the hoof wall.

Such changes occurring during acute laminitis are quite difficult to determine without radiographs although depressions palpable at the coronary band are indicative of movement of P3 within the hoof capsule. Depression behind the dorsal wall coronary band indicate rotation while depression all around the coronary band reflects sinking of the bony column throughout the hoof capsule; this can occur at acute or chronic stages (Eustace, 1992). Often, the hair on the coronary band stands up rather than lying flat as the limb sinks through the foot (Figure 1.20)

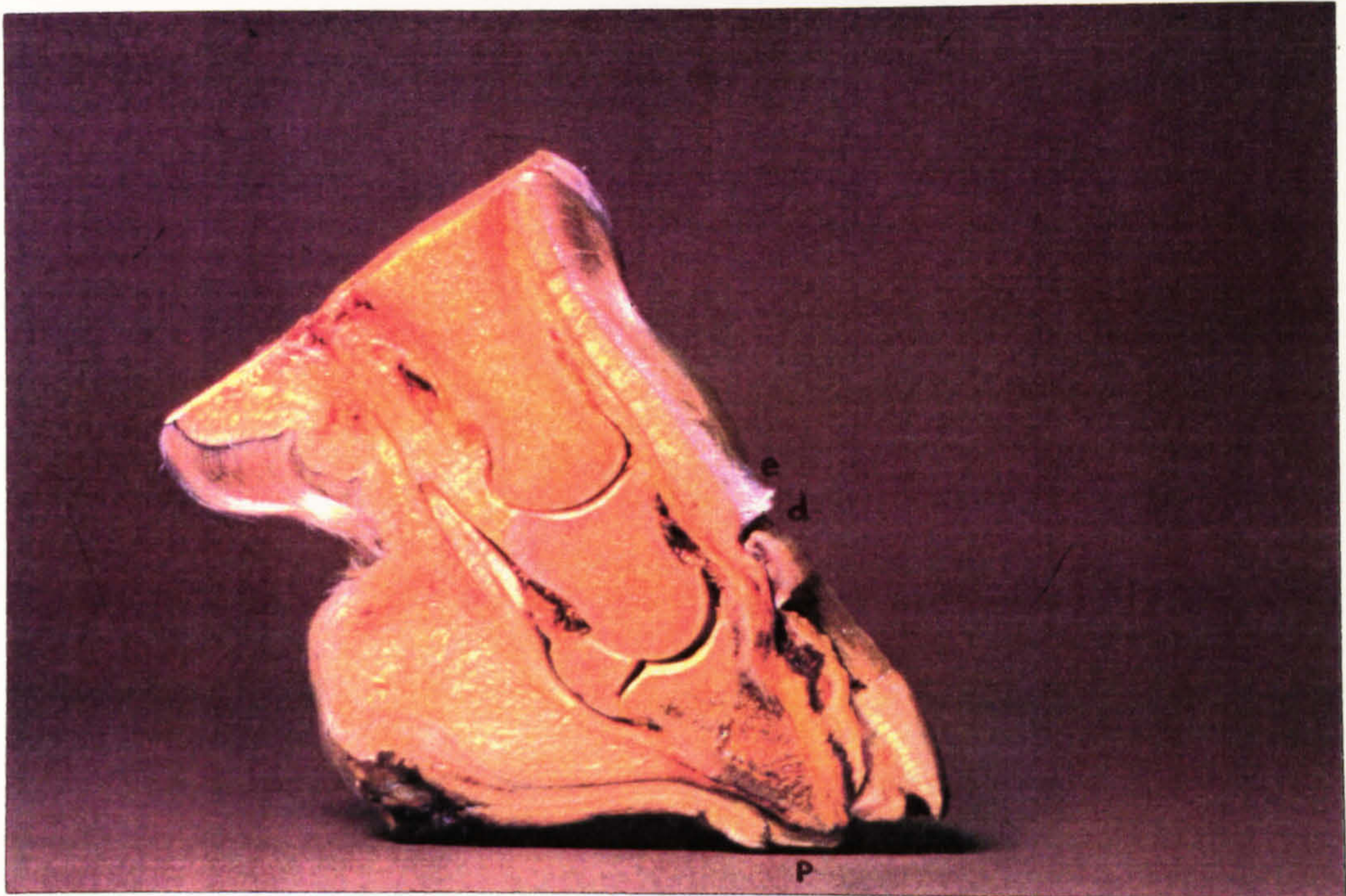
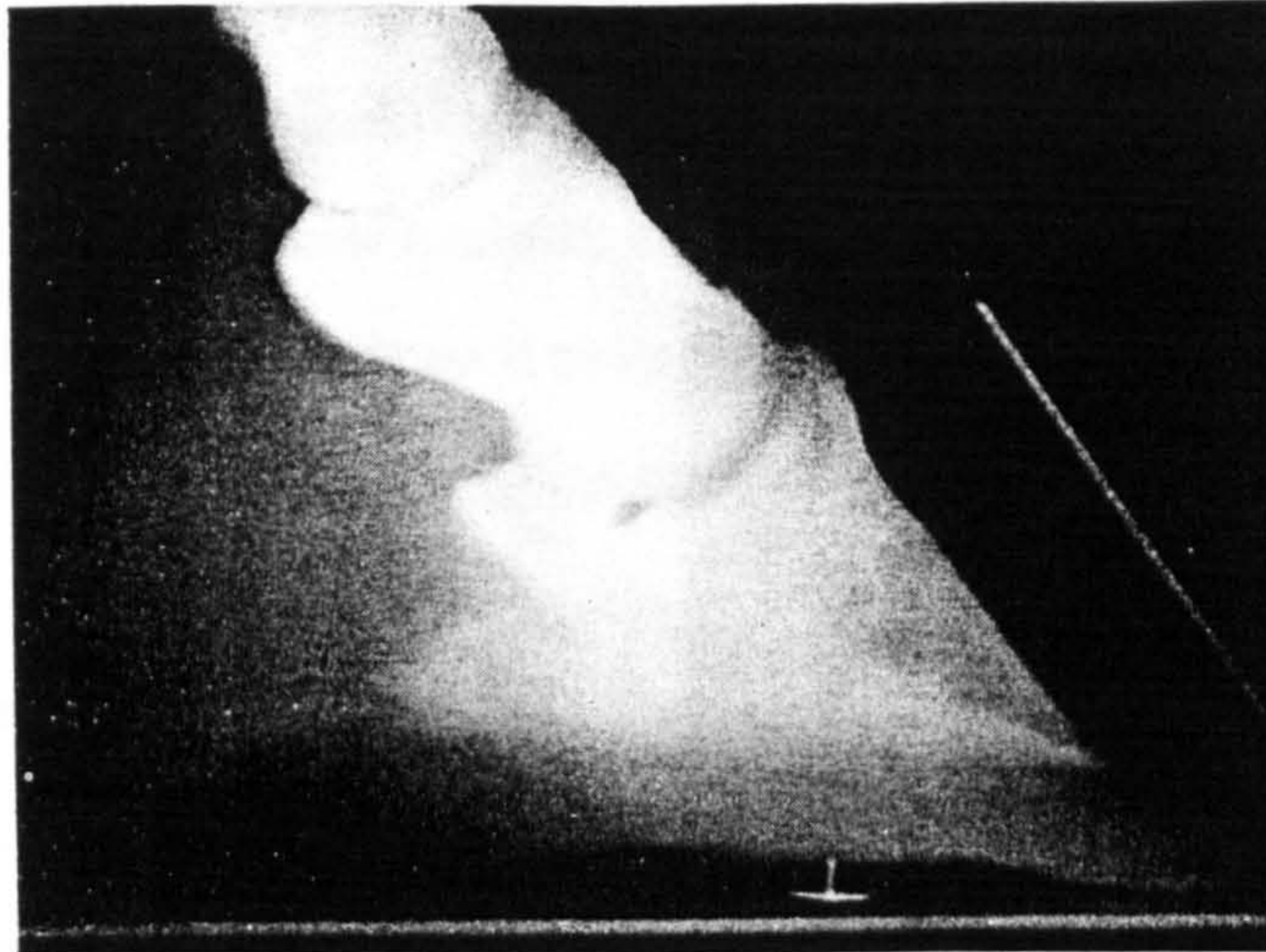


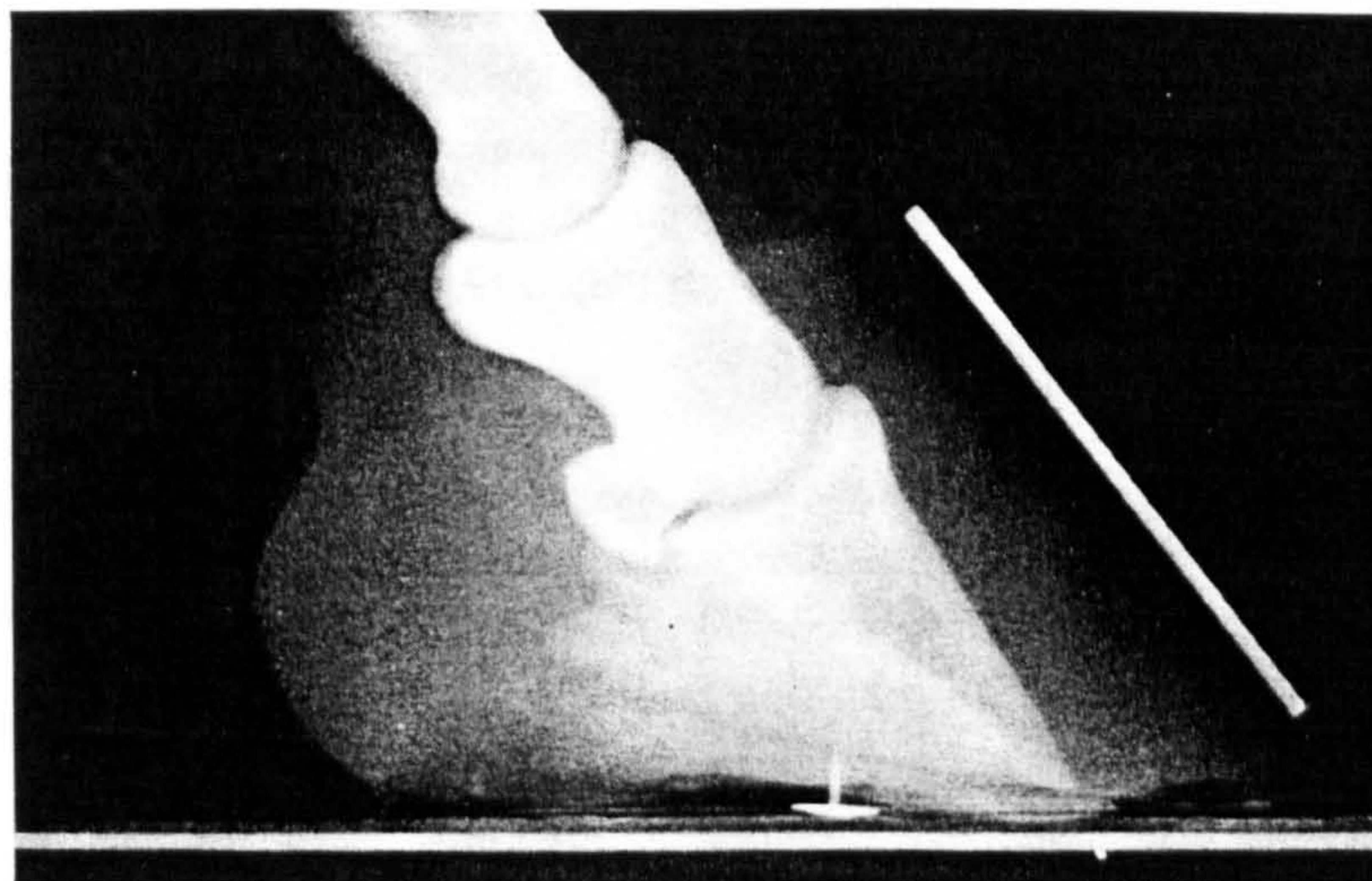
Figure 1.20 Saggital section of the hoof of a clinical case of acute laminitis showing sinking of the bony column through the hoof capsule and P3 prolapsed through the sole (p). Elevation of the hair (e) at the coronary band is visible and a depression (d) above the coronary band is apparent.

Radiography therefore defines the changes within the foot and is the basis for surgical treatment, farriery or the decision of euthanasia. Figure 1. 21 compares medial -lateral radiographs of the left digit of i) a normal horse, ii) a case of chronic laminitis and iii) a case of sinking 6 weeks after acute laminitis. If the top of the marker pin, which is taped onto the dorsal wall of the hoof, is well above the most proximal face of the pedal bone then the horse is 'sinking' and the prognosis is very poor. Radiographs can distinguish between rotation of P3 , which can be treated surgically by dorsal wall resection and /or support shoes, and 'sinking' which cannot be treated. Humane destruction is the invariable outcome when sinking is diagnosed.



X-ray of a normal foot. The phalanges are in a straight line, the wire marker is parallel to the front of the pedal bone, the top of the wire is in a normal position just above the top of the pedal bone and the pedal bone is at a normal angle to the ground.

Figure 1.21a Medial -lateral radiographs of the left digit of i) a normal horse with coronary band marker (Eustace, 1992) compared with Figure 1.21b



X-ray of a foot of a sinker. The founder distance (between the top of the pedal bone and the top of the wire,) is greatly increased from normal

Figure 1. 21b Medial -lateral radiographs of the left digit of ii) a case of sinking after acute laminitis (Eustace, 1992). There is a marker on the dorsal wall of the hoof, the top of which is level with the coronary band and the drawing pin marking the tip of the frog

Management of acute and chronic laminitis

The proper management of laminitis presents a challenge to horse owners and clinicians alike. First, the cause(s) of acute laminitis must be addressed and removed. For example, the horse should not remain at grass if acute laminitis is grass induced; retained placental membranes removed and uterine irrigation instigated if endometritis is implicated.

a) General care of the hooves. Affected animals should be given a very deep bed to provide support for their feet and to alleviate pain; this will also encourage them to lie down, minimising the weightbearing load on the hooves. Ideally, river sand should be used to a depth of 1 - 2 feet but peat and shavings are also effective and can be covered with straw. A deep bed provides better support than shoes. Hoof support can be provided by glue on shoes but in acute stages it is very difficult to lift a foot and impossible to nail shoes on. Shoes are not advised during acute laminitis since they aggravate pain. Rubber frog supports ('Lily pads') and rolled bandage support are ineffective since, if the horse is in severe pain, it will inevitably lie down. This author's experience is that rolled bandages and 'Lily pads' are rarely properly applied and if incorrectly placed will restrict an already compromised circulation. Acute laminitis should not be exercised unless perhaps swimming is possible locally; this has not been assessed but intuitively may benefit circulation and healing. If laminitis in acute or refractory stages have to be moved, they should be transported 'door to door' and not have to walk any distance. It is essential to encourage such animals to maintain an optimistic outlook; grooming and massage are appreciated and are beneficial to the circulation.

Chronic laminitic equines require regular trimming of their feet, at least every 4 weeks. Support shoes may be necessary. Heart bar shoes provide invaluable support (Eustace and Caldwell, 1989; Eustace, 1992) but must be applied by an expert farrier as incorrectly fitted they produce extreme pain. Correct balancing of the feet by trimming is essential, especially if shoes are to be fitted. Trimming and balancing ensures the correct

alignment of P3 and the weight bearing surface allowing proper circulation and regrowth of horn. Radiographs are of great assistance and an expert farrier is essential.

b) Nursing. Management at the acute stage can be equine intensive care nursing. Recumbent laminitics will require turning every 90 minutes, night and day to prevent respiratory complications and to minimise decubitus sores. Pressure sores can be treated topically and dressed if severe. Urine should be washed regularly from recumbent animals as it irritates the skin. If the soles of the hooves have prolapsed, or if the pedal bones are exposed, the feet should be bathed gently in tepid normal saline if clean, or dilute 'Hibiscrub ' or 'Pevidine' solution if dirty or septic, remembering that exposed tissues are sensitive. Many horses enjoy 'hot soaks' and will stand in buckets of wash solutions ! Abscesses should be irrigated with dilute sodium peroxide as infecting organisms are invariably anaerobes and treated topically with metronidazole (Torgyl, RMB Animal Health, Dagenham; Borgal Hoechst, Milton Keynes, Bucks). Systemic treatment with antibiotics is not recommended as the agents are unlikely to reach the areas of need. Chronic cases often have 'under -run 'soles which can be carefully removed by clinician or farrier. If it is unwise to remove the sole then it can be irrigated with solutions - a cat urinary catheter is a very useful tool. Soft horn can be dressed with eucalyptus Oil BP and iodine solution which is both antiseptic and a hardener. It should not be applied to sensitive tissue during the healing processes. After treatment, feet should be dressed with non-stick gauze dressing and gamgee tissue. The dressing is held in place with adhesive elastic tape (Treatplast, Animalcare, York) surrounding the whole hoof. Rubber adhesive tape (Vetwrap) should never be used in these cases as the foot sweats and softens beneath the dressing. Water should be brought to the horse and offered frequently to prevent dehydration; this is especially important if the animals is receiving NSAIDS which may cause renal dysfunction (Higgins and Wright, 1995).

c) *Feeding.* Animals should be fed hay alone and if considered necessary a balanced compound feed can be provided. Horse and pony 'cubes' are a high fibre compound that provides trace elements and vitamins. Dried alfalfa is a good choice too as it contains chelated calcium but is low in carbohydrates. Laxatives used to be recommended but there is little advantage in forced administration of mineral oil by nasogastric intubation as this will further upset an already disturbed digestion. Bran, sometimes recommended for its laxative properties, should be avoided by all horses but particularly laminitics as it contains high levels of phosphorous and very low quantities of calcium, usually required for laminitic patients (Colles, 1991b). The provision of salt is controversial; some recommend it (Colles, 1991b) whereas others suggest it be restricted (Stashak, 1987). Supplements containing calcium, sodium, and other electrolytes help replace those electrolytes that are deficient or lost during acute stage. Dietary methionine, zinc and biotin assist hoof growth (Larson *et al.*, 1956; Eustace, 1992; Reilly, 1995). Obese animals should have a diet that gradually reduces weight but they should not be starved which causes hyperlipidaemia, ketosis and does not help the morale of an already sick animal. Animals losing weight after *post partum* or surgical laminitis can be offered freshly cut grass, 3 times a day, but obviously this should not be offered to ponies with a grass induced attack.

1.5 Aetiology and pathogenesis

The possible aetiologies involved in the pathogenesis of equine laminitis have been reviewed by Hunt (1991) and Hood (1993). Unfortunately, the pathogenesis of the disease is both complex and multifactorial. Laminitis can be initiated by a number of seemingly unrelated factors, such as infection or iatrogenic effects of corticosteroids (Stashak, 1987). More usually, there also seems to be a strong association between dietary habit and the onset of the disease. Anecdotal evidence suggests that excessive intakes of rich grass, high protein or carbohydrate are principle causes of the laminitis, especially amongst ponies (Garner *et al.*, 1975; Colles, 1977). Carbohydrate overload has been used to induce the disease experimentally ('Special Laminitis Diet' Theracon Inc., Kansas, USA) or accidentally when an animal gains unauthorised access to the feed shed.

Post parturient complications often cause laminitis, especially in large mares, and the condition is always serious. Complications include retained placenta, uterine prolapse, vaginal tears, uterine infection after assisted foaling etc. Other causative factors include trauma, post surgical aetiology (especially after abdominal surgery), stress and allergic reactions. Little is known of the most proximate aetiology of the condition that eventually leads to compromised blood flow in the feet (Colles and Jeffcott, 1977). Whatever the cause, the mechanisms precipitating the disease are in principle, undefined.

It is agreed that vascular disruption accompanies the acute stage of laminitis, even in developmental phase but exactly how blood flow within the foot is altered is subject to debate. Therefore, arguments arise because experimental methods used to assess pedal haemodynamics are either invasive or semi invasive; these are likely to introduce artefacts.

Early reports described increased blood flow throughout the major digital vessels (Coffman *et al.*, 1970; Robinson *et al.*, 1976; Robinson, 1990). However, when regional blood flow was assessed by angiography, areas of hypoperfusion were evident (Coffman *et al.*, 1970; Hood *et al.*, 1978). These findings were not necessarily contradictory and were explained by blood preferentially flowing through arteriovenous anastomoses (AVA) (Hood, 1979; Hood and Stephens, 1981) causing regions of ischaemia. Real contradictory evidence appeared when Trout, (1990) evaluated blood flow during developmental and acute laminitis by scintigraphy using tc99 radiolabelled albumin. Trout's work suggested that total blood flow and transit time increased but there were no areas of ischaemia. However, both Trout (1990) and Robinson (1990) suggested that these results should be treated with caution. The size of the macromolecules are 10 - 40 μm and the size of the capillaries and AVA are the same diameter so there is the distinct possibility that the molecules lodged in the vessels at an early stage and mask ischaemic regions. Similarly, Trout could have missed transient ischaemia as the measurements were only taken every 24 hours (Robinson, 1990). It is clear that a truly non-invasive method of determination of pedal haemodynamics would be ideal and would obviate or at least minimise the risk of artefacts. This thesis evaluates two novel non-invasive methods in Chapter 4- thermography and near infrared spectroscopy.

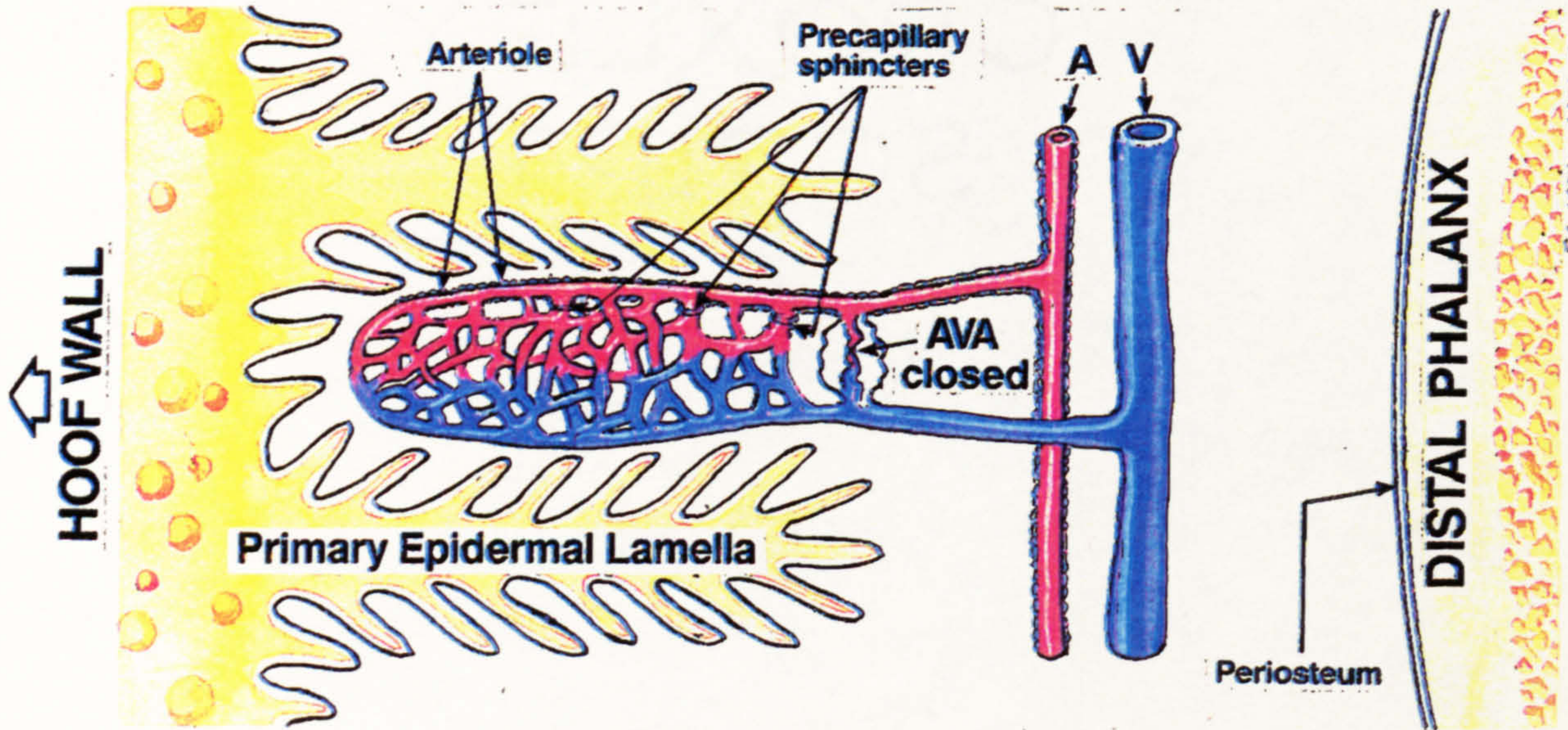
It is now generally accepted that, although total blood flow to the foot is increased, vasoconstriction occurs in the arterioles, which opens arteriovenous shunts (Hood *et al.*, 1978; Pollitt and Molyneux, 1990; Molyneux *et al.*, 1994) to result in ischaemia and eventual breakdown of dermal tissues (Coffman *et al.*, 1970; Pollitt, 1995) (Figure 1.22).

Hypotheses. Various theories are proposed to explain the vascular derangement and the breakdown of dermal and epidermal laminae (Hood, 1979; Hunt, 1991; Hood *et al.*, 1993; Molyneux *et al.*, 1994). The various theories stem in part from detailed histological studies which describe diseased and normal laminae. Histological studies on dermal and epidermal laminae were undertaken first by Obel (1948) then followed by others (Larson *et al.*, 1956; Roberts *et al.* 1980; Ekfalck *et al.*, 1985; Ekfalck *et al.*,

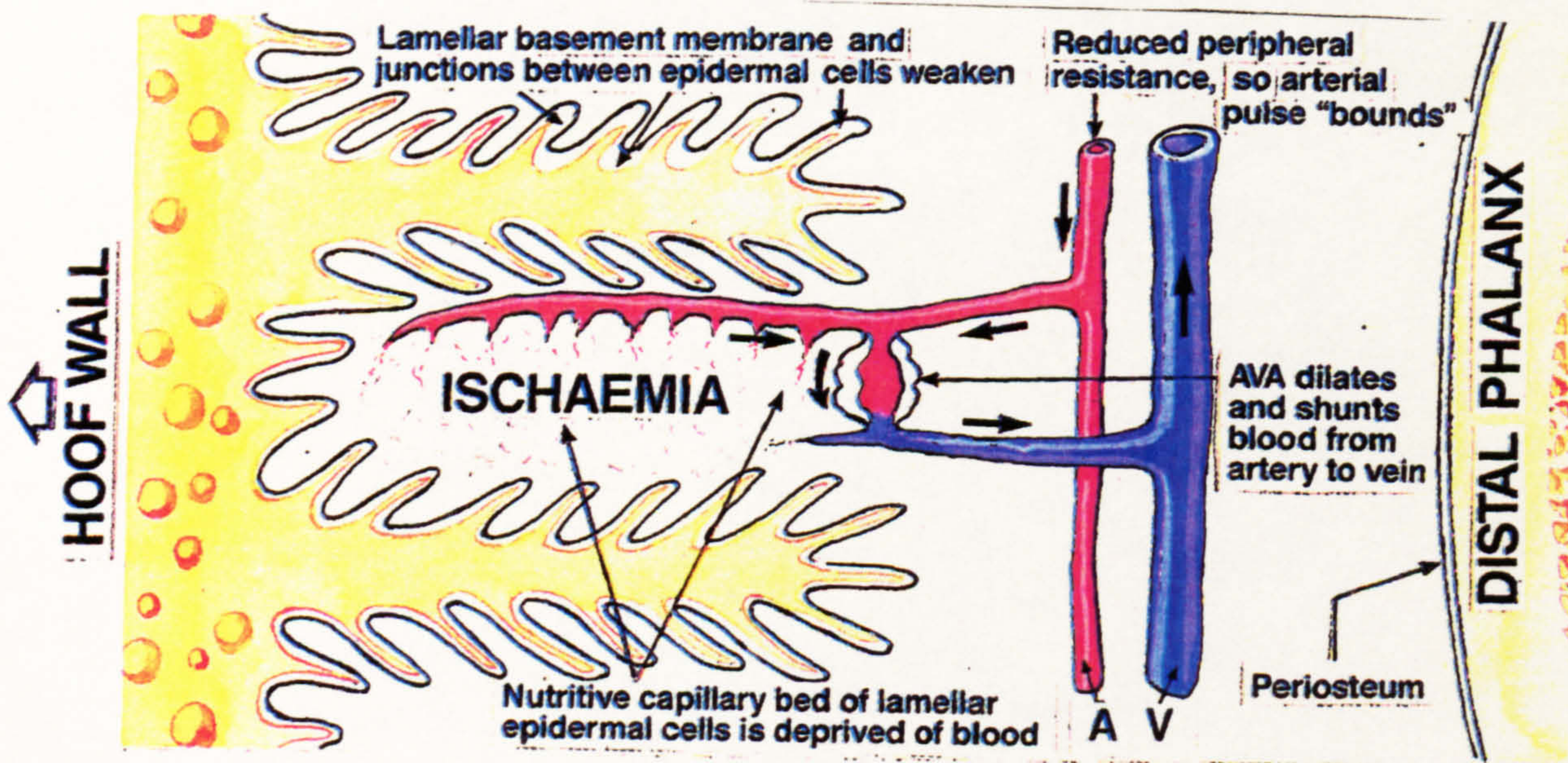
1992; Pollitt, 1995). The breakdown in dermal and epidermal connection is shown in Figure 1.22. Similar changes are evident in the closely related vascular beds of the chestnut (Flunquist, 1992). These studies are the basis for the 'metabolic' theory, that a metabolic disturbance is the initiating factor and that failure of protein synthesis in these tissues allows breakdown of the hoof (Hood *et al.*, 1993).

Other theories address the known haemodynamic disturbances within the foot to explain the pathogenesis. The 'endotoxaemia' theory suggests that endotoxins are responsible for the circulatory failure. Endotoxins (lipopolysaccharides) enter the blood stream during sepsis or digestive upset and restrict the circulation either directly or through an unknown secondary mechanism (Stashak, 1987; Hood *et al.*, 1993). However, injection of endotoxins does not necessarily cause laminitis (Fessler *et al.*, 1982). Microthrombi have been claimed to occlude small bore vessels (Weiss *et al.*, 1994). It has been suggested that vasoconstriction in digital capillaries, or compromised venous return, would inhibit arteriolar supply causing haemostasis and secondary local oedema (Robinson, 1990; Hunt, 1991). It may also be that regions of ischaemia are caused by the inappropriate opening of the AVA's by vasoactive peptides such as vasoactive intestinal peptide (VIP), calcitonin gene related peptide (CGRP) or Substance P (Molyneux *et al.*, 1994). Finally, it has been suggested that a period of pronounced vasoconstriction occurs in the arterioles within the hoof and that this is responsible for the cascade of events that so severely damage the dermal and epidermal laminae (Hood, 1993). It may be that all theories are in part correct and that all described events are implicated in the pathogenesis. Clearly, vasoactive processes are at work and some of these are the subject of this thesis.

Figure 1.22 The normal microcirculation of the dermal laminae (i) and during the ischaemia of acute laminitis (ii) (Pollitt 1995).



(i)



(ii)

1.6 Equine laminitis in the context of modern endocrinology

Endocrinology is a discipline which has grown in size and complexity since its inception some 150 years ago when Berthold conducted the first endocrinological experiments in cockerels (Hadley, 1992). At the beginning of the century, Starling introduced the word hormone (meaning to arouse or excite) and the names of the substances and the endocrinologists associated with these are now legend to include: Bayliss and Starling (secretin); Loewi (acetylcholine); Sanger (insulin); Harris (hypothalamic hormones) and Schally and Guillemin (growth hormone) (see Hadley, 1992). Endocrinology has burgeoned in terms of complexity and is recognised as being central to all physiological processes. Recently, the knowledge of peptide hormones, the advent of measurement of these hormones by radioimmunoassay and the application of molecular biology techniques has led to improved understanding of physiological regulation and homeostasis.

Several recent concepts in endocrinology (summarized by Baulieu and Kelly, 1993) may be noted in the context of understanding the pathophysiology of diseases such as equine laminitis. The classical ideas of the hormone being delivered in the blood to their target organs remain perfectly valid. Knowledge of the key events that follow this arrival are essential components, among them: interactions of the hormones with their specific receptors either on, in the cell or with the genome itself; the signals that stem from these interactions be it changes in cyclic nucleotides, inositol phosphates or genomic expression; the regulation of a receptor function by its own hormone or by other hormones (up and down regulation); the synergistic, antagonistic, agonistic, additive actions between hormones.

Advances in the understanding of the cellular and molecular biology of hormonal synthesis lay the basis for the idea that potentially hormones need not enter the blood circulation - neighbouring cells could interact with one another in a paracrine fashion, a

cell could communicate with itself in an autocrine manner and indeed, intracellular, intracrine “talk” could take place (O’ Malley, 1989; Henderson, 1990; Bern, 1990). In this area a second important concept appeared which in essence stated that “everything is made everywhere” ; all cells could potentially produce all hormones, especially peptide-like materials (Niall, 1982). In other words, it should not be surprising to find hormones being produced outside their classical sites of origin.

A whole new endocrine arena appeared when some of these locally produced hormones were classified as “growth factors” of which there are now upwards of 30 identified belonging to several “superfamilies”. It is held today that these substances not only control growth and differentiation locally but they may also have profound influences in hormonal receptor biology to affect some of the events that follow hormone-receptor binding.

The discovery that the vascular endothelium synthesised and secreted vasoactive regulators led to particularly intense investigations (see Änggård, 1990; Vane *et al.*, 1990). Local endothelial derived relaxing factor (EDRF) (Furchgott and Zawadski, 1980) and endothelium derived contracting agents (Hickey *et al.*, 1985) were discovered and later identified as nitric oxide (Palmer *et al.*, 1987; Palmer *et al.*, 1988) and endothelin (Yanagisawa *et al.*, 1988) respectively.

These observations have burgeoned investigations into the systemic, paracrine, autocrine and intracrine interactions between the vascular endothelium and other vasoactive hormones to regulate tissue haemodynamics at all levels. Many other regulators of blood pressure, plasma volume and vascular tone are involved at systemic, paracrine, autocrine and intracrine levels (Carmine and Fleming, 1990; O’ Malley, 1990; Vanhoutte *et al.*, 1993; Lüscher, 1994; Liu and Barnes, 1994).

Equine endocrinology is not a subject that has been extensively studied, except with respect to reproduction, and it is not surprising that the endocrinology of equine laminitis is poorly documented. Some endocrinological investigations have been undertaken in the past during induced laminitis *in vivo* and these have been the basis for most of the assertions made in the veterinary literature. Changes in vital signs such as blood pressure, heart rate, PCV, diarrhoea and reduced plasma electrolyte concentrations have been observed after deliberate induction of acute laminitis by intragastric carbohydrate overload (Garner *et al.*, 1975; Harkema *et al.*, 1978). Increased renin activity, plasma aldosterone and cortisol have been recorded during experimental induction of the disease (Miller, 1981; Clarke *et al.*, 1982; Hood *et al.*, 1979). Other steroids are implicated in the endocrine associations of laminitis. Plasma testosterone is elevated in all genders during acute laminitis (Miller 1981) and exogenous corticosteroids have been implicated in the pathogenesis of laminitis (Stashak, 1987; Eustace, 1992). Hood (1979) suggested that if hypertension was a secondary response to pain then digital / volar nerve blocks would largely alleviate the hypertensive response. Local application of lignocaine to nerves of the fore limbs lowered heart rate and systemic blood pressure; administration of high doses (4g / 45 min) of phenylbutazone reduced blood pressure and heart rate to near normal levels (Hood 1979). In the same study, treatment regimes of α and β blockade (with acepromazine and propranolol respectively) were responsible for some reduction of blood pressure, but not heart rate, although the effects were less profound than large doses of phenylbutazone (Hood, 1979).

These data support the hypothesis that systemic hypertension is secondary to pain mediated by the central nervous system. Despite these results, Purohit (1981) suggested that the catecholamines adrenaline and noradrenaline are primarily involved in the vasoconstriction of microvascular beds within the hoof and showed that infusion of catecholamines vasoconstricts vessels in equine limbs *in vivo*. Similarly, infusion of α and β agonists promotes vasodilatation in normal horses. The treatment of laminitis with α -adrenergic agonists such as acepromazine and β agonists like isoxsuprine is based on

these observations. Unfortunately the huge numbers of clinical cases that are killed or crippled each year bear witness to the lack of efficacy of this strategy and it is clear that these agents are incapable of prophylaxis.

Acute laminitis often follows endotoxaemia as a post operative complication, usually after strangulating infarctions or dystocia, but the mechanisms involved remain speculative. Increased levels of circulating endotoxins have been recorded during carbohydrate induced laminitis (Sprouse *et al.*, 1987). Inflammatory mediators such as arachidonic products - prostaglandins, interleukins, thromboxane A₂ are likely to be involved (Ganjam and Garner, 1981; Field and Jeffcott, 1989). Inhibition of cyclooxygenase by flunixin meglumine is a therapeutic measure suggested by Templeton *et al.*, (1985) and has been effective in reducing production of eicosanoids in endotoxic pathologies in horses (Moore *et al.*, 1986; Semrad *et al.*, 1987). Eicosanoids are not implicated in the lameness involved in chronic laminitis even after mild concussion (Owens *et al.*, 1995).

Laminitis often accompanies pituitary adenoma in ponies and the animals may be affected by diabetes mellitus and/or the equine equivalent of Cushing's syndrome. Affected animals present with hirsutism, supraorbital fat, polydipsia, polyuria and sweating, even during cold weather or when clipped. Other symptoms seen in human patients such as striations and thin skin are not easily determined in horses. Anecdotally, fat ponies are prone to laminitis; laminitic horses are indeed often obese, hyperglycaemic and hyperinsulaemic (Hintz, 1990; Freestone *et al.*, 1992). The relationship between dietary factors and endocrinology is clearly relevant to the pathogenesis of equine laminitis.

Dietary factors include ingestion of black walnut (*Julens nigra*) bark which is present as fencing or as wood shavings bedding in the U.S.A. (Stashak, 1987; Stick, 1987; Galey *et al.*, 1990). This method is used to induce laminitis without the side effects of a carbohydrate overload. The active ingredient of black walnut toxaemia is unknown but bark extracts do not cause vasoconstriction of equine digital rings *in vitro* (Galey *et al.*, 1989). Similarly, endophytes, a parasitic fungal infection of ryegrass has been associated with increased incidence of laminitis in the U.S. (Rohbach *et al.*, 1995); again the mechanisms are uncertain. Table 1.1 outlines the endocrine studies that have been undertaken on the horse in relation to laminitis -they are clearly very limited

Table 1.1 Endocrine changes in acute equine laminitis

Hormone	Change + or -, ↔	Reference
Adrenaline	+	Hood, 1979
Aldosterone	+	Clarke <i>et al.</i> , 1982
Angiotensin II	+	present study
Atrial Natriuretic Factor	+	present study
Corticosterone	+	Hood, 1979
Cortisol	+	Hood, 1979
Dehydroepiandrosterone (DHEA)	+	Hood, 1979
Eicosanoids (chronic laminitis)	↔	Owens <i>et al.</i> , 1995
Endothelin	- or ↔	present study
Insulin (chronic laminitis)	+	Hintz, 1989, Freestone <i>et al.</i> , 1992
Noradrenaline	+	Hood, 1979
Progesterone	↔	Hood, 1979
Renin Activity	+	Miller, 1981, Clarke, 1982
Testosterone	+	Hood, 1979
Thromboxane A2	+	Field and Jeffcott, 1989

Key: + increase; - decrease; ↔ no change

There have only been a few studies reflecting the paucity of information available. Histamine, oestrogen, prostacyclin, prostaglandin, vasopressin, bradykinin and thyroid hormones are among the many candidates that have not been determined in relation to equine laminitis.

Advances in the understanding of vascular endocrinology in general have led to more focused investigations *in vitro* of specific hormones that may be involved in equine laminitis. Table 1.2 outlines the vascular reactivity studies that have been done on equine digital vessels with reference to laminitis.

Table 1.2 *In vitro* reactivity studies on equine digital arteries and veins

Agent tested	Reference
α -adrenergic agonists	Elliot <i>et al.</i> , 1994
11 alpha, 9 alpha	Baxter, 1989
Acetylcholine	Baxter, 1994a; Schneider <i>et al.</i> , 1994
Adrenaline	Baxter, 1989; Baxter, 1994a
Angiotensin II	Baxter, 1989
Betamethasone	Eyre <i>et al.</i> , 1979
Bradykinin	Baxter, 1994a
Calcium channel blockade	Galey <i>et al.</i> , 1989
Catecholamines	Eyre <i>et al.</i> , 1979
Dopamine	Baxter <i>et al.</i> , 1991
Endothelin	Baxter, 1994b
Endotoxins	Schneider <i>et al.</i> , 1994; Baxter, 1994b
Histamine	Eyre <i>et al.</i> , 1979
Hydrocortisone	Eyre <i>et al.</i> , 1979; Galey <i>et al.</i> , 1989; Baxter, 1994
Ibuprofen	Elliot <i>et al.</i> , 1994
Isoxsuprine	Galey <i>et al.</i> , 1989; Elliot <i>et al.</i> , 1995
L-Arginine	Elliot <i>et al.</i> , 1994
L-NAME	Elliot <i>et al.</i> , 1994; Schneider <i>et al.</i> , 1994
Nifedipine	Galey <i>et al.</i> , 1989
Noradrenaline	Baxter, 1994a
Prazosin	Galey <i>et al.</i> , 1989
Prostaglandin F ₂ α	Baxter, 1989
Serotonin (5 HT)	Baxter, 1989; Baxter, 1994a
Tumor Necrosis Factor	Baxter, 1994a

Some limited receptor/binding studies have been undertaken on digital tissues and laminal tissue taken from normal and diseased horses. Epidermal growth factor receptors were identified in chronic laminitic ponies (Grosenbaugh *et al.*, 1991) but clearly major endocrine investigations remain to be undertaken on normal horses *in vivo* and *in vitro* as well as the study of endocrine changes in disease states.

1.7 Specific aims and objectives of this thesis

The central objective of this thesis was to further understanding of the complex haemodynamic and endocrine processes that partake in the pathogenesis of equine laminitis. In carrying out these investigations, a real secondary objective was to develop and apply therapy. Several avenues of investigation were undertaken to achieve these objectives :

A preliminary epidemiological survey was carried out to gain insight into the incidence/prevalence of laminitis in horses in the U.K.

Systemic and digital haemodynamics were assessed using novel non-invasive techniques in acute and chronic laminitis. Pedal vascular responses were related to degrees of lameness and treatment given during the acute phases of laminitis.

Based on the findings above, the possible involvement of selected vasoactive hormones was examined. Specifically, this involved the study of paracrine and systemic hormones - angiotensin II and atrial natriuretic peptide; nitric oxide and endothelin. Normal plasma levels of angiotensin II, atrial natriuretic peptide and endothelin, not been previously determined in the horse, were compared with values for different stages of the disease. The possible role of nitric oxide was assessed *in vivo* by providing substrates for nitric oxide synthesis. The effects of nitric oxide donors and their inhibitory analogues were also assessed, and their efficacy as therapeutic agents evaluated.

Serum and urinary composition of normal and laminitic horses were compared with respect to electrolytes and hippuric acid to evaluate changes in these parameters associated with laminitis.

In the final sections the findings are discussed in the context of plasma levels of vasoactive hormones measured and a testable hypothesis for the aetiology is presented.

Chapter 2. Epidemiology of equine laminitis

		Page
2.1	Introduction	55
2.2	Collection of Data	57
2.3	Results	63
2.4	Discussion	76

"A great multitude which no man could number ..."

"Let him that has understanding count the number of the beast"

Revelations 7.9 and 18

2.1 Introduction

The epidemiology of equine laminitis relies largely on apocryphal accounts and there is no definitive and comprehensive analysis available.

'Epidemiology' based on the Greek for epidemic $\nu\phi\sigma\eta\mu\alpha$ (Edwards, 1912), means 'the science of epidemics' in which an epidemic is 'a disease prevalent among a community at a special time' (Oxford English Dictionary). It is almost received knowledge, although in reality anecdotal, that laminitis has a seasonal character and is one of the most common non-infectious diseases of horses. Epidemiological data to support such assertions are singularly lacking. This thesis is concerned with laminitis and it is obviously both interesting and relevant to have insight into the incidence of the disease. Accordingly, two small preliminary epidemiological surveys were undertaken. In addition to collecting these data, anecdotal information on the observed seasonality, aetiology, current veterinary and owners' treatments, breed predisposition, and sex or age differences accrued.

It is remarkable that solid data on the equine population are lacking. There is no national register of horses and numbers of horses and ponies in Britain can only be estimated. Polls of horse riders and farmers suggest that the equine population is 550,000 (Mr Syd Emmerson, Ministry of Agriculture, personal communication 1991; Peat Marwick Survey, British Horse Society, 1988). However, unofficial estimates of the population give considerably higher figures. One pharmaceutical company estimates the population to be 2,000,000 in the British Isles (Dr Bartram, Janssens UK,

personal communication) Extrapolation of the data gained in the present survey to the general population is thus fraught with problems when considering the total incidence of laminitis.

The fates of affected horses were assessed and estimates were made of those horses making a full recovery, those permanently disabled and those destroyed. Horse owners potentially lose their horses and incur substantial bills for veterinary treatments. From a financial standpoint, laminitis is costly. The magnitude of such losses has also been estimated in this investigation.

2.2 Collection of Data

Two questionnaires were designed respectively for veterinary surgeons and members of the Irish Draught Horse Society (GB) (Figure 2.1 and 2.2) and were mailed together with a prepaid envelope for their return. Recipients were asked to answer all questions and encouraged to make comments.

The questionnaire aimed to assess the modes of treatment and the occurrence of laminitis. Firstly, veterinary clinicians and practices were targeted. The animals in the care of the members of the British Equine Veterinary Association (BEVA), an organisation of veterinary equine specialists, comprise random selections of horses and ponies of all types.

Horses registered with the Irish Draught Horse Society (GB) (IDHS) are specifically a population of horses, rather than ponies. Animals are either purebred Irish Draught or partbreds - Irish Draught crossed with Thoroughbred. This population contains little, if any, pony breeding. The population of IDHS animals consists of general purpose horses which are used for riding, showjumping, hunting and breeding.

The geographical areas of Irish Draught Horse Society returns were determined either by the address given in the comment section, or by the postmark. Areas were divided into South of England; Midlands; North of England; Scotland; Wales and Northern Ireland (Table 2.1).

Table 2.1 Division of English Counties into regions for BEVA and IDHS surveys.**South of England**

Avon	Berkshire	Buckinghamshire	Cornwall
Devon	Dorset	East Sussex	Essex
Greater London	Hampshire	Hertfordshire	Isle of Wight
Kent	Oxfordshire	Somerset	Surrey
West Sussex	Wiltshire		

Midlands

Bedfordshire.	Cambridgeshire.	Cheshire	Derbyshire
Gloucestershire	Hereford and Worcestershire,	Leicestershire	Lincolnshire
Norfolk	Northampton	Nottinghamshire	Shropshire
Staffordshire	Suffolk	Warwickshire	West Midlands

North of England

Cleveland	Cumbria	Durham	Greater Manchester
Humberside	Isle of Man	Lancashire	Merseyside
Northumberland	North Yorkshire	South Yorkshire	Tyne and Wear

Rigorous statistics have not been applied to this preliminary study since numbers surveyed, members responding and the often subjective nature of the data render conclusions essentially qualitative.

All data from this preliminary study are regarded as indicative, rather than definitive, of the epidemiology of equine laminitis.

Figure 2.1 Questionnaire sent to BEVA members

SURVEY ON THE INCIDENCE AND AETIOLOGY OF EQUINE LAMINITIS*Please answer questions 1-3 by giving the appropriate number in the box provided.*

1. Total number of horses in the care of your practice		[]
2. Number of chronic cases of laminitis in your care		[]
3. Estimated number of new laminitis cases during	1992	[]
	1993	[]

*Please answer questions 4-10 by giving the appropriate % in the box provided.***4. Of the new cases of laminitis i.e. 1992 -1993, please give the % which :-**

a) Returned to full use without treatment	[] %
b) Returned to full use with treatment	[] %
c) Suffered permanent unsoundness	[] %
d) Were destroyed because of laminitis	[] %

5. Please estimate the percentage of acute cases which are caused by :-

a) Excess grass	[] %	d) Foaling complications	[] %
b) An excess of hard feed	[] %	e) Following colic surgery	[] %
c) Pituitary adenomas	[] %		
f) Iatrogenic effect, please state which drug			[] %
g) other			[] %

6. What percentage of laminitis cases do you treat with these treatments ? :-

a) phenylbutazone	[] %	f) corticosteroids	[] %
b) flunixin meglumine	[] %	g) antibiotics	[] %
c) acetylpromazine	[] %	h) frog support / shoes	[] %
d) isoxsuprine	[] %	i) dorsal wall resection	[] %
e) antihistamines	[] %	j) feed supplement	[] %
g) other, state which			[] %

7. Please give an estimated percentage of the cases that occur in following months

:-

a) January -February	[] %	d) July - August	[] %
b) March -April	[] %	e) September - October	[] %
c) May - June	[] %	f) November - December	[] %

8. Please give an estimated percentage of the breed /types in your cases of

laminitis:

a) Heavy horse	[] %	f) Riding horse	[] %
b) Warmblood	[] %	g) Welsh Cob	[] %
c) Hunter	[] %	h) Welsh Pony	[] %
d) Thoroughbred	[] %	i) Shetland	[] %
e) Arab	[] %	j) Other native breeds	[] %

9. Please divide your cases of laminitis into the following groups :-

a) mare	[] %	c) stallion	[] %
b) gelding	[] %		[] %

10. Please divide your cases of laminitis into the following age groups :-

a) up to 4 years	[] %	c) 11 years and above	[] %
b) 5 - 10 years	[] %		[] %

COMMENTS

.....

Figure 2.2 Questionnaire sent to members of the Irish Draught Horse Society
SURVEY ON THE INCIDENCE AND CAUSES OF EQUINE LAMINITIS IN THE
IRISH DRAUGHT HORSE

SECTION A

- 1 In which county do you live ?
2. How many Irish Draught Horses (pure and Part-bred) do you have in your care??
3. Have any of the Irish Draught Horses you care for had/have laminitis ? YES/NO
4. If yes, how many ?

The following questions, 5 - 25 only apply to those who care for a laminitic horse.

5. What sex is the horse in your care ?	Mare/Gelding/stallion
6. How old was the horse when it first developed laminitis?	years
7. Has your horse had more than one attack ?	YES / NO
8. If yes, how many attacks has it had ?	
9. Did a veterinary surgeon visit ?	YES / NO
10. If yes, how many visits were made ?	
11. What was the estimated total cost of treatment ?	£
12. Was your horse destroyed because of laminitis ?	YES / NO
13 If yes, what was the value of the horse lost ?	£
14. Did laminitis cause permanent unsoundness in your horse ?	YES / NO
15. If yes, did you claim insurance for the loss of use ?	YES / NO
16. If yes, how much was claimed ?	£
17. Did laminitis cause temporary unsoundness in your horse ?	YES / NO
If yes, for how long ?days.....weeks.....months.....years

Please ask for additional forms if you have more than one horse

SECTION B Please tick the appropriate statement. You may tick more than one statement if applicable

19. Was the recovery of your horse

Complete recovery, no treatment	
Complete recovery with treatment	
Permanent lameness	
Destroyed	

20. What do you think caused the original attack ?

Excess grass	
Excess hard feed	
Side effects of drugs	
Pituitary tumour	
Foaling complications	
Other	

21. At which time of year did the disease develop?

Jan -Feb.		Aug. - Sept.	
Mar - Apr.		Oct. -Nov.	
Jun. - Jul.		Nov. - Dec.	

COMMENTS.....

.....

22. Mark the treatments used

Drugs	
Special farriery	
Change of diet	
Rest	
Exercise	
Cold hosing	
Hot tubbing	
Other	

23. Was your horse in ..

Heavy work (competitions)	
Light work	
No work	

24. Prior to laminitis management was....

Grass kept	
Stable kept	

25. After laminitis management was ...

Grass kept	
Stable kept	
Some dietary change	
Starvation diet	
Additional feed supplement	

2.3 Results

1. Responses

There was a 6.1 % return (92 replies/1500 questionnaires) of the BEVA survey and a 33.1 % return (398 replies / 1200 questionnaires) of the IDHS survey.

2. Numbers of horses in the study

Responding BEVA veterinary practices had a total of 111,643 horses in their care. The IDHS return involved 1,198 horses giving an overall total of 112,841 horses to analyse. Given that the British equine population is 550,000 (Peat Marwick survey, British Horse Society, 1988), the horses in this study represented approximately 20% of the total equine population (but see below).

3. Numbers of horses suffering acute or chronic laminitis

Equines that had an initial attack of the disease were described as 'acute'. Those that had suffered at least one attack in the past and had evidence of secondary changes in the hooves requiring special veterinary care or management were considered 'chronic' cases.

Table 2.2 Percentage of the equine population affected by either chronic or acute laminitis.

No.		Percentage of population
1,444	horses and ponies suffered <i>acute</i> laminitis in 1992	1.29%
1,678	horses and ponies suffered <i>acute</i> laminitis in 1993	1.5%
2,063	horses and ponies suffered <i>chronic</i> laminitis in 1993	1.84%
	Total percentage of the population affected by acute or chronic laminitis	3.34%

The mean number (\pm SE) of horses and ponies (in the care of BEVA practitioners) suffering *acute* laminitis each year is 1562 ± 118 . In all, a total of 3,741 horses in the care of the BEVA practices during 1993 could be described as having laminitis. This figure should be regarded as a minimum because of the low response rate of BEVA members.

4. Response of IDHS members

Returns from the IDHS members indicated that 36 horses suffered laminitis (chronic and acute) out of a total of 1198 horses surveyed, an incidence of 3%, a figure very similar to that arrived at from the BEVA practitioners survey.

5. Percentage of equine population affected by laminitis

Each year an average 1.39 % of the equine population suffer attacks of acute laminitis. This is in addition to the 1.84 % of the equine herd that have chronic laminitis. Therefore 3.2% of the equine herd suffer laminitis in either acute or chronic forms. Given that the two surveys obtained information from different sources it is of interest that both gave a 3% incidence.

6. Extrapolation of percentage incidence to the whole population

Extrapolated figures from a population of 550,000 would give figures for incidence of laminitis (acute and chronic) in the region of 16,000 animals. Acute laminitis accounts for approximately 6950 animals each year but only 16% of these acute laminitis cases join the herd of 'chronic laminitics' (*vide infra*).

If unofficial estimates of the population are correct and the equine population is much higher, then in a population of 2,000,000 horses and ponies, some 30,000 will suffer acute laminitis each year and a total of 60,000 will have the chronic form of the disease. Table 2.7

(below) shows extrapolation of such an incidence to the whole population and the financial implications.

7. Acute cases

The outcome of cases of acute laminitis seen by BEVA veterinarians is summarized in Table 2.3.

Table 2.3 Outcome of acute cases (BEVA)

<i>Year</i>	<i>1993</i>	<i>1992</i>	<i>Outcome</i>	<i>Percentage in each category</i>
<i>Numbers</i>	117	101	recovered fully without treatment	7%
	1,139	981	returned to full work after treatment	68%
	268	231	remained permanently unsound	16%
	134	115	were destroyed during acute phase	8%

The IDHS returns are summarized in Table 2.4. There is a higher proportion of fatalities than the BEVA returns. Differences in the outcome of acute cases probably reflects differences in the aetiology and in the bodyweight of the ID horses. All those that recovered had done so within 12 months, the majority within 6 months.

Table 2.4 Outcome of acute cases (IDHS)

<i>Year</i>		<i>1993</i>	<i>Outcome</i>	<i>Percentage in each category</i>
<i>Numbers</i>		9	recovered fully without treatment	25%
		12	returned to full work after treatment	33%
		4	remained permanently unsound	11%
		11	were destroyed during acute phase	30%

It is impossible to arrive at precise or even reasonable numbers for the UK since such an extrapolation requires, as noted, knowledge of the total horse population. However, extrapolation from percentages from the IDHS and BEVA returns are shown in Table 2.5. The figures for outcome have been extrapolated from the BEVA returns which are more representative of the population. Chronically lame animals may be kept for many years and contribute to the total number of chronic laminitics (*vide supra*). The numbers of chronic laminitics destroyed each year are unknown.

Table 2 .5 Epidemiology of equine laminitis, a preliminary survey 1992/1993
Incidence and outcome

The equine population has been estimated at between 550,000 and 2 million

	<u>550,000</u>	<u>2 million</u>
Number of acute cases / year	8,250	30,000
Number killed / year	660	2,400
Number permanently unsound / year	1,320	4,800
Total chronic laminitic herd	16,500	60,000

8 Treatments

The therapies applied are summarized in Table 2.6

Table 2.6

<i>Percentage of cases</i>		<i>Drug</i>
82%	of cases received	Phenylbutazone
43 %	of cases received	Acepromazine
28%	of cases received	Flunixin meglumine
12%	of cases received	Antibiotics
11%	of cases received	Other treatments (*)
6%	of cases received	Isoxsuprine
2%	of cases received	Corticosteroids
1%	of cases received	Antihistamines

(*) Other treatments include : meclofenamic acid; carprofen; heparin; warfarin; butorphanol/detomidine; pethidine; thyroxine; aspirin; liquid paraffin by nasogastric intubation; and homeopathy .

Feed supplements, especially those containing methionine and biotin were often given. Physical and surgical treatments were also given; 40% of cases received frog support and 8% of cases received dorsal wall resection. Treatments total more than 100% as horses usually receive more than one treatment. It is clear that therapies applied are for the most part rather empirical and subject to "fashion".

9. Financial aspects

Irish Draught Horse owners reported that the cost of treatment ranged between £30 and £1800 (mean £280). The value of the horses killed ranged from £850 to over £7500 (mean £3,785). Taking the mean cost of treatment from the IDHS survey at £280, and using the numbers of horses affected from the BEVA survey, treatment of acute laminitis costs clients of BEVA members around £437,360 each year. This figure does not include the loss of their horses. If these figures are extrapolated to a population of 500,000, then the annual cost of treatment is £1,960,000, again without the loss of the animals. The financial losses (excluding the costs of radiography and farriery) are summarized in Table 2.7 The average cost of a horse/pony is taken to be £1500. Treatment of chronic laminitis i.e. radiographs, remedial farriery, analgesics, supplements and management, are not included in these estimates.

Table 2.7

	Financial impact of equine laminitis	
	<u>550,000</u>	<u>2 million</u>
Cost of veterinary treatment #*	£2,310,000	£8,400,000
Cost of horses killed / year**	£990,000	£3,600,000
Cost of loss of use	£1,980,000	£7,200,000
Combined Total	£5,280,000	£19,200,000

(excluding radiography, farriery)

* Mean value for cost of treatment : £280

** Mean value of horse £1,500

10. Appraisal of causes of laminitis.

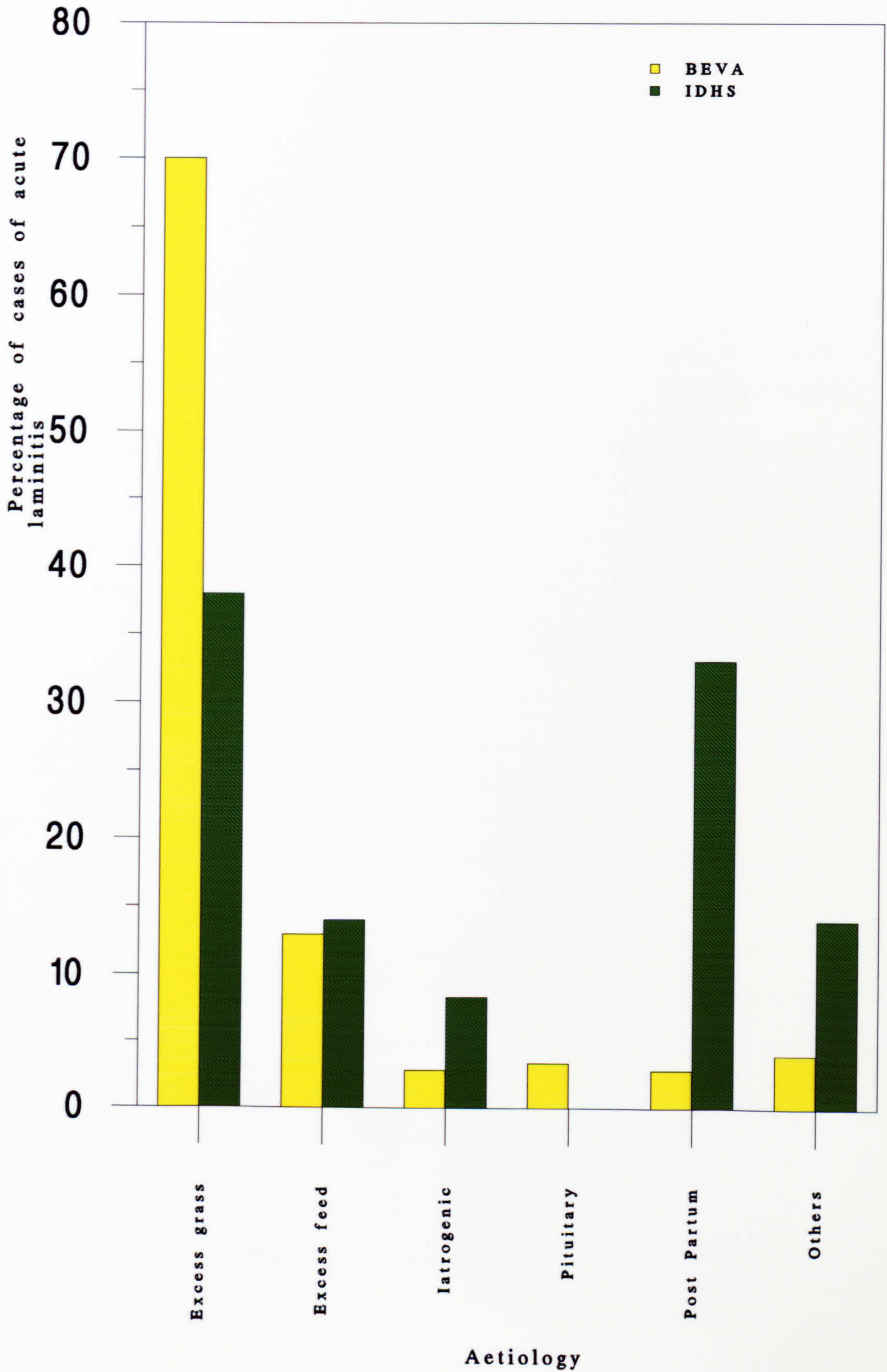
Veterinary surgeons were asked to give an opinion on the aetiology of acute laminitis and options were listed on the survey form. The options were :- excess grass; excess hard feed; pituitary adenomas; foaling complications; a sequel of colic surgery; iatrogenic effects; or other possible reasons. Ingestion of grass was considered to be the main cause of laminitis (70 % cases) but ingestion of hard feed (carbohydrate) was also considered a common causative agent (13% cases). Other causes had a much lower incidence of around 3%. Other causes not included on the questionnaire were mentioned by veterinary clinicians. These included: trauma (fast work on hard ground); bearing too much weight on one limb after a fracture of the contralateral limb; lack of exercise; septicaemia and in one case, anaphylaxis after a bee sting ! Exogenous corticosteroids, administered as treatments for other diseases such as infected dermatitis (sweet itch), were recorded as sometimes causing laminitis.

The IDHS returns indicated that ingestion of grass and foaling complications were to the fore in the aetiology of the disease (38% and 33% respectively). Iatrogenic effects were indicated in 4 of the cases (8%), 2 animals reacting to administration of steroids and another to administration of hormones for reproductive purposes and another seemingly contracting laminitis after a booster vaccination against equine influenza and tetanus. Other factors accounted for the remaining percentages; stress responses to travel were thought to be responsible for the onset of an attack and infections (systemic viral and systemic /local bacterial) were also concurrent with the onset of laminitis.

These data are summarised in Figure 2 3.

Figure 2.3 Aetiology of acute equine laminitis in the national population of horses and ponies, surveyed by the BEVA members, compared with the aetiology of acute laminitis in Irish Draught Horses, surveyed by IDHS(GB) members.

Aetiology of acute laminitis from surveys of the BEVA practitioners and IDHS members



11. Seasonality

Both veterinary surgeons and IDHS members judged May and June to be the most common time for laminitis (38 % cases and 58% cases respectively). A comparison of the results are outlined in Figure 2.4

12. Breed, Sex and Age Differences

Most veterinarians commented that the highest proportion of cases were native breeds of ponies and commented that these breeds may be more susceptible to laminitis. Fewer cases of laminitis were seen in horse breeds. The distribution of cases between the different breeds or types is shown in Figure 2.5.

Veterinary opinion considered laminitis to be evenly distributed between mares (48%) and geldings (50%) and stallions accounted for a very small proportion of cases (2%). IDHS returns showed a slightly different picture. Of the 36 cases, 30 (83%) were mares and 6 (17%) were geldings. No RID stallions had suffered laminitis but it should be noted that there are only 57 Irish Draught stallions in England !

Veterinary clinicians reported that 48% of their cases were over 11 years old, 43% were aged 5 to 10 years and only 8% were 4 years old or under. These data are similar to the results given by the IDHS - 48% of laminitis cases were aged 11 years or older; 34% were 5 - 10 years old and 18 % were 4 years or under.

Figure 2.4 Seasonality of acute equine laminitis in the national population of horses and ponies, surveyed by the BEVA members, compared with the seasonality of acute laminitis in Irish Draught Horses, surveyed by IDHS(GB) members.

Seasonality of cases of acute laminitis in Irish Draught Horses and in the national equine population

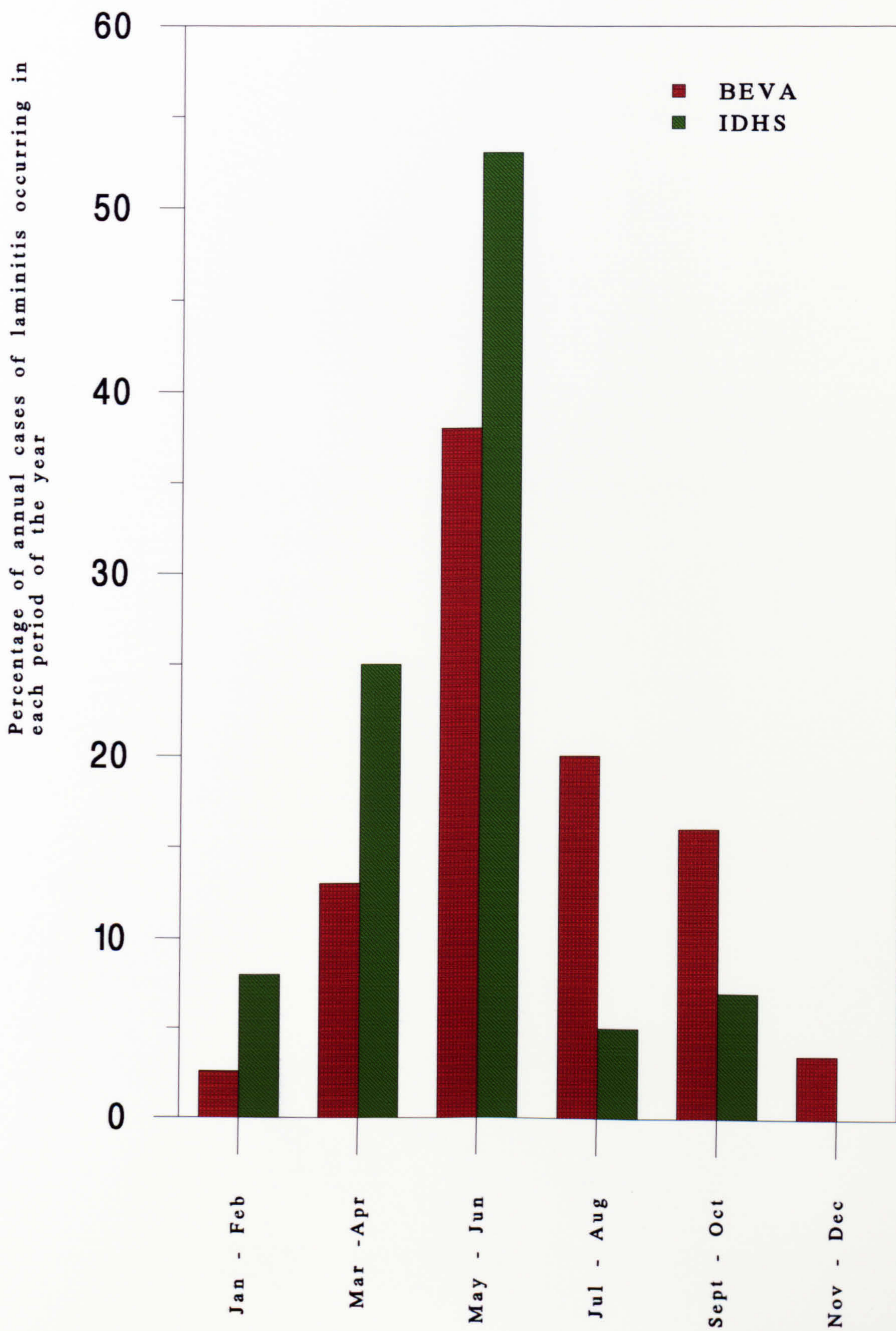
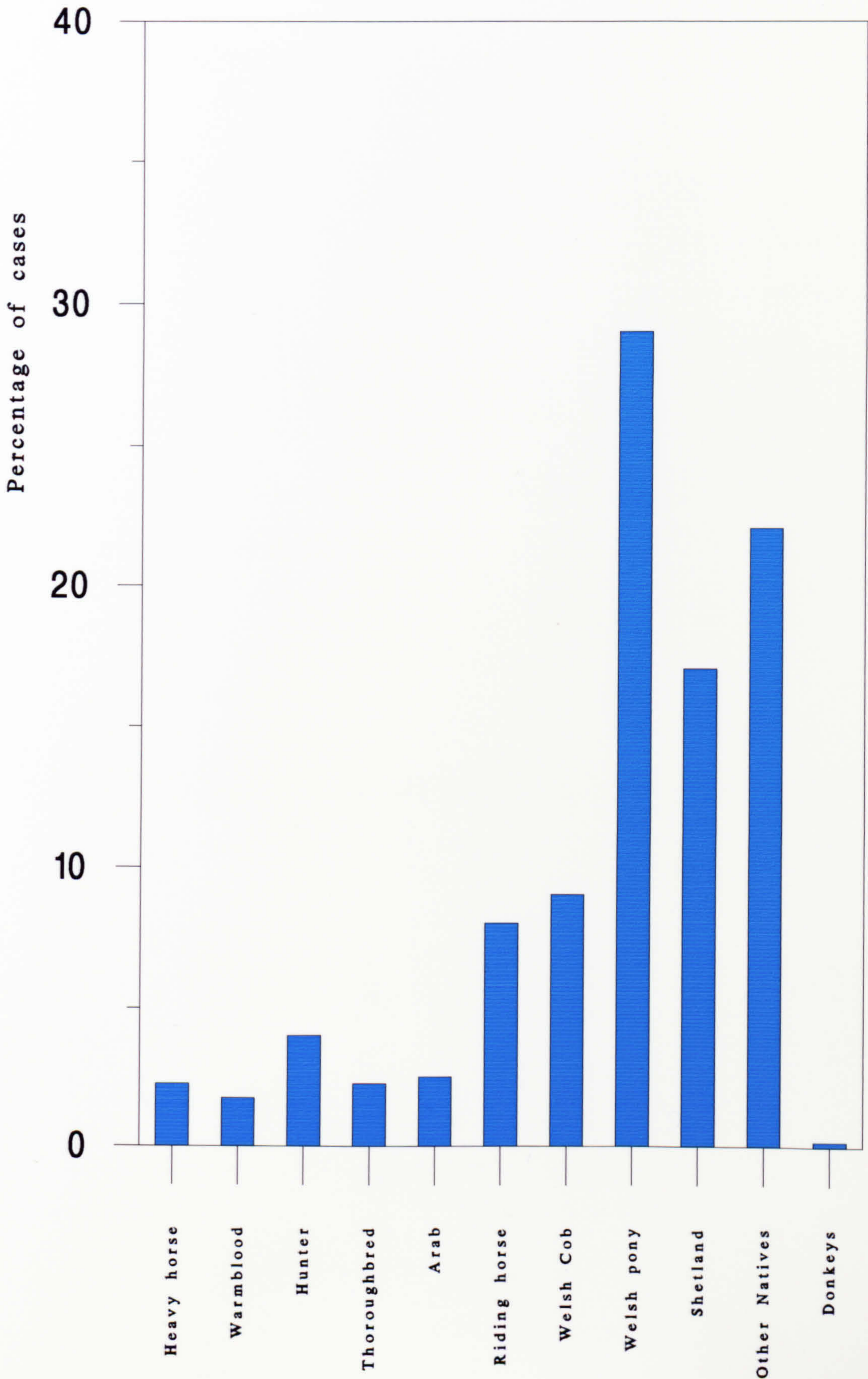


Figure 2.5 Comparison of different breeds that make up the cases of acute equine laminitis nationally, assessed by the BEVA practitioners.

The percentage of breeds/types of horses and ponies that make up the total number of acute cases of laminitis nationally (from BEVA survey) (eg. of every 100 cases, 30 were Welsh ponies).



13. Management before and after laminitis

Most of the cases of laminitis in the IDH herd (63%) occurred in horses that were not in work compared with 12 (33%) in light work and only 2 (5%) in hard work. Prior to an attack of laminitis, 61% were kept at grass, 30% were on a combined system and 9% were stabled. Management was changed after an episode of laminitis. About half of the horses that had been at grass before the onset of disease were stabled afterwards or put on a combined system of grass and stabling. Dietary changes were also implemented. Five cases were put on a weight reducing diet. Nine cases were given feed supplements usually methisal (Intervet, UK) Farrier's Formula (EFS, Wiltshire UK) or dried alfalfa (Dengi HI -Fi, UK). Similar feed supplements were advised for cases in BEVA members care.

14. Geographical Aspects

The population distribution of the Irish Draught herd, and the incidence of laminitis was distributed as shown in Table 2.8

Table 2.8. Distribution of the Irish Draught herd in the UK and the percentage affected by acute or chronic laminitis.

Area	Distribution of herd (% total)	Number of horses	Percentage affected by laminitis
South of England	22 %	275 horses	3.64 %
Midlands	39 %	477 horses	3.14 %
North of England	19 %	238 horses	2.56 %
Scotland	7 %	88 horses	2.53 %
Wales	9.7 %	117 horses	2.27 %
Northern Ireland	0.25%	3 horses	0.0 %

2.1.4 Discussion

This is the first epidemiological study of equine laminitis in Britain. All surveys have inevitable restrictions - response rates are usually low and the sample studied is only a fraction of the whole. This, coupled with possible bias introduced into the questionnaire itself, means that such analysis is not always reliable. However, there are few other feasible methods to collate information about the incidence of laminitis.

A survey of this sort gives a very approximate estimate of the incidences and percentages in the areas under study. Documented information is difficult to obtain as horse registration is not compulsory in this country. Little information exists on horses and their owners, let alone on diseases and veterinary care. It is difficult to collect veterinary information. Veterinary care of the national equine herd is divided between numerous small animal veterinary practices in addition to those affiliated to BEVA. Few veterinary practices, if any, were able to retrieve the information requested from databases and several of the respondents said "these figures are guesstimates", "based on impression rather than fact" and that the replies were "not accurate enough for statistical analyses". This survey gives information, but it is important to note that replies were invariably from memory rather than documented sources.

Response rates were low, but this is usual for polls - the first response rate to postal questionnaires is usually around 20% (C.True, personal communication; D. Mellor, personal communication). The low response rate of BEVA members may reflect the fact that several members in one practice have received questionnaires but only one person replied. Similarly, no responses would come from university or research workers or those in second opinion referral clinics. Indeed, many veterinary practitioners may not simply be interested. The response rate of the IDHS was higher than that of the veterinary profession, although

perhaps surprisingly low. This may be because several members of the Society are in one family or that members who do not own horses did not reply.

Large numbers of horses recorded on the BEVA returns indicates that a significant proportion of the equine population was studied. The numbers of IDH horses in the survey results compares well with the total number of horses currently recorded with the IDHS. A study of the numbers of horses in the care of veterinary practices in Scotland and the border counties gave equivocal results of the numbers of horses in the area. Veterinary surgeons listed owners as having 2.5 horses each but when these owners were contacted, the mean number of horses owned was 5 (D. Mellor, personal communication). This discrepancy may be explained by suggesting that horse owners use more than one veterinary practice or, and this is more likely, that there are many horses that are never seen by veterinary surgeons for such horses do not receive routine prophylactic veterinary treatments. Figures for the population of horses in Britain are therefore likely to be higher than estimated by veterinary surgeons.

Both surveys gave remarkably similar results for the incidence of laminitis:- 3% of the equine population suffers either acute or chronic laminitis. The similarity of the figures in both surveys gives a degree of confidence in the information gained in this study. The figure is identical to one featured in an earlier study in the U.S. The incidence of laminitis in different breeds referred to New York Veterinary College, Cornell University, Ithaca, New York, was assessed between January 1st 1966 and January 1st 1972 (Hintz, 1990). The incidence of acute and chronic laminitis in all breeds of horses and ponies was 3.04% ; 215 animals had acute or chronic laminitis out of a total of 7072.

The IDHS returns gave a higher proportion of fatalities than the BEVA returns. 30% had been destroyed compared with 8% nationally. This may reflect differences in the aetiology since a higher incidence of *post partum* complications (these are most severe) were implicated in the aetiology of the IDH returns. The great bodyweight of Irish Draught horses may worsen the fate of the animal after an acute attack.

The comparison of conservative with more aggressive treatments gave insight into the sequelae of the disease (Peremans *et al.*, 1991). Ponies treated with usual therapeutic regimes (but not dorsal wall resection) had a 20% chance of returning to soundness. In a similar study of horses referred to a veterinary hospital, only 25 % of cases are able to return to an athletic career after laminitis (Hunt, 1993). Many of those that survive the original attack are permanently crippled and are thereafter effectively useless. In the studies mentioned above, 40% of ponies are intermittently or remain permanently lame afterwards (Peremans *et al.*, 1991) and 20% of horses are permanently unsound after laminitis (Hunt, 1993).

Slightly more horses than ponies are killed because of laminitis; 48% horses and 40% ponies are destroyed (Hunt, 1993; Peremans *et al.*, 1991). Comparison of the fates of the horses and ponies surveyed by BEVA and the horses in the IHD surveys are compared in Table 2.7

Both veterinary studies (Hunt, 1993; Peremans *et al.*, 1991) were performed at veterinary hospitals and so were certain to receive treatment. The dorsal wall resection (DWR) was performed by experienced clinicians and the nursing and aftercare of plaster of Paris casts which were replaced every three days would clearly be outside the scope of even the most capable horse owner. Although DWR seems to minimise the rotation of the distal phalanx and so considerably improve the prognosis, it is probably not a suitable routine treatment, or

for horses, who endure greater pain and which have a poorer eventual outcome (Colles and Ware, 1995).

In a survey of the prevalence of equine diseases undertaken in Scotland and in the border counties, 50% of veterinary surgeons said that "laminitis is a serious problem" (D. Mellor, personal communication). One veterinary surgeon commented that if the animal was still receiving analgesics 6 weeks after the onset of the disease, then it was unlikely to return to soundness. Another veterinary surgeon commented that if the cost of veterinary treatment exceeded £250 then it was unlikely that the animal would make a full recovery (R. Herdman, personal communication).

Veterinary treatment and loss of capital value of animals killed are costly. Laminitis is clearly responsible for considerable expenses and losses. On a national scale the losses are huge. Insurance companies must face an enormous total in claims each year; not only for humane destruction but also for veterinary fees and loss of use. In addition to financial aspects the refractory nature of the disease and the insurmountable suffering of the animals involved takes an enormous emotional toll on horse owners.

Table 2.7

The fate of horse and ponies after acute laminitis, recorded by BEVA and IDHS surveys. The present study compared with studies of horses* (Hunt, 1993); ponies** given usual therapy (Peremans *et al.*, 1991) and those given dorsal wall resection ***(DWR) (Peremans *et al.*, 1991)

BEVA	IDHS	Horses*	Ponies**	Ponies DWR***	Outcome
7%	27%	0%	0%	0%	recovered fully without treatment
68%	33%	32%	20%	100%	returned to full work after treatment
16%	11%	20%	40%	0%	remained permanently unsound
8%	30%	48%	40%	0%	were destroyed during acute phase

There was nothing surprising about the results on the types of treatments which are virtually identical to those listed in various reviews and textbooks (Stashak, 1987; Baxter, 1992; Hunt, 1993; Slater *et al.*, 1995). Anecdotally, phenylbutazone (PBZ) is often given to both acute and cases of chronic laminitis. PBZ, non-steroidal anti-inflammatory agent (NSIAD), works as an analgesic and reduces synthesis of prostaglandins and thromboxanes from arachidonic acid by inhibition of cyclo-oxygenase. PBZ therefore reduces inflammation and oedema (Baxter, 1992). No comments were made about the duration of PBZ treatment but in practice it is common for chronic laminitics to receive oral PBZ medication for the rest of their lives.

Other treatments, especially acepromazine and flunixin meglumine are well documented (Baxter 1992). Acepromazine is another α -adrenergic receptor blocker hence having a slight vasodilator action; it lowers blood pressure as a secondary effect. Flunixin meglumine is known to have an anti-endotoxic effect (Templeton *et al.*, 1985) in addition to its analgesic actions.

Heparin has also been listed as a possible prophylactic agent in horses that have undergone surgery for duodenitis / proximal jejunitis; there were no instances of laminitis in treated horses (n=12) compared with an incidence of 28% in the group not given heparin (n=104) (Cohen *et al.*, 1994) Other reports have been wary of the administration of heparin clinically because of adverse reactions including shock (Stashak, 1987). Of those treatments not listed by BEVA clinicians, only dimethyl sulphoxide (DMSO) was missing. Baxter (1992) lists this as a therapy because of its free radical scavenging qualities which may be helpful in reducing reperfusion injury. However, DMSO is difficult to obtain and it is usually mixed with dexamethasone for topical application for other diseases; of course, dexamethasone, like all glucocorticoids, is contraindicated for laminitis. Aspirin is used for its anti-platelet aggregating qualities but very large doses are required. All these therapies are palliative and

none address the precipitating factors, namely vasoconstriction of afferent vessels or venous pooling . The current pharmaceutical remedies do not restore the digital vascular system to normality.

The aetiology of laminitis was judged to be similar in both groups studied, although some differences were apparent. The small number of IDH horses affected by laminitis within the survey may account for these differences. However, the increased incidence of foaling complications as a causative factor in the IDH survey reflects a high proportion of the herd that is used for breeding. Hunt (1993) studied horses sent to a veterinary clinic and revealed a strong association between gastro-intestinal malfunction, endotoxaemia and laminitis. A very different aetiology has been described in Texas, USA, where 49% of cases were of unknown origin and only 3% were the result of ingestion of grass and 0.57% of cases metritis (Hood *et al.*, 1993). Different environmental and / or genetic factors are suggested. Of course the more usual, and less severe, cases of equine laminitis are not referred to veterinary hospitals .

Seasonal incidence of laminitis showed similar patterns in both surveys reinforcing the idea that the disease has annual zeniths and nadirs. The seasonal occurrence mirrors the aetiology of most cases. March and June are the months for spring grass and foaling. Peremans *et al.*, (1991) noted that among ponies the peak incidence occurred during these months. Autumn cases may coincide with migration of parasitic worm larvae and the associated problems of colic.

The highest proportion of cases seen by BEVA members were native breeds of ponies and it was commented that such breeds may be more susceptible to laminitis. Previous studies show up to four times as many ponies as horses develop laminitis (Colles, 1991b) and that, in the U.S., Shetland ponies and Morgan horses have an incidence of 5.2% and 6%

respectively (Slater *et al.*, 1995). Fewer cases of laminitis were evident amongst horse breeds in this study. However, these differences may merely reflect the population distribution of horse and pony breeds. The incidence of laminitis in pony breeds may not be a genetic predisposition but simply reflect greater numbers of Welsh ponies within the national population. Similarly, although horse breeds did not make as much contribution to the BEVA clinicians' caseload, there may be fewer horses than ponies in this country. Further studies, perhaps a similar survey of Welsh Pony Society members, could yield data on the incidence of laminitis in ponies compared with horses and resolve such questions.

Age and sex relationships were similar in both surveys. Again, any differences in results for disease incidence are likely to mirror the distribution of stallions, mares and geldings within the population. The Irish Draught Society returns probably are slightly biased. Registered stock (IDHS) are predominately mares as the organisation is concerned with breeding horses. There are only 57 RID stallions and these are usually kept stabled. The lack of laminitis in this group thus reflects the small numbers of stallions and their management. Although two studies recorded a difference in incidence of laminitis between mares, stallions and geldings (Dorn *et al.*, 1975; Amoss *et al.*, 1979) a subsequent larger study revealed no gender differences (Hunt, 1993). As in the latter report, the present study finds no difference in the incidence of laminitis when population composition is taken into account. This assertion is supported by a recently published study which showed no differences in breed, age, sex or weight (Slater *et al.*, 1995). Chronic cases tend to be older and female, perhaps because they can still fulfill a purpose as brood mares despite their disability (Slater *et al.*, 1995).

The highest incidence of laminitis occurs in animals over 10 years; one veterinary surgeon said "laminitis tends to be a disease of geriatrics". The mean age for acute laminitis was 9 years in one study (Slater *et al.*, 1995). Animals in the age group of 5 - 10 years had the

next highest incidence. Chronic cases of laminitis are older than other animals presented at veterinary practices and hospitals in the U.S.A. (Slater *et al.*, 1995). Laminitis was rarely seen in young animals. The reasons why young animals do not develop laminitis are numerous. In essence, young animals use carbohydrates for growth, exercise and maintenance and do not become obese. Young animals take more exercise. Their vascular system is likely to have better tone. The vascular system will not have been exposed to potential damage, for example by long term exposure to sugars. Exposure to ingested sugars is addressed in Chapter 10. Despite these considerations, there was a higher incidence of laminitis in animals under 4 years old within the IDH herd compared with the national herd. This may reflect *post partum* complications encountered by young brood mares who are pregnant in their second year.

Feed intake and exercise have some bearing on the reoccurrence of laminitis. If an animal developed laminitis at grass, then the animal was stabled to avoid further ingestion of grass. Correct feeding was important in recovery. If overweight, the horse's weight should be reduced to minimize mechanical breakdown of laminar tissue. Most cases of laminitis had not been in regular exercise. Management of these aspects has a considerable influence on nutrient metabolism, on maintenance of vascular health and the predisposition to laminitis (Freestone *et al.*, 1990); this is discussed in Chapter 10.

There seemed to be no geographical differences in incidence of laminitis. The percentage of the population affected was around 3% in most regions, like other results. The method for assessing geographical area was not sensitive enough to detect any differences in the onset of disease. Maybe there are no differences in timing of disease. After all, farming methods and grassland composition are fairly uniform throughout the country. Anecdotally, it is said that laminitis does not occur on the Isle of Wight (C.M. Colles, personal communication), but there were no survey returns from the Isle of Wight - maybe there are no horses there!

More detailed epidemiological studies are beyond the scope of this project but, if undertaken, would throw light on aspects of many equine diseases and be of enormous benefit. In spite of the obvious limitations of work of this sort, these preliminary findings are of interest and place the physiological studies in a context of equine welfare.

Chapter 3 Blood Pressure and Laminitis

	Page
3.1 Introduction	87
3.2 Materials and Methods	97
3.3 Results	102
3.3.1 Normal horses and ponies	102
3.3.2 Horses and ponies with acute laminitis	109
3.3.3 Horses and ponies with chronic laminitis	117
3.4 Discussion	123

*

3.1 Introduction

Vertebrates have a closed vascular system which together with the heart is responsible for transport of oxygen, heat, nutrients, vitamins and hormones to organs and tissues and also ensures rapid removal of waste products (Levick, 1992). The cardiovascular system must obviously be under delicate endocrine, neural and reflex control. Unlike low pressure/ low resistance systems of many other vertebrates mammals, together with birds, employ a higher pressure/ high resistance system to provide rapid and efficient transport of vital molecules to and from tissues (Schmidt-Neilson, 1991). Relatively high pressure guarantees circulatory function in all tissues, especially to those most distal to the heart, and ensures non static circulation in the smallest vessels - the arterioles (resistance vessels) and capillaries (capacitance vessels). Peripheral resistance of the vessels to cardiac output creates the familiar concept of blood pressure which is controlled by changes of arteriolar tone in particular. Capillaries offer little resistance to the bolus type of flow and as the thickness of the walls is thinner than that of arterioles (Levick, 1992).

Blood pressure itself is generated by the heart and is regulated by many mechanisms. Cardiac output is defined as the volume of blood ejected by one ventricle in one minute and depends on the stroke volume (volume ejected during with each contraction) and heart rate (number of contractions each minute) (Levick, 1992).

The heart beat is myogenic but superimposed are the central and peripheral neural controls. The parasympathetic system has a major influence to regulate heart rate (vagal inhibition) and distribution of the cardiac output. Sympathetic nervous control dominates during physical or mental emergencies when heart rate increases.

Parasympathetic and sympathetic systems in a sense oppose each other to give rapid control of cardiac activity and selective distribution of its output (Starr and Taggart, 1992).

Blood pressure, is a function of heart rate and peripheral resistance. Increased systolic pressure is detected by baroreceptors in the carotid sinuses and the aorta. Sensory fibres run from the carotid artery via the glossopharyngeal nerve to the cardioinhibitory centre of the brain and from the aorta via the vagus nerve (Starr and Taggart, 1992). If blood pressure increases, afferent signals travel to the medulla and parasympathetic efferent signals slow heart rate to vasodilate and decrease peripheral resistance. Conversely, if blood pressure decreases, sympathetic signals speed cardiac activity to vasoconstrict resistance vessels. Arterioles are richly innervated and under direct and indirect sympathetic and parasympathetic control. The release of catecholamines: acetylcholine at nerve endings, and noradrenaline from nerve synapses and chromaffin cells in the adrenal medulla, is under direct nervous control. Adrenaline and noradrenaline bind to specific adrenergic receptors in cell membranes. Ahlquist (1948) suggested two types of adrenergic receptors: α -adrenergic receptors, associated with vasoconstriction, and β - adrenergic receptors which are mostly associated with vasodilatation and inhibitory functions. Both are further divided into subgroups 1 and 2. Classically, the neurotransmitters from parasympathetic and sympathetic nervous systems are of course acetylcholine and noradrenaline. Parasympathetic nerve synapses release the neurotransmitter acetylcholine which binds to muscarinic receptors in cell membranes. Sympathetic nerves release the neurotransmitter noradrenaline which binds to β 1 adrenoreceptors causing cardiac stimulation and vasodilatation (Ganong, 1991). Heart rate is homeostatically controlled by the sympathetic nervous system and the parasympathetic nervous system working in opposition (Starr and Taggart, 1992). Increased activity of the sympathetic nerves results in increased heart rate (tachycardia) whereas increased activity of parasympathetic, or vagal, nerves slows the heart (bradycardia) (Starr and Taggart, 1992) . Adrenaline increases heart rate and both

adrenaline and noradrenaline increase the force of myocardial contractions (inotropic action). The catecholamines also have a chronotropic effect, shortening the time taken to reach threshold and shortening all phases of the cardiac cycle. Chronotropic actions are mediated by receptor activation which affects the Ca^{2+} pumps enhancing the inward transport across the cell membrane of free Ca^{2+} ions. The activation of receptors and secondary messengers, and the activation in intracellular cascades following receptor activation, is an area of very active research. The activation of the β 1-adrenoreceptor is mediated by an intramembrane protein, a Gs-protein, which in turn activates adenylate cyclase. Adenylate cyclase activates an intracellular secondary messenger, cyclic adenosine monophosphate (cAMP). cAMP activates protein kinase which is phosphorylated and then influences the number of calcium channels and active calcium pumps (Levick, 1992). Electrolyte concentrations in extracellular fluid affect normal cardiac homeostasis. Even a small shift in either calcium or potassium concentration will dramatically affect the heart, altering both myocardial contractility and the resting membrane potential.

β 1-antagonists (e.g. Propanolol) block β 1 receptors reduce cardiac output and are commonly used in the treatment of hypertension and angina. Pharmaceutical therapeutics for equine laminitis work on a similar principle. α -antagonists such as acepromazine and non-steroidal anti-inflammatory agents, such as phenylbutazone, counter central nervous vasoconstrictive mechanisms and prevent a positive feedback mechanism from pain. Blood pressure is therefore controlled on an immediate or short term basis by neural mechanisms together with neurohypophyseal and other systemic hormones, however other paracrine and systemic hormones are involved in longer term regulation of blood pressure.

Neurohypophyseal peptides have clear effects upon blood pressure; arginine vasopressin increases blood pressure. Other systemic hormones include angiotensins, bradykinin and

example, parathyroid hormone, vasoactive intestinal peptide (VIP), calcitonin gene related peptide, urodilatin, endothelin) affecting vascular homeostasis but their exact roles are not fully defined. The role of some of these hormones is investigated in other Chapters and the interaction between them more fully discussed in the Introduction (Chapter 1).

Recent discoveries have highlighted paracrine control of vascular tone, by local factors such as endothelial derived relaxing factor (nitric oxide) and endothelin; this control can be immediate (seconds or minutes) or longer term (hours). The possible role of these paracrine hormones in equine laminitis is studied in Chapter 5. Longer term control (days or months) involves adrenal steroids which affect electrolyte regulation.

Blood pressure is closely linked to plasma volume in that the pressure in a vascular circuit depends upon the the volume and viscosity of the circulated fluid. Serum electrolytes will be discussed in Chapter 9. Cardiovascular homeostasis is maintained by a shibboleth of intricate neurohormonal and endocrine mechanisms. Despite the complexity of cardiovascular regulatory mechanisms, measurement of blood pressure itself gives a useful insight into physiological status. Blood pressure, hormones and plasma biochemistry, may provide an insight into the interactions between many factors and give an overall picture of the physiology / pathology of the animal involved.



Figure 3.1 First demonstration of blood pressure by Stephen Hales 1733 (from Lyons and Petrucelli, 1987)

This thesis specifically concerns the horse and it is therefore significant that blood pressure was first measured by Stephen Hales in 1733 (Statistical Essays). Using a 9 foot glass tube inserted into the carotid artery via a goose trachea, arterial pressure in the horse was measured directly by observing the height to which the blood rose in the tube (Figure 3.1). Human blood pressure was measured using a mercury manometer by J. L. M. Poiseuille 100 years later and after another 100 years electronic methods of detections (transducers) were developed by Lambert and Wood (Levick, 1992). Although the horse was responsible for the first demonstration of blood pressure other methods - palpation, auscultation and oscillometry - have been less successful (Schilling,

1919; Covington and McNutt, 1931; Taylor, 1981; Bailey, 1994). Modern measurement of equine blood pressure is usually only done under anaesthesia. An indwelling catheter placed in the facial, or transverse facial artery, is connected to a pressure transducer and a chart recorder. Measurement of blood pressure in conscious horses is not easy for several reasons. First, there are few sites in the horse where an available artery can be occluded successfully; limbs possess several accessible arteries but these cannot be occluded properly because of the relatively deep setting of the artery in the architecture of the limb (Hinckley and Henderson, unpublished observations; Chapter 4.2). Even if the artery is occluded successfully, a further difficulty involves trying to listen to the reoccurrence of cardiac sounds, since it is somewhat risky, even with the quietest of subjects to be putting a stethoscope to its distal limb after the blood supply has been stopped ! The only other part of the equine anatomy that readily lends itself to the measurement of blood pressure is the tail. A small section of the coccygeal artery is near the surface on the ventral fascia of the 'dock' where the skin is not covered by hair. Unfortunately, a sphygmomanometer and stethoscope cannot be used at this site as the return of heart beats cannot be heard without amplification. However, advances in technology for the measurement of human blood pressure has made techniques available for use on horses, especially those based on Doppler ultrasound which have been used in this study. Caudal blood pressure is taken at the coccygeal artery in the horse's tail. This position of measurement at the horse's tail is at a higher level (relative to the ground) than the level of the heart (Figure 3.2). Gravity dictates that readings at a higher level to the heart are of lower pressure and those below the level of the heart are at a greater pressure. Therefore blood pressures measured at the coccygeal artery will be less than direct measurements taken by indwelling catheters in the atrium or aorta. Direct measurements of BP taken through the facial or transverse facial artery will be equivalent to indirect measurements taken at the tail.

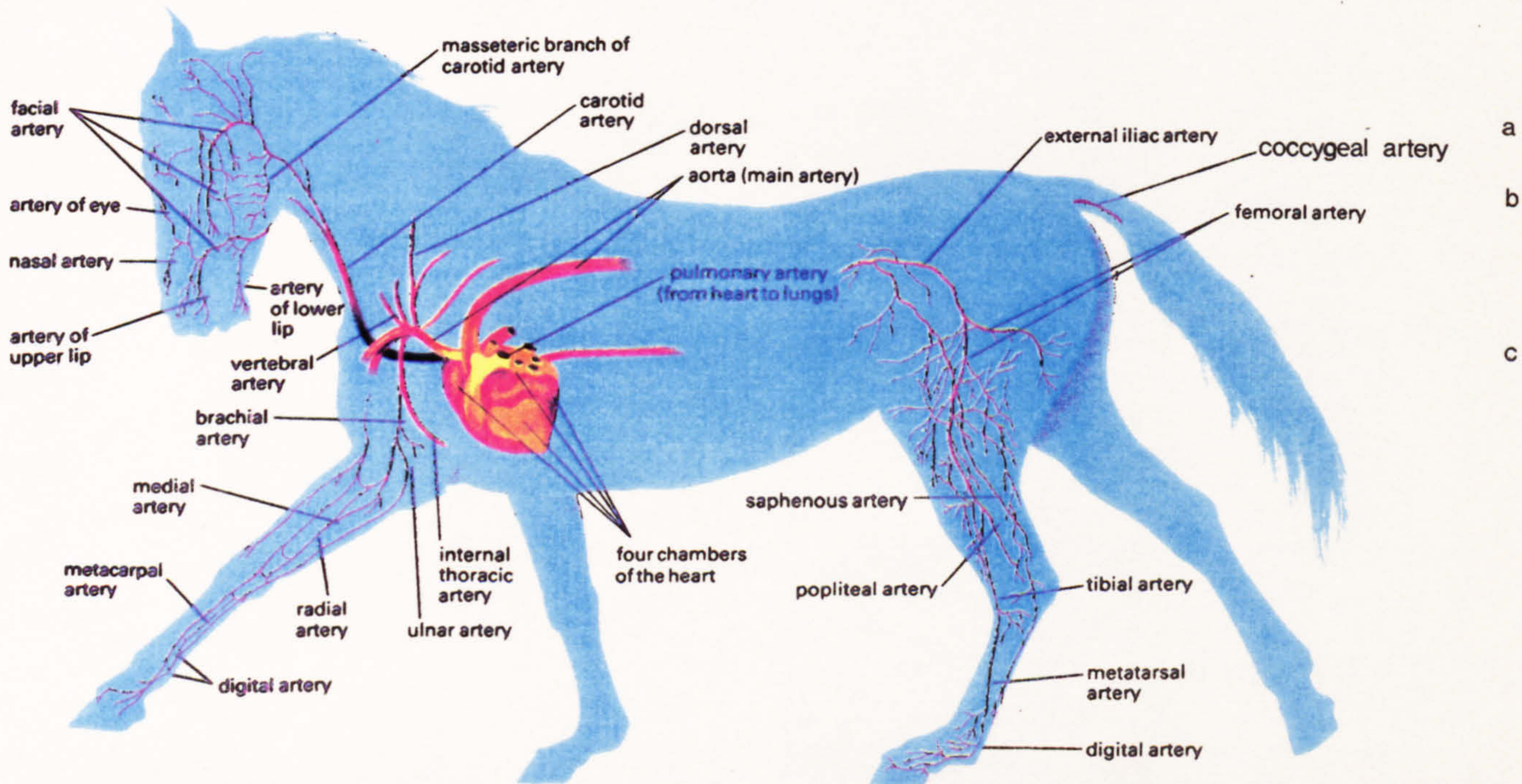


Figure 3.2 Diagrammatic representation of the major arteries of the horse showing positioning of tail cuff on the coccygeal artery (Hastie, 1983) and the relative positions for BP measurement at a) direct facial artery b) indirect tail cuff method and c) direct measurement from a catheter in the aorta or atria.

Some preliminary investigations were carried out to assess methods of measuring blood pressure. Doppler ultrasound equipment (Parks Medical) has been used on the horse (Kvart, 1979) and this method was tried in this study. Inherent difficulties with this method were found, not least that the sensor acted as an aerial and picked up radio signals when used outside; the machine amplified Radio 4 instead of heart sounds! It was also extremely difficult to occlude the coccygeal artery while listening to heart sounds as the exposed area of artery is so small. Doppler methods are essentially the same as the standard manual sphygmomanometer and stethoscope, but a sensitive sound receiver senses the heart sounds and these are amplified onto a radio set. The sensitivity of detection of systolic heart sounds leads to falsely elevated high readings for systolic BP compared with manual calibration on human subjects (Hinckley and Henderson, unpublished observations). For this reason, the accuracy of the Doppler method has been questioned because differences of some 20.2 mm Hg between consecutive measurements have been noted (Bailey, 1994). To some extent this has cast doubt on all indirect methods.

Equine blood pressure has not been extensively studied. Reported values for normal horse blood pressure are:

Systolic 112 ± 16 ; Diastolic 77 ± 14 ; with a heart rate of between 39 - 42 beats / minute (Blood and Radostits, 1989; Vaala *et al.*, 1995). Other breed values, together with age relationships, are outlined by Ostlund *et al.*, (1983) where systolic pressures ranged from 83 - 120 mmHg (Swedish Warmbloods); 85 - 140 mmHg (Arabian horses); and 95 - 135 mmHg (ponies).

Comparison of non-invasive measurements with direct measurements of normal equine BP are given below, regrettably the authors did not specify the sites of measurement:

Direct arterial

Systolic	110 - 160 mm Hg
Diastolic	70 - 90 mm Hg
Mean	80 - 110 mm Hg

Indirect arterial

Systolic	100 - 135 mm Hg
Diastolic	70 - 97 mm Hg
Mean	82 - 110 mm Hg

Table 3.1 (Vaala *et al.*, 1995)

Environmental influences upon blood pressure include variations in intake of dietary electrolytes especially sodium which may vary because of feed variations, because horse owners add salt to the feed, or because 'salt licks' are provided irrespective of requirement. Exercise, degree of fitness, and other factors such as temperature, hypoxia and so on will also affect blood pressure. Pain, disease, state of hydration, and pregnancy are also influential. Psychological influences are often responsible for blood pressure being labile in human and equine subjects - the 'white coat phenomenon' (Moseley *et al.*, 1995). Abnormally high readings may be recorded if a veterinary surgeon, or clinician, is associated with other stressful or painful procedures. Horses are herd animals and are likely to be excitable if separated from their group; some animals are also stressed by handling particularly if the attendant is not their usual handler.

In addition to these factors, more difficulties arise because apparatus suitable for the measurement of blood pressure is invariably housed in veterinary hospitals. Animals that attend veterinary hospitals are generally diseased and have travelled in a horsebox to the

clinic. Direct measurement of blood pressure under anaesthesia is the usual approach (which is in itself rather stressful and not without risk) and this coupled with handling stress and the excitement of the occasion are not ideal circumstances in which to try to establish basal values for normal equines . Normal blood pressures are difficult to acquire and it will not be surprising to find that very few studies of normal equine blood pressure have been performed. To the author's knowledge, blood pressure has not been measured outside veterinary hospitals or when laminitis arises spontaneously. The present data are "stress free", under controlled long term management, and reflect the disease that arises from a variety of normal pathogeneses, rather than in the artificial induction of the disease.

Indirect methods of measuring blood pressure were evaluated and normal blood pressure values for normal horses and ponies of different ages and sex, kept under strictly controlled conditions regarding feed (especially sodium intake) and exercise were established. When normal values were known, comparisons were made with diseased animals during acute and chronic laminitis. In the acute stage, blood pressure was used to establish the severity of laminitis, and related to Obel Grade of lameness. Subsequently, the efficacy of the treatments given was gauged by blood pressure values and later the relationship of the vasoactive hormones under investigation were assessed in this context.

The aims of this section on equine BP were to: i) establish the usefulness of indirect BP measurements in field conditions; ii) establish basal BPs of horses and ponies using the Dinamap apparatus; iii) investigate the assertions that both acute and chronically laminitic horses and ponies are hypertensive, and provide measurements for both groups under typical conditions and those related to different aetiologies. BP results from this section are related to plasma vasoactive hormones and substances affecting vascular tone in later chapters. BP is correlated with Obel grade of lameness and is used to gauge the efficacy of treatment of acute laminitis in Chapter 5.

3.2 Materials and Methods

Animals The animals studied were either Registered Irish Draught, aged 4 to 26 years, and weighing 500 - 750 Kg; cross bred hunters aged 17 - 18 years, weighing 550 Kg; or cross bred Welsh ponies aged 4 - 20 years, weighing 250 - 350 Kg. The Irish Draught mares and hunters were classed as 'horses' and the remainder as 'ponies', according to size. Each group was subdivided into normal animals and those suffering acute or chronic laminitis (Table 3.2). An animal was defined as 'chronic laminitic' if it had suffered at least one attack of acute laminitis in its history. Acute laminitis is sudden and painful; it is an obvious clinical condition requiring immediate treatment. All but one case of acute laminitis were grass induced. The other case of acute laminitis was in an Irish Draught mare that suffered endometritis after retention of placental membranes. Medication of the *post partum* case of laminitis is listed in Appendix. Obel Grades of lameness 1 - 4 (see Chapter 1) were subdivided into intermediate Grades of 1.5, 2.5, 3.5 to define the slight differences of lameness between the grades.

Table 3.2--Numbers of animals in each group for blood pressure assessment:

			Mares	Geldings
Normal ponies	n = 7		5	2
Normal horses	n = 15		13	2
Chronic laminitis (ponies)	n = 5		4	1
Chronic laminitis (horses)	n = 2		2	
Acute laminitis (ponies)	n = 5		4	1
(11 cases of acute laminitis)				
Acute laminitis (horses)	n = 1		1	

Some of the normal horses (Irish Draught mares) were pregnant when tested, so BPs during gestation were compared with other non-pregnant mares before basal values were established.

Procedure boxes were used for measurements and during winter, the box was heated so the ambient temperature was nearer to summer values. This also ensured consistent functioning of the Dinamap apparatus (see below). Measurements were taken at the same time of day to minimise circadian or diurnal variations.

Animals were always quietly handled and any stress of the procedure kept to a minimum. No physical or medical restraint was necessary. Animals had been accustomed to the routine over the previous 3 months.

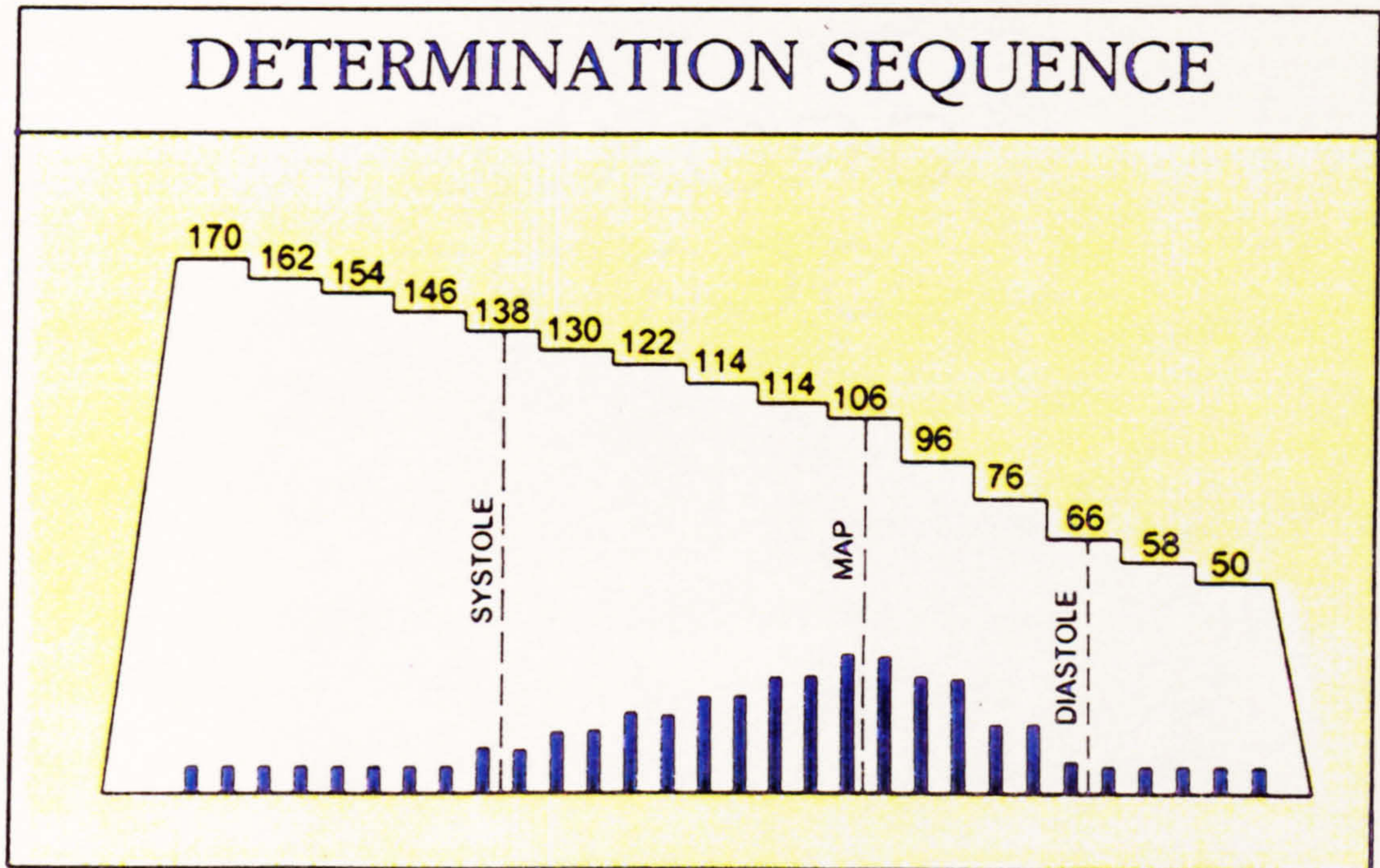
Blood pressure and heart rate. Systolic, diastolic and mean blood pressures and heart rates were measured non-invasively using a tail cuff attached to a Dinamap Vital Signs Monitor Model 8711 (Critikon, Johnson and Johnson, Ascot, UK.). The cuff used was the human adult standard arm cuff which was an appropriate size to ensure accuracy on horses' and ponies' tails (Figure 3.3). This machine works on similar a principle to the manual sphygmomanometer. The cuff is inflated until heart sounds are transmitted to the monitor transducers; the cuff is then further inflated until heart sounds are no longer detected. The pressure inside the cuff is progressively deflated and the systolic reading recorded when heart sounds (Korotkov sounds) reappear (Levick, 1992). Diastolic pressure is recorded when there is no heart sound at the lower pressure and mean blood pressure is calculated from the curve produced by the Monitor's software (Figure 3.4).

Mean blood pressures presented on tables represent multiple readings (on any one occasion the mean value of 3 measurements was recorded). Basal blood pressures of normal horses and ponies were taken during winter, spring and summer months. Blood pressures of normal Irish Draught mares were recorded at different stages of pregnancy during winter and spring.

Figure 3.3 Dinamap Vital Signs Monitor in use under field conditions.



Figure 3.4 Method of determination of systolic, diastolic and mean blood pressures using the Dinamap Vital Signs Monitor 8711.



Systolic Pressure is the cuff pressure at which oscillations in pressure begin to increase in amplitude.

Mean Arterial Pressure is the lowest cuff pressure at which the maximum oscillations in pressure occur.

Diastolic Pressure is the cuff pressure at which the oscillations stop decreasing in amplitude.

Values were established for those animals that had suffered an attack of laminitis in the past and are described as 'chronic' laminitis cases. Some of these animals, and one of the normal ponies, suffered acute laminitis when at grass so blood pressures during the acute stage of the disease could be established. In addition, one of the normal Irish Draught brood mares suffered acute laminitis of endotoxic aetiology following endometritis. The weeks that followed this attack were described as post acute or refractory (Hood 1995); where the animal neither substantially improves nor worsens clinically but is inherently unstable (see Chapter 1). Lateral-medial radiographs were taken of the front digits of two long term chronic laminitic mares - Isadora and Rose Marie, and the *post partum* case of acute laminitis - using Phillips C-Arm apparatus (Phillips, UK).

Statistics. Individual values presented on tables represent at least three readings taken on the same occasion. Values are Means \pm Standard Error (SE). Standard deviation (S.D.) and standard error (S.E.) were calculated on computer software (Microsoft Excel Microsoft). Three readings of blood pressure were taken on each occasion of measurement and the mean value for the individual determined (see Appendix I). Group mean values were calculated from individual mean values. The determination of S.E was part of the statistics package and was confirmed by hand using the formula

$$\text{S.E.} = \frac{\sqrt{\text{S.D.}}}{n} \quad \text{where } n \geq 9 \text{ or } n-1 \text{ if } \leq 9 \text{ and } n \text{ is the number of animals.}$$

Statistical significance was determined by a Student's t - test (Microsoft Excel, Microsoft). Statistical tests used the individual values rather than the mean values for each individual, and this results in slight variations in SE seen in the Tables. Differences considered significant when $p < 0.05$.

3.3 Results

Section 3.3.1 gives basal values for normal horses and ponies during winter, spring, and summer months. Section 3.3.2 lists the mean blood pressures of animals with chronic laminitis and Section 3.3.3. those during acute laminitis, but before treatment.

3.3.1 Basal values of blood pressures and heart rates of normal horses and ponies

Normal ponies. Mean BP values of normal ponies are listed below in Table 3.3. and there were no striking variations. Details of individual ponies mean values are given in Table 3.4 (winter), Table 3.5 (spring) and Table 3.6 (summer). Group mean values are shown beneath each table. Full details of individual values are listed in the Appendix .

Table 3.3 Basal blood pressures and heart rates of normal ponies

	Blood pressure (mmHg)			Heart rate (bts/min)
	Systolic	Diastolic	Mean	
Normal pony (winter)	104 ± 7	59 ± 2	73 ± 3	43 ± 1
Normal pony (spring)	108 ± 3	68 ± 2	83 ± 3	45 ± 4
Normal pony (summer)	109 ± 6	58 ± 1	76 ± 2	43 ± 2
Normal pony values	107 ± 2	62 ± 3	77 ± 3	44 ± 1

Table 3.4 Mean BP values and heart rates of normal ponies taken during winter months

	Blood pressure (mmHg)			Heart rate
	Systolic	Diastolic	Mean	(bts/min)
Cracker II	99	54	70	41
Despina	86	58	69	39
Jasper	115	61	78	39
Marron	90	55	69	40
Selluci	103	55	72	43
Snowflake	132	68	89	49
Group Mean	104	59	73	43
Standard Error	7	2	3	1
Range	(86 - 132)	(54 - 68)	(69 - 89)	(39 - 49)

n = 6

Number of tests = 18

Table 3.5 Mean BP values and heart rates of normal ponies taken during spring months

	Blood pressure (mmHg)			Heart rate
	Systolic	Diastolic	Mean	(bts/min)
Cracker II	105	68	89	47
Despina	-	-	-	-
Jasper	-	-	-	-
Marron	100	66	77	41
Selluci				
Snowflake	116	73	87	38
Tess	111	63	80	54
Group Mean	108	68	83	45
Standard Error	3	2	3	4
Range	(100 - 116)	(63 - 73)	(77 - 89)	(38 - 54)

n = 4

Number of tests = 11

Table 3.6 Mean BP values and heart rates of normal ponies taken during summer months

	Blood pressure (mmHg)			Heart rate
	Systolic	Diastolic	Mean	(bts/min)
Cracker II	110	59	77	44
Despina	95	58	71	39
Marron	107	58	74	40
Selluci	125	56	80	48
Group Mean	109	58	76	43
Standard Error	6	1	2	2
Range	(95 - 125)	(56 - 59)	(71 - 80)	(39 - 48)

n = 4

Number of tests = 21

Normal Horses Basal mean values for normal horses, are shown in Table 3.7 Individual means are shown in Table 3.8 (winter values) and Table 3.9 (summer values). Data on individual animals are shown in the Appendix. Although diastolic pressure appeared to be lower there were no significant differences.

Table 3.7 Mean basal blood pressure and heart rate for normal horses

Normal horse (winter)	95 ± 4	51 ± 3	65 ± 2	52 ± 2
Normal horse (summer)	94 ± 3	52 ± 2	66 ± 3	47 ± 1
Normal horse	95 ± 1	52 ± 1	66 ± 1	50 ± 3

Table 3.8 Mean BP values and heart rates of normal horses taken during winter months

	Blood pressure (mmHg)			Heart rate
	Systolic	Diastolic	Mean	(bts/min)
Albert	87	41	57	65
Amanda	99	51	65	43
Annabella	88	44	65	43
Beckford	101	61	74	45
Biscuit	100	55	68	48
Blossom	93	57	70	57
Dolly	89	68	66	52
Emily	108	62	81	50
Maree Gray	80	39	54	58
Melody	118	50	63	46
Poppy	79	41	54	52
Tango	104	58	73	59
Group Mean	95	51	65	52
Standard Error	4	3	2	2
Range	(79 -118)	(39 -68)	(54 -81)	(43 -65)

n = 12

Number of tests = 36

Table 3.9 Mean BP values and heart rates of normal horses taken during summer months :

	Blood pressure (mmHg)			Heart rate
	Systolic	Diastolic	Mean	(bts/min)
Amanda	90	53	67	48
Annabella	82	48	63	57
Biscuit	94	50	62	45
Blossom	84	44	55	53
Bryn	120	64	81	46
Dolly	96	61	72	51
Emily	102	58	77	43
Maree Gray	88	42	57	40
Melody	94	43	57	45
Poppy	86	49	57	41
Rose	90	51	70	41
Tango	100	56	75	48
Group Mean	94	52	66	47
Standard Error	3	2	3	1
Range	(82 -120)	(42 -64)	(55 -81)	(40 -57)

n = 12

Number of tests = 38

There are no significant differences in BP or heart rate of normal horses during summer and winter months.

Comparison of BP of pregnant and non-pregnant mares.

Mean values for BP of pregnant mares (in the last trimester) are compared with non-pregnant mares. Mean values and group means are shown in Table 3.10

Table 3.10

	Blood pressure (mmHg)			Heart rate
	Systolic	Diastolic	Mean	(bts/min)
<i>Non-pregnant</i>				
Bryn	120	64	81	46
Dolly	96	61	72	51
Maree Gray	88	42	57	40
Rose	90	51	70	41
Tango	100	56	75	48
Group Mean	99	55	71	45
Standard Error	6	4	4	2
Range	(88 - 120)	(42 - 64)	(57 - 81)	(40 - 51)
No of tests =15				
n=5				
<i>Pregnant</i>				
Amanda	90	53	67	48
Annabella	82	48	63	57
Biscuit	94	50	62	45
Blossom	84	44	55	53
Emily	102	58	77	43
Melody	94	43	57	45
Poppy	86	49	57	45
Group Mean	90	49	63	48
Standard Error	3	2	3	2
Range	(82 - 102)	(43 - 58)	(55 - 77)	(43 - 57)
No of tests =21				
n=7				

There are no significant differences between pregnant and non-pregnant mares ($p > 0.5$).

Age related BP Observations on normal horses aged 4 - 20 years, on two separate occasions, showed no obvious differences in blood pressure with age (Table 3.11). Older horses did not show an increase, indeed some of the lowest readings were on the oldest horses ; conversely, some of the highest readings were recorded on young animals. There is no significant difference.

Table 3.11

	Age (years)	Blood pressure (mmHg)			Heart rate (bts/min)
		Systolic	Diastolic	Mean	
Albert	18	87	41	57	65
Amanda	5	99	51	65	43
Annabella	14	88	44	65	43
Beckford	17	101	61	74	45
Biscuit	5	100	55	68	48
Blossom	5	93	57	70	57
Dolly	11	89	68	76	52
Emily	4	108	62	81	50
Maree Gray	20	80	39	54	58
Melody	7	118	50	63	46
Poppy	7	79	41	54	52
Tango	4	104	58	73	59

	Age (years)	Blood pressure (mmHg)			Heart rate (bts/min)
		Systolic	Diastolic	Mean	
Amanda	5	90	53	67	48
Annabella	14	82	48	63	57
Biscuit	5	94	50	62	45
Blossom	5	84	44	55	53
Bryn	7	120	64	81	46
Dolly	11	96	61	72	51
Emily	4	102	58	77	43
Maree Gray	20	88	42	57	40
Melody	7	94	43	57	45
Poppy	7	86	49	57	41
Rose	7	90	51	70	41
Tango	4	100	56	75	48

Basal values for normal equine blood pressure and heart rate.

The basal values for BP and heart rate of normal horses and ponies throughout the year which were established in this study are

Normal pony BP	107 ±2 / 62 ± 3 mmHg mean 77 ± 3 mmHg; heart rate 44 beats /min
Normal horse BP	95 ± 1 / 52 ±1 mmHg mean 66 ±1 mmHg heart rate 50 ± 3 beats /min
Equine Blood Pressure	101 ± 6 / 57 ± 5 mmHg mean 72 ± 6 mmHg
Heart Rate	47 ± 3 beats/min

These data provide a solid basis for normal animals upon which to judge changes following disease.

3.3.2 Acute laminitis - Blood pressures, heart rates and Obel Grade of acute cases

Table 3.12 gives mean blood pressures and heart rates of individual ponies suffering spontaneous grass induced laminitis, during the summer of 1994, together with the Obel Grades of lameness 1 - 4. The original Obel grade was not sufficiently sensitive to distinguish between the slight differences of lameness observed, so the grades were subdivided into 'half grades' of 0.5, 1.5, 2.5 and 3.5. Blood pressures and Obel grades, obtained prior to treatment, are shown in Table 3.12. Mean blood pressure and heart rate of the *post partum* case of acute laminitis is also recorded on the first two days; in this case, treatment was given and is listed in the Appendix IV. Results are shown in Table 3.11. Individual data are tabulated in the Appendix II.

Grass induced acute laminitis in ponies before treatment

Table 3.12 **Mean BP and heart rates during grass induced acute laminitis 1994 and the relationship to Obel Grade of lameness**

		Systolic	Diastolic	Mean	Ht rate	Obel Grade
	Animal code	mmHg	mmHg	mmHg		
	Case 1	183	97	138	70	4
	Case 2	188	86	123	56	3.5
	Case 3	165	75	106	54	3
	Case 4	171	88	129	61	3.5
	Case 5	155	94	121	75	3.5
	Case 6	177	86	125	57	4
	Case 7	131	81	98	51	3
	Case 8	164	79	115	52	2
	Case 9	165	90	118	71	3
	Case 10	155	84	117	62	2
Mean		165	86	119	61	3
Standard Error		5	2	4	3	
Range		(131-188)	(75-97)	(98 -138)	(51- 75)	(2 -4)
Number of Cases	10					
Number of tests	23					

Table 3.13 Mean BP and heart rates of ponies with acute laminitis (1994), before treatment, values for normal ponies, during Spring months, are also presented. Slight differences in mean values from Table 3.3 arise because individual values, rather than individual means, were used (see Material and Methods above).

			Blood pressure (mmHg)				Heart rate		Obel Grade
	Systolic	%inc	Diastolic	%inc	Mean	%inc	(bts/min)	%inc	
Normal	107± 4		62 ± 2		78 ± 2		44 ± 1		0
(n= 8)									
Number of tests = 63									
Acute laminitic	165 ± 5*	54%	86 ± 2*	39%	119 ± 4*	52%	61 ± 3*	38%	3
(n=10)									
Number of tests = 23									

* (p≤ 0.001)

Individual cases of acute grass induced laminitis was associated with changes in basal BP from previous months, with animals acting as their own controls. These changes together with those in heart rate were related to the Obel grade of lameness. There was an increase of up to 124% in mean blood pressure during grass laminitis in Beau, for example, (Table 3.13).

Table 3.14 Individual case of grass induced laminitis 1995

			Blood pressure (mmHg)				Heart rate		Obel Grade
	Systolic	%inc	Diastolic	%inc	Mean	%inc	(bts/min)	%inc	
<i>Beau</i>									
Basal	104 ± 1		58 ± 1		69 ± 5		50 ± 5		0.5
Day 1	197 ± 6	89%	113 ± 8	94%	155 ± 11	124%	79 ± 1	58%	3.5

A case of atypical grass laminitis

One case was observed (Domino 1 1995) which was an atypical case of acute laminitis. Unusually, there was not a significant increase in BP during the untreated acute stage. Domino had an elevated heart rate during basal assessment but this fell during the acute phase. The lameness was not severe in this case (Obel 2). Although blood pressure increased, heart rate actually fell. Data are shown in Table 3.15

Table 3.15 Atypical BP and heart rate during grass laminitis

	Blood pressure		(mmHg)		Heart rate		Obel Grade
	Systolic	%inc	Diastolic	%inc	Mean	%inc	
<i>Domino</i>							
Basal	90 ± 9		56 ± 8		68 ± 8		0.5
Day 1	108 ± 4	20%	63 ± 6	12%	75 ± 2	10%	2

Individual case of post partum laminitis in an Irish Draught Mare

There was a increase of 88 mmHg systolic pressure, 45 mm Hg diastolic pressure and 43 mmHg increase in mean BP from basal values to the onset of Obel grade 4 lameness.

Heart rate nearly doubled from 45 beats /min to 88 beats at this stage.

Table 3.16 Mean BP \pm SE during *post partum* acute laminitis in an Irish Draught Mare 1995

<i>Biscuit</i>				Blood pressure (mmHg)				Heart rate (bts/min)		Obel Grade
Day	Time	Systolic	%inc	Diastolic	%inc	Mean	%inc		%inc	
Basal		94 \pm 1		50 \pm 3		62 \pm 3		45 \pm 0		0
No tests =6										
Day 1	12.30am	132 \pm 3	40%	77 \pm 4	54%	97 \pm 7	56%	79 \pm 3	75%	3
	7.30pm	127 \pm 4		79 \pm 4		101 \pm 6		73 \pm 3		3
Day 2	7.0am	182 \pm 2	93%	95 \pm 4	90%	127 \pm 6	104%	88 \pm 1	95%	4
	11.0am	151 \pm 2		86 \pm 2		104 \pm 7		103 \pm 2		4
	7.0pm	134 \pm 1		87 \pm 5		107 \pm 0		83 \pm 2		4
	11.0pm	137 \pm 4		93 \pm 1		108 \pm 2		87 \pm 1		4
	Mean	144 \pm 8	53%	86 \pm 3	72%	107 \pm 4	72%	86 \pm 4	91%	4
n =1										
No tests = 30										

This mare was treated with nitrovasodilators during the first weeks of the disease (see Appendix IV). and also received analgesics, phenylbutazone, flunixin meglumine iv, and the α -adrenergic blocker acepromazine iv. The changes in blood pressure over the course of the first week are shown in Figure 3.5. The next 6 weeks are summarized in Figure 3.6. A gross elevation of BP was seen in the sixth week when tearing of the dermal laminae and 'sinking' of the bony column through the hoof capsule occurred (Figure 3.7). Additional analgesics were given but although BP was slightly reduced, it was considered that pain was uncontrollable and the mare was destroyed.

Figure 3.5 Changes in blood pressure and heart rate over the course of the first week of *post partum* laminitis.

Blood pressure and heart rate in the first week of post partum acute laminitis

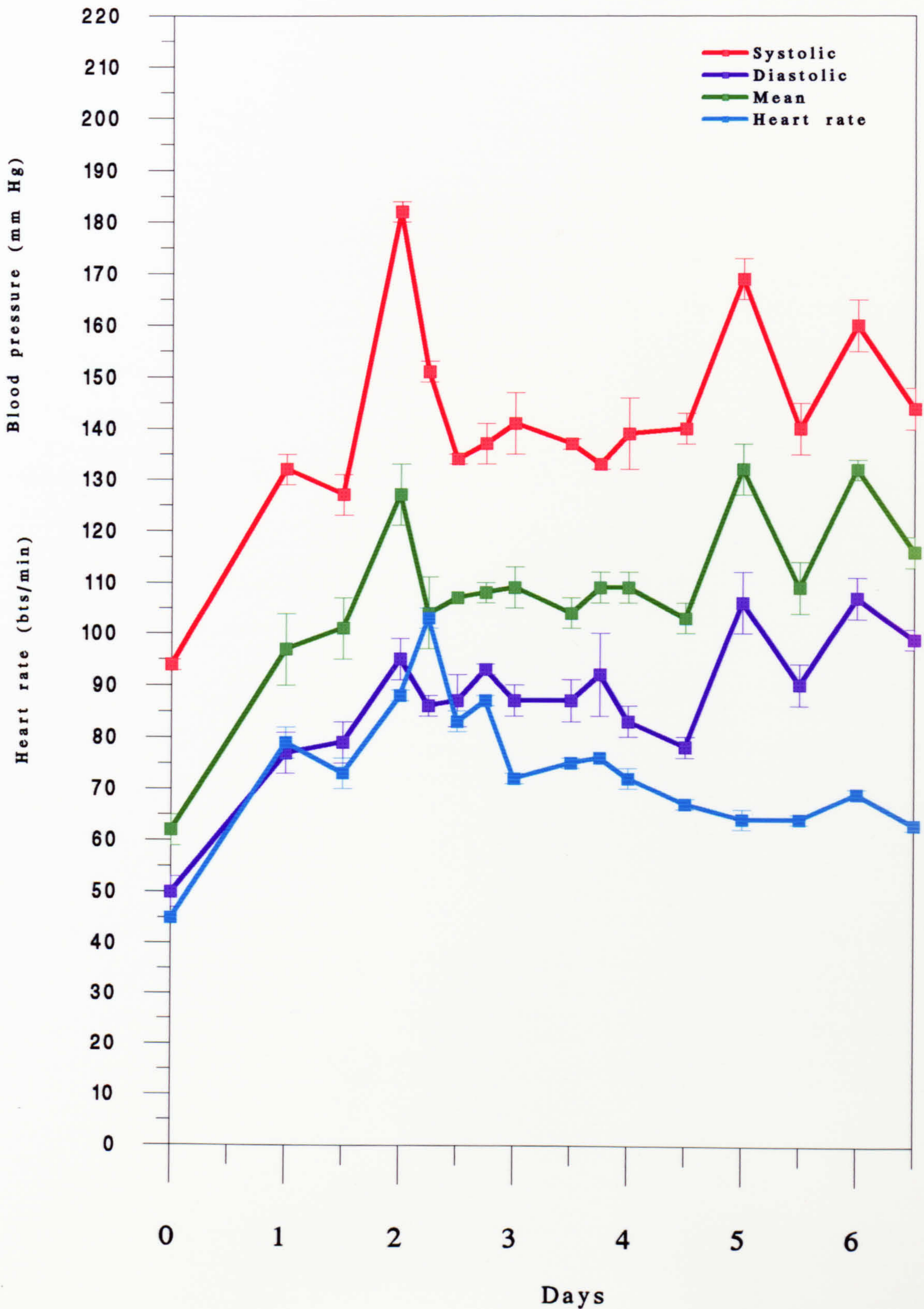


Figure 3.6 Changes in blood pressure and heart rate of a *post partum* case in refractory stage of laminitis in the five weeks following acute laminitis.

Blood pressure and heart rate during the course of post partum laminitis

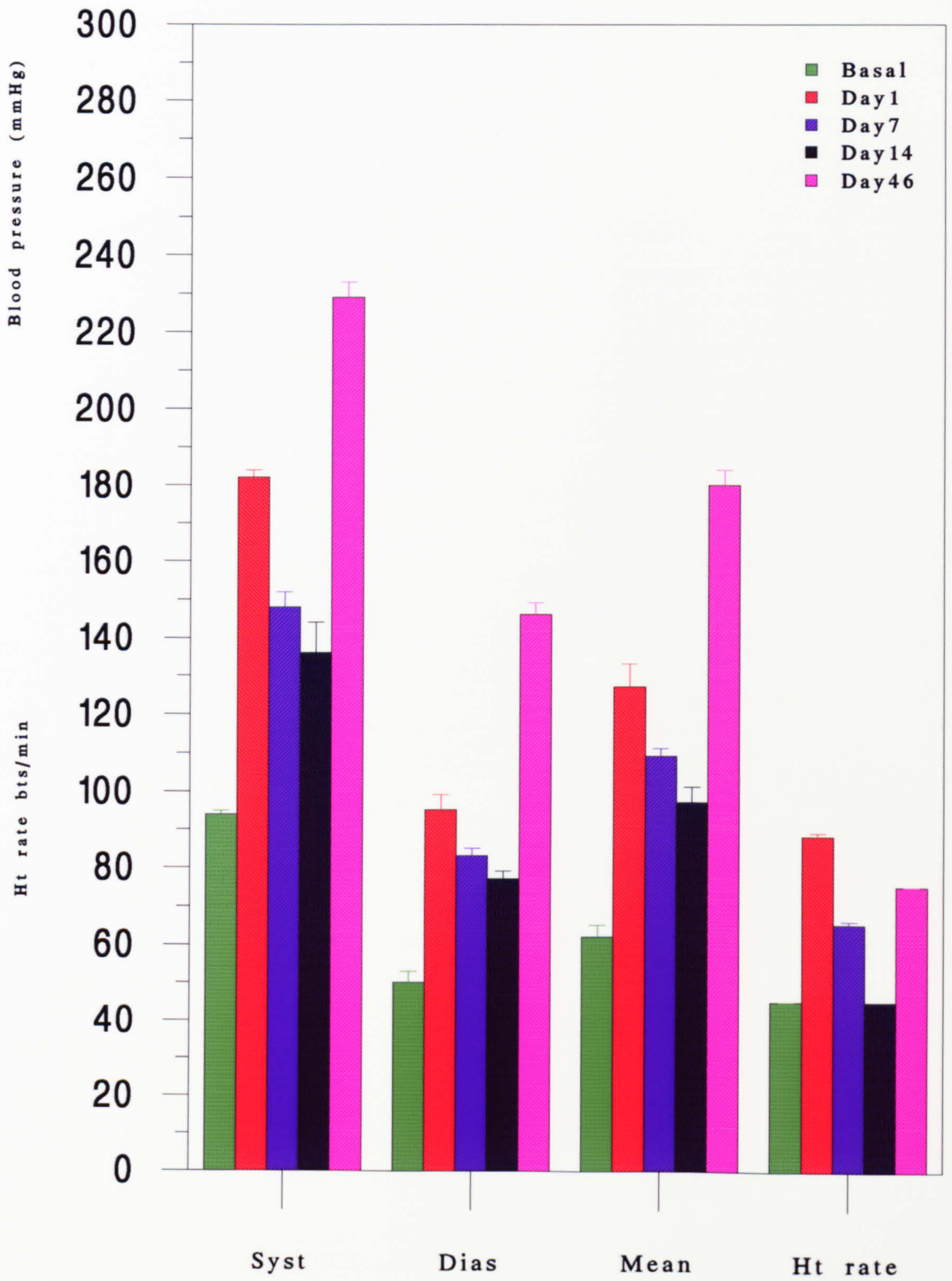


Figure 3.7 Radiograph (lateral - medial) of left digit of the *post partum* case showing 'sinking' of the weight bearing bony column through the hoof capsule, taken in the sixth week after the acute stage.



Table 3.17 A summary of Mean BP and heart rates of treated post partum acute laminitis over the first two days was compared with normal horse's basal values.

	Blood pressure (mmHg)				Heart rate			Obel Grade
<i>Biscuit</i>	Systolic	%inc	Diastolic	%inc	Mean	%inc	(bts/min)	%inc
Normal	95 ± 1		52 ± 1		66 ± 1		50 ± 3	
(n=15)								
Number of tests =87								
Acute laminitic	144* ± 8	51%	86* ± 3	65%	107* ± 4	62%	86* ± 4	72%
(n=1)								
Number of tests =30								

Significant difference * ($p \leq 0.001$).

3.3.3 Chronic laminitis

Blood pressures and heart rates of horses and ponies with chronic laminitis

Horses and Ponies Mean values of BP and heart rates of horses and ponies with chronic laminitis during winter months and summer months are shown in Tables 3.17 and 3.18 respectively. None of the mares was pregnant. Horses and ponies were generally combined in one group as numbers were small. Blood pressures of chronically laminitic ponies are compared with normal ponies in Figures 3.8. Individual data are shown in Appendix I.

Table 3.17 Mean BP and heart rates of horses and ponies with chronic laminitis taken during winter months.

	Systolic	Diastolic	Mean	Ht rate	Obel Grade
C.Laminitic	mmHg	mmHg	mmHg		
Rose Marie	95	58	70	40	4
Beau	109	56	77	40	1
Domino	88	52	62	39	1
Tess	109	60	81	39	0
Misty	97	55	72	47	0
Cobweb	98	57	72	44	0
Mean	99	56	72	42	
SE	3	1	3	1	
Range	(88 - 109)	(52 - 60)	(62 - 77)	(39 - 47)	
n = 6					
Number of tests = 18					

Table 3.18 Mean BP and heart rates of chronic laminitic ponies taken during summer months

	Systolic	Diastolic	Mean	Ht rate	Obel Grade
C.Laminitic	mmHg	mmHg	mmHg		
Cobweb	125	61	81	55	
Domino	105	54	72	50	
Misty	122	68	95	64	
Tess	107	43	54	48	
Group Mean	115	57	76	54	
Standard Error	5	5	9	4	
Range	105 - 125	43 - 68	54 - 95	48 - 64	
(n = 4)					
Number of tests = 12					

Comparison of chronically laminitic ponies with normal controls. BP and heart rates, during winter and summer months, of ponies with chronic laminitis were compared with normal values. The results are shown in Tables 3.19 and 3.20 respectively. Although there was no difference between the groups during winter, there was a significant increase in heart rate of chronically laminitic animals during summer months ($p > 0.05$).

Table 3.19 Comparison of mean BP and heart rates of chronic laminitic ponies with normal ponies during winter months.

	Blood pressure (mmHg)			Heart rate (bts/min)	
	Systolic	(% change)	Diastolic	Mean	
Normal (n= 6) Number of tests = 19	104 ± 7		59 ± 2	73 ± 3	43 ± 1
Chronic laminitic (n= 6) Number of tests = 21	99 ± 3 (-5%)		56 ± 1 (-5%)	73 ± 3	42 ± 1

There are no significant differences.

Table 3.20 Comparison of mean BP and heart rates of chronic laminitic ponies with normal ponies during summer months.

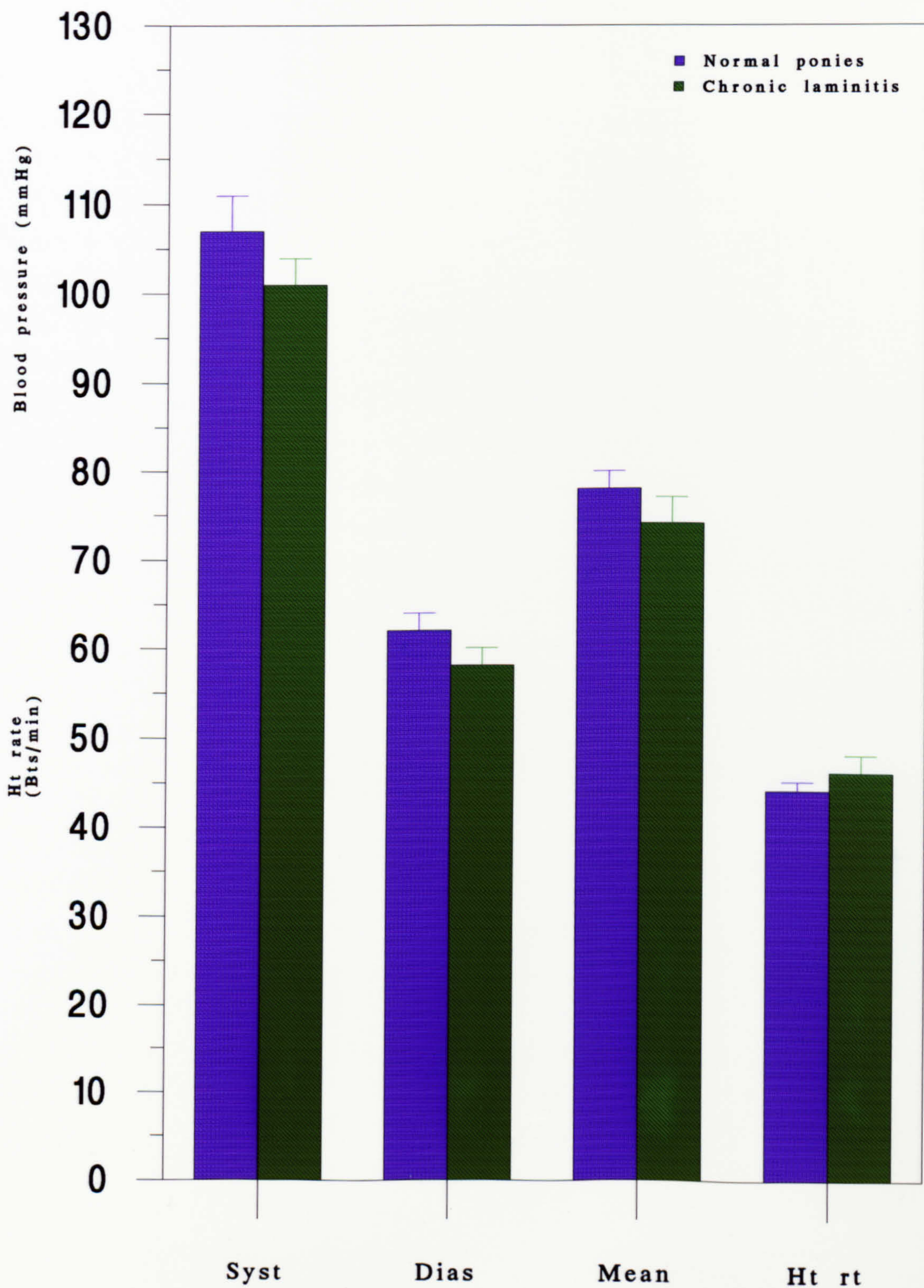
	Blood pressure (mmHg)			Heart rate (bts/min)
	Systolic	Diastolic	Mean	
Normal (n= 4) Number of tests = 12 Range	109 ± 6 (95 -125)	58 ± 1 (56 - 59)	76 ± 2 (71 - 80)	43 ± 2 (39 - 48)
Chronic laminitic (n= 4) Number of tests =12 Range	115 ± 5 (105 - 125)	57 ± 5 (43 - 68)	76 ± 9 (54 - 95)	54 ± 4 (48 - 64)

There is a significant difference in only in heart rate ($p < 0.05$)

BP and heart rate of ponies with chronic laminitis are compared with normal controls in Figure 3.8. Chronic laminitis cases are not hypertensive and there is no significant difference between the groups.

Figure 3.8 Blood pressure and heart rates of normal ponies and those with chronic laminitis.

Blood pressures and heart rates of normal ponies and those with chronic laminitis



Comparison of two cases of long term chronic laminitis in horses

Despite lameness both animals were normotensive and had normal heart rates. Figure 3.9 compares blood pressures and heart rates with normal horses. A comparison of the radiographic findings and Obel Grade is shown in Table 3.21. Radiographs of medial lateral views of the front hooves are shown in Figure 3.10.

Table 3.21 Comparison of BP of two cases of chronic laminitis in horses and comparison of Obel Grade of lameness and radiographic findings (a) and (b)

Case	Type	Obel Grade	BP	Radiographic findings
<i>Isadora</i>	Chronic laminitic	2	normal	rotation (a)
<i>Rose Marie</i>	Chronic laminitic	4	normal	rotation / remodelling P3 osteomyelitis (b)

Figure 3.9 Blood pressure and heart rates of normal horses and those with chronic laminitis.

Blood pressures and heart rates of normal horses and those with chronic laminitis

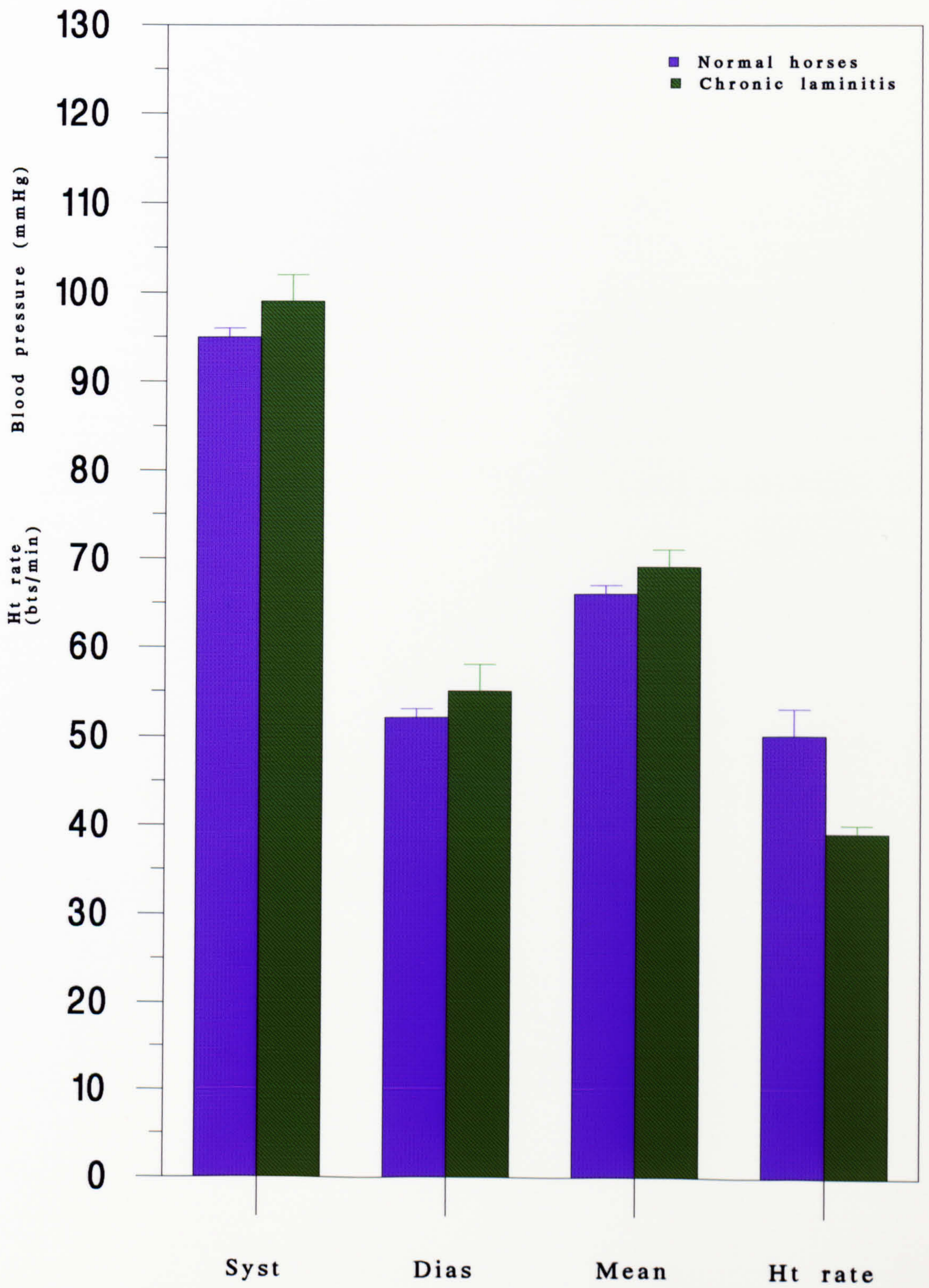


Figure 3.10 Radiography of lateral medial views of the distal portion of the front limbs of two chronically laminitic mares (a) Isadora (b) Rose Marie

a



b



3.4 Discussion

This is probably one of the most comprehensive, thoroughly controlled examination of blood pressure and heart rate in equines to date, with over 2000 measurements taken. Other reports suffer from lack of controlled management or stress of handling at veterinary hospitals. Normal basal blood pressures for both ponies and horses showed small variation which probably reflects lack of stress, and apprehension, during handling and the ponies were relaxed during the measurements. The homogeneity of the groups probably also reflects careful management of the animals and / or reduced number of variables and artefacts because of varying sodium intake or other dietary factors seen in other studies.

Normal equine BPs established here as 101 / 57 (72) - ranges 95 - 107 / 52 - 62 (66 - 77) are slightly lower than recently published values of 100 -132 / 70 -97 (82 -110) taken by other indirect methods (Vaala *et al.*, 1995). The reasons for the differences may be several. First, handling stress may have caused BP readings taken by veterinary practitioners to be slightly higher than values for ponies at home taken by their usual handlers in familiar surroundings. This assertion is supported by the fact that when experimental ponies first arrived at the field station in the Spring of 1994, apprehension caused a wider range of BPs (eg. 'Domino'). Secondly, the indirect method used in other studies may give generally higher readings than the Dinamap apparatus.

Published indirect values and those of the present values, are less than direct measurements. The cuff site, on the median/ ventral coccygeal artery is at a higher level relative to the heart and BP values obtained at this site would be lower than direct measurement at the aorta simply because of the effect of gravity (Ganong, 1991; Levick, 1992). The facial artery site for direct measurement is roughly between the two sites and would be roughly equivalent to the pressure readings taken at the tail site. The difference in level would be less in ponies than in horses i.e the height in metres between

the respective sites is smaller in those animals with a smaller height and body size (ponies) than in horses. Quite simply, the difference in level between facial and coccygeal arteries is less in ponies and greater in horses. Therefore, indirect readings would be closer to the direct readings in smaller animals. This observation is supported by the fact that in this study basal values for ponies are nearer than horse values to published values, which are taken from small Thoroughbred horses. The Thoroughbred, which has a body size somewhere between ponies and Irish Draught horses, provides normal equine blood pressure of 112 /77 mm Hg given in a standard veterinary texts (Blood and Radostits, 1989); these direct values were obtained from Thoroughbred horses (Parry, 1984). BP values in the present study are greater for ponies - 107 / 62 mm Hg than horses 95 / 52 mm Hg . The differences between published values and those in this study are a combination of the differences between direct and indirect measurement methods and the differences due to relative distances between measurement sites.

Measurements at different sites are not necessarily equivalent in physiological terms. As the pressure 'wave' moves along the vessels, the narrowing and branching of the arteries and arterioles, together with wall resistance (Poisuille's Law) initially increases pressure. This eventually decreases with distance from the heart (Berne and Levy, 1981; Levick, 1992) and when vessels are narrowed - Bernouilli's Principle (Ganong, 1991). Peripheral arterial pressure is therefore less than that at the aorta. Which blood pressure is the 'real' blood pressure - direct or indirect ? As Dr L Young (1994) notes " Blood pressure - more questions than answers ?". This question is often asked and largely remains unanswered (Ganong, 1991; Levick, 1992). Nonetheless, blood pressure is a most useful and immediate way of assessing clinical normality and abnormality. The Dinamap apparatus could be calibrated with direct cardiac measurements and at other sites, to find a relationship between direct and indirect values, so that a constant value for data adjustment could be developed. Unfortunately, the variation in methods of assessment and the difficulties mentioned above (body size and gravity; peripheral and central

pressure) make any calibration of direct and indirect measurements is at best inconclusive, even superfluous. Perhaps the most practical and scientific protocol is to establish basal values using the equipment that will be used in the study to compare diseased and control BP; that is relative changes. The Dinamap machine was used to establish basal values for normal animals which in many cases acted as their own controls. Therefore, changed BP could be identified when they occurred.

Measurements taken at the same time of day minimised diurnal variations that are evident in man (O'Brien *et al.*, 1991; Staesson *et al.*, 1992). The present study did not reveal differences in summer or winter BPs of normal horses or ponies or between pregnant and non-pregnant mares. However, there were significant differences in BP, but not heart rate, between the horse group and the pony group. It has been suggested that larger animals have lower BP because of body size and possibly lower metabolic rates (Schmidt - Neilsen, 1991). One study of blood pressure of Swedish Warmblood horses and Arabian horses and cross bred ponies did not find a correlation between bodyweight and blood pressure (Ostlund *et al.*, 1983) Although, the basal BP values of ponies were lower than those of horses, and may be explained by smaller body mass, the differences in relative heights of measurement sites are a more likely explanation, *vide supra* .

No age related differences were observed although these have been described in horses (Covington and McNutt, 1931; Ostlund *et al.*, 1983), humans and other mammals (Ganong, 1991).

Acute laminitis

Desleins (1935) was the first to associate hypertension with equine laminitis but this observation was not pursued for forty years (Garner *et al.*, 1975). Since then studies, using a variety of methods, on the experimentally induced form of the disease have determined blood pressures (Garner *et al.*, 1975; Harkema *et al.*, 1978) cardiac output (Templeton *et al.*, 1985; Harkema *et al.*, 1978) and plasma volume (Clarke *et al.*, 1982; Harkema *et al.*, 1978) during the acute stage. In addition, digital blood pressures have been measured using direct methods (Robinson *et al.*, 1976). Increases in systolic and diastolic pressures and plasma volume occur when Obel Grade 3 lameness is evident following experimental induction of the disease. Hypertension during acute laminitis has been suggested to be because of increased renin activity and plasma aldosterone (Clarke *et al.*, 1982). Angiotensin values, extrapolated from plasma renin activity, have been suggested to be part of hypertensive processes in laminitis (Hood, 1979). On this basis, and perhaps because the 'bounding digital pulses' are clinically obvious, hypertension has been accepted as a clinical occurrence (Yelle, 1986; Stashak, 1987).

Systemic hypertension during acute laminitis is now 'horse-lore' in veterinary circles and there has been much discussion on the possible causes of hypertension (Hood, 1979; Clarke, 1982; Purhohit, 1981.; Yelle, 1986; Stashak, 1987) In reality, the evidence is scant and arguable (Garner *et al.*, 1974) and often equivocal (Hood *et al.*, 1993). Documented studies only refer to experimentally induced laminitis (Garner *et al.*, 1975; Harkema *et al.*, 1978) which is not equivalent to laminitis of other aetiologies. Even before laminitis was induced, basal values of arterial pressure and heart rate were higher than stated normal values, even for normal controls. For example, the basal heart rate of normal ponies prior to the experiment was 84 beats /min (Harkema *et al.*, 1978) while normal equine heart rate is usually 38 - 42 beats /min. Increased basal BP values reflect stress at the start of the experiment which influences plasma hormones, especially catecholamines and angiotensins.

When laminitis is induced experimentally, only approximately 50 % develop laminitis and subsequent hypertension; 25 % remain clinically normal; and 25 % die of shock (Garner *et al.*, 1975; Garner *et al.*, 1978; Hood *et al.*, 1982; Trout, 1990). In fact, 10 - 15 % of horses given carbohydrate overload suffer fatal circulatory collapse within 32 hours of induction (Sprouse *et al.*, 1987) ; the possible reasons for this are discussed in Chapter 5. It is rare for horses to die of shock during laminitis when the disease is spontaneous, suggesting that the progress of the disease differs under the two circumstances.

In the investigations of spontaneous laminitis presented in this thesis, hypertension was observed at the onset of the acute phase and considerable increases in BP were seen when compared with the animal's own BP recorded in the months before the disease. There was a marked increase in systolic, diastolic and mean BP at the onset of clinical signs and Obel Grade of lameness. Lameness (Obel Grade) is closely correlated with an increased BP during the acute stage. Mean BP increased between 104% and 124 %.

One unusual case of acute laminitis was not particularly hypertensive, compared with established blood pressures, but still exhibited clinical signs of laminitis. This mare was usually hypotensive and showed an increase of mean BP of just 10 % from her own data prior to the onset of disease. The BP of this case was still within normal range even when elevated during the acute phase. This animal however had an Obel grade of 2 and the case was mild. This mare had a lower than usual heart rate during the acute phase and it may be that relative bradycardia lowered blood pressure with compensatory CNS mediated peripheral vasoconstriction to maintain blood pressure. It is possible that adrenergic vasoconstriction of peripheral vasculature could compromise the laminar circulation and resulting in low grade ischaemia. The assessment of the case was complicated by the fact that this mare also suffered chronic obstructive pulmonary disease (COPD). Other possible causes of local peripheral vasoconstriction, without systemic hypertension, include endothelin. This could be a key factor since hypoxia would cause synthesis of endothelin which would have a specific local effect on pedal

vasculature without being apparent in systemic measurements. Alternatively, laminitis under these conditions could be purely mechanical and the hoof morphology could be changing without any systemic response being evident. Such changes are usually found in the chronic stages.

It is hard to compare results with data accumulated from acutely diseased animals after induction of laminitis, collected at veterinary hospitals or experimental stations (Harkema *et al.*, 1978 ; Hood *et al.*, 1978; Templeton *et al.*, 1985) and there are no data for comparison which have been recorded under carefully controlled non-hospital conditions and with a spontaneous aetiology. There are enormous physiological variations inherent in the induction of the disease by oral carbohydrate overload. The process of induction by nasogastric intubation / administration of carbohydrate overload is very stressful and the method of induction invariably involves diarrhoea; both of these inevitably alter endocrine status and plasma electrolytes. Results taken under such conditions are hardly typical of the more usual aetiologies of laminitis. An exchange at the AAEP Congress 1974 distils the argument on the similarities or differences between spontaneous and induced laminitis:

"Is the pathogenesis of founder in ponies on spring pasture the same as seen in your model ?"

"I believe there may be some common denominator. However, one has to be very careful in extrapolation. I think it is a form of alimentary laminitis." (Garner, 1974)

In cases of acute laminitis of mild to moderate severity, hypertension is transient and in most ponies blood pressures and heart rate return to normal within days, or a week, if treated with nitric oxide donors and/or phenylbutazone (see Chapter 5). It is usually assumed that all stages of laminitis involve hypertension (Yelle, 1986; Sprouse *et al.*,

1984) Very few data are available for blood pressures taken immediately after the acute phase of the disease .

Refractory laminitis

The only hypertension that was refractory to treatment in this study was that of the *post partum* case. Although treatment with analgesics, vasodilators (nitric oxide donors) and sedation using α -adrenergic blockade was continued, the hypertension was unresponsive. Blood pressure and heart rate decreased over the first 4 weeks but were still considerably higher than normal (Figure 3.6). If catecholamines and CNS responses are mediating pain, vasoconstriction and an increase in heart rate, then it is strange that α -adrenergic blockade appears to be so ineffective. It is also strange that despite some signs of clinical improvement (the mare was able to be kept at grass) that BP and heart rate did not significantly reflect this improvement. It is possible, indeed likely, that other factors pertained. It is possible that baroreceptors are 're-set' at a higher range during prolonged hypertension. Alternatively, increased circulating catecholamines and / or administration of adrenergic blockade might cause up-regulation of α -adrenergic receptors to increase responsiveness to catecholamines. Under these circumstances an ever increasing amount of α -adrenergic blockade would be necessary to relieve pain. Receptor binding studies are obviously necessary to investigate this possibility. It is also possible that prolonged high doses of all drugs (except glyceryl trinitrate) led to renal damage and increased circulating angiotensin. It is impossible to quantify the mare's pain that was suffered despite treatment but the breakdown of pedal structures must be profoundly painful. Acute hypertension was recorded during mechanical breakdown of hoof structures (sinking) some 7 weeks after onset of laminitis of endotoxaemic aetiology, when there was uncontrollable pain despite analgesia. In one study when each limb received a digital 'nerve block' of lignocaine topically, lameness improved and blood pressure fell (Hood *et al.*, 1979). The *post partum* case of laminitis was receiving substantial amounts of analgesics, but her heart rate and blood pressure were nonetheless high, suggesting that pain is not the only consideration. At this point

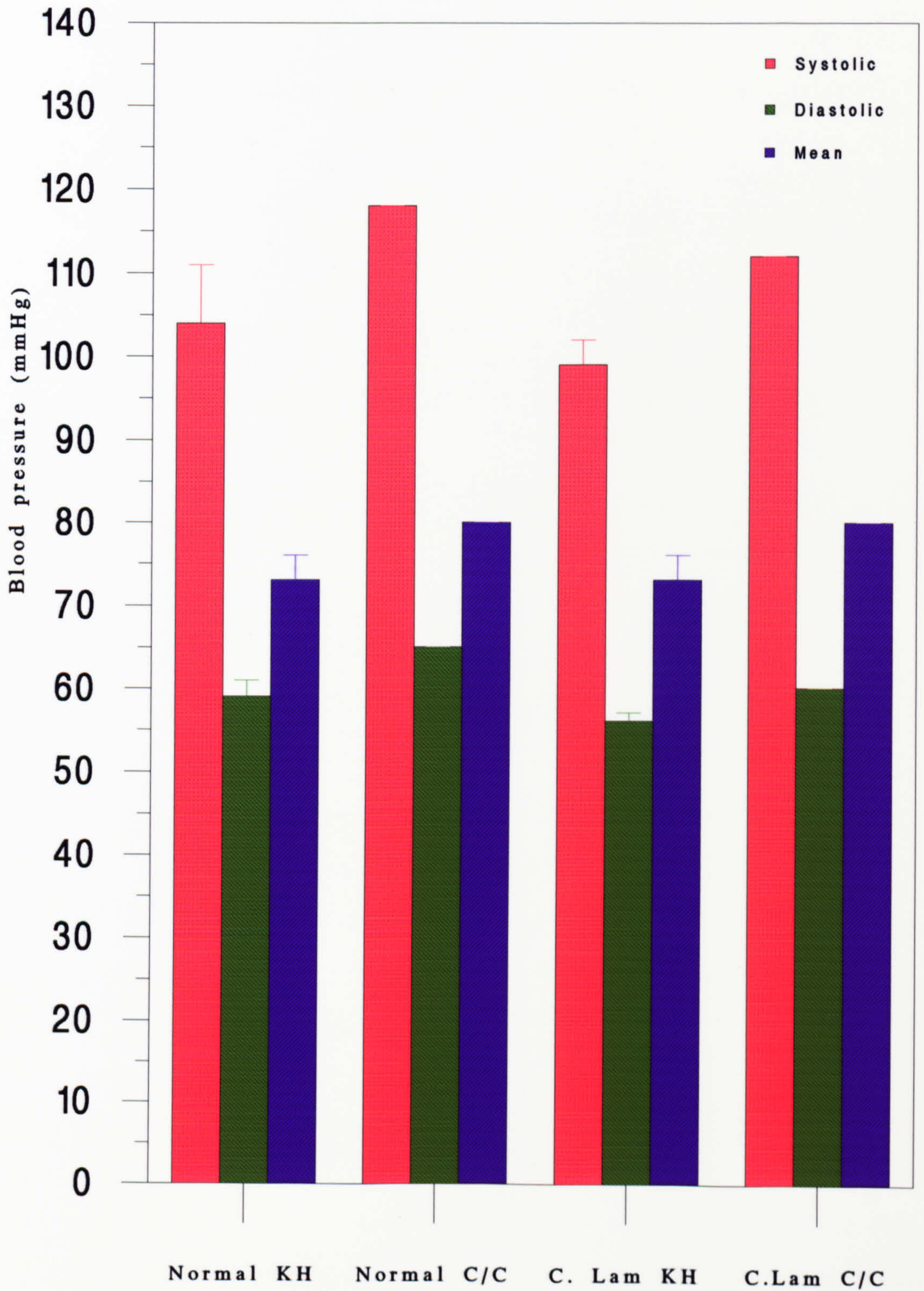
the highest ever readings for blood pressure and heart rate were recorded. The simplest explanation is that the observed hypertension was, at least for the most part, a response to pain. There may be variation in the individual's capacity to deal with pain. However, pain responses alone may not account for increased BP. Other possible causes of hypertension were investigated, in relation to plasma levels of angiotensin (Chapter 6); endothelin (Chapter 8) and serum electrolytes (Chapter 9).

Chronic laminitis Blood pressures of chronic laminitis cases under normal conditions (that is outside veterinary hospitals) have never been established. The data presented in this thesis shows that chronically laminitic horses and ponies are not hypertensive. If anything, chronic laminitis cases were slightly hypotensive during winter compared with normal controls kept under the same conditions. These data are contrary to the findings of Sprouse *et al.*, (1984) who suggested that chronic laminitic ponies are hypertensive. His ponies had BPs of $161 \pm 11 / 99 \pm 6$ mm Hg compared with $128 \pm 2 / 76 \pm 3$ mmHg of normal controls, but these ponies were relatively recent arrivals at a veterinary hospital and may have been stressed (particularly if they remembered the pain of previous encounters during an acute phase). Similarly high, but not significantly different, BP were reported in normal and chronically laminitic ponies of $131 \pm 6 / 83 \pm 3$ mm Hg and $146 \pm 11 / 82 \pm 5$ mm Hg respectively Rugh *et al.*, (1984). Rugh *et al.*, (1984) found that chronically laminitic ponies (n=6) had very similar BP to normal ponies (n=5) but this changed if both groups were fed a high carbohydrate diet; under these circumstances chronic laminitics became hypertensive compared with normal controls, showing increases of $32 \pm 8\%$ and $26 \pm 5\%$ respectively. The present findings in this thesis are concur with those of Rugh *et al.*, 1984 who found that chronically laminitic horses and ponies are normotensive.

One report on a slightly different but related subject (Coffman and Colles, 1982) recorded similar BP results for normal and laminitic groups of ponies using indirect Doppler shift methodology. There were slight, but not significant differences, in BP

Figure 3.11 Comparison of blood pressures of chronically laminitic ponies in the present study with those at the Animal Health Trust, (Coffman and Colles 1982).

Comparison of BP of normal and CL ponies in this study (KH) and at the Animal Health Trust (Coffman et al., 1972)(C/C).



between normal and chronically laminitic groups with the laminitic group having slightly lower BP than normal controls. However, it is noticeable that the differences between the winter readings in this study and those in the Coffman report, seem to be of the same magnitude i.e. 5 and 3 mm Hg for systolic and diastolic respectively in this study, compared with 6 and 5 mmHg respectively. One concludes that there are no real differences between the blood pressures of normal and chronic laminitic ponies.

Results in this study show a significant increase in heart rate between chronic laminitic ponies at grass in the summer and their normal counterparts. There are many possible mechanisms for the increased heart rates. It is possible that these animals are developing the disease at a very early stage which is reflected in the increased heart rate and subclinical pain. There were one or two occasions when hypertension was noticed in chronic laminitics during the summer but as they were stabled there was no possibility of telling if this was the developmental stage of laminitis. It may be that these ponies have a different metabolism and response to water soluble carbohydrates which is shown in obesity and a greater stress on the heart.

If BP is correlated with Obel Grade during the acute phase then it is logical to assume that this may be the case in chronic stages too. Reports from the AAEP Congress 1974 suggest that this is the case:

"Did you say that blood pressure stays elevated throughout the course of chronic laminitis for days , weeks or months ?"

"If the horses are lame due to laminitis, they are hypertensive" (Garner, 1974)

Unfortunately , no data were provided to support this assertion.

In the present investigation, BP in chronic laminitis was not related to Obel Grade. Chronic laminitics are normotensive whatever the Obel grade of lameness. In individual cases of long term chronic laminitis, with Obel Grades 2 and 4, both cases studied were normotensive. Radiographic evidence revealed that the first case, Isadora, who had

repeated attacks of laminitis over the previous 4 years had rotation and some remodelling of P3. The latter case had suffered one attack 6 years previously but none since. This case had rotation evident on radiography and considerable osteomyelitis and necrosis of P3. There were visible signs of morphological changes to the hooves and this mare was very lame. However, the fact that they are normotensive despite lameness suggests that there are different mechanisms responsible for the lameness and that CNS mediated pain responses are not primarily involved. Long term chronic laminitis have orthopaedic changes which cause lameness (eg pedal bone remodelling) which causes lameness and immobility but does not result in hypertension. The lameness in long term chronic laminitis cases should therefore be considered to be a disability rather than profound pain. The assessment of BP under clinical conditions would be a useful means of distinguishing between laminitic disability and the lameness that is associated with another acute attack - which needs urgent treatment.

In conclusion, hypertension during acute laminitis is usually transient when the disease is treated. The worst acute cases stay hypertensive despite all types of treatment. Refractory cases often maintain tachycardia and hypertension and their prognosis is poor. Heart rate may be an accurate indication of the eventual prognosis. The prognosis for those animals that have a high heart rate even when lameness improves must be guarded and in clinical situations these may be the ones that need the closest attention.

Whilst there is a correlation of Obel Grade of lameness, in acute and refractory stages, with blood pressure, the original change in BP does not give any indication of the prognosis. The grass induced laminitis had a greater increase in mean BP than the *post partum* case, but the grass induced case recovered with treatment whereas the *post partum* case could not be saved whatever the treatment and management. Chronic laminitis cases are not hypertensive whatever the lameness.

The role of vasoactive hormones and plasma/serum biochemistry in the regulation of blood pressure are considered in later chapters (see Chapters 6, 7, 8 and 9).

Chapter 4 Haemodynamics of the equine hoof

	Page
4.0 Introduction	133
4.1 Infra Red Thermography	
4.1.1 Introduction	136
4.1.2 Materials and Methods	137
4.1.3 Results	138
4.1.4 Discussion	145
4.2 Near Infra Red Spectroscopy	
4.2.1 Introduction	149
4.2.2 Materials and Methods	149
4.2.3 Results	157
4.2.4 Discussion	163
4.3 General Discussion	170

" And Life is Colour and Warmth and Light...."

Julian Grenfell 1888 - 1915 'Into Battle' 1915

4.0 Introduction

The anatomically elaborate and complex vasculature within the equine hoof has been considered in Chapter 1. The equine digital vascular system is sufficiently robust to tolerate the obvious mechanical stresses of weight bearing and to dissipate concussive forces during movement consonant with appropriate haemodynamics of tissue perfusion.

The gross anatomical arrangements of the digital arterial tree and venous drainage (Kruger, 1934; Schummer, 1951; Mishra, 1982) alongside the microanatomy of the smaller digital vessels have been described, using a combination of angiographic perfusion and electron microscopy (Mishra and Leach, 1983 a,b; Pollitt and Molyneux, 1990). These studies describe the dermal laminar tissues and reveal the presence of arterio-venous anastomoses which potentially shunt blood away from the terminal portions of the hoof vasculature. Electron microscopy of perfusion casts have revealed a number of macroscopic vascular lesions within the laminitic hoof. These descriptions have all employed disarticulated hooves and clearly may not necessarily reflect the arrangement *in situ*.

To place the anatomical arrangements into a functional context, qualitative and quantitative blood flows within the hoof should be assessed to define local and systemic factors that influence pedal haemodynamics. With varying success, a variety of invasive and non-invasive techniques have been applied to determine blood flow to,

and distribution within, the hoof. The techniques and approaches applied have included radiography / angiography, using radioactively labelled materials and contrast media, electromagnetic flow probes, dye dilution and extrapolation of data obtained from the measurement of vascular resistances (Coffman *et al.*, 1970; Robinson, 1976; Hood *et al.*, 1978; Scott and Sandler, 1978; Trout, 1990). The problem with such invasive methods is that they require anaesthetics, surgery to the digital vessels (which may cause vasospasm, or thromboembolism) and the sizes of the molecules used to track vessels are sometimes similar to the lumen of the vessels under study. If smaller particles are used these may permeate into the interstitial fluid in the area of study (Robinson, 1990). Similarly injection of exogenous agents may produce secondary vascular responses or modify mechanical relations within the pedal microvessels (Robinson, 1990). Invasive studies can be criticised adversely since the procedures produce artefacts from secondary cardiovascular responses. (Robinson, 1990). At least in part, for these reasons, there is no consensus as to the precise nature of pedal vascular perfusion and, more especially, what occurs in either the acute or the chronic laminitic hoof.

Most recently Hood *et al.* (1994) and Adair *et al.* (1994) have used semi-invasive methods, radioactively labelled macromolecules and Laser Doppler Flow Probes respectively to assess pedal blood flow. The Doppler system, successfully applied in human clinical practice, involves subcutaneous implantation of the probe and has been used in horses to assess muscle blood flow (Sertyn *et al.*, 1986). If used in the equine hoof then a series of small holes have to be drilled in the hoof wall to expose laminar tissue; this procedure is semi-invasive and therefore not ideal under field conditions. Two other non-invasive methods have been applied - thermography (Turner, 1981; Turner, 1991) and, to disarticulated limbs, nuclear magnetic resonance imaging (Denoix *et al.*, 1993). Magnetic resonance potentially has greater usefulness but is

expensive and not presently available for *in vivo* studies. Infra red thermography has been used to investigate lameness (Turner 1980; Turner 1991), tendon damage in racehorses (Webbon,1978), and to assess the effects of neurotomy and administration of adrenaline to horses (Purohit, 1980). Thermography has not been used to evaluate putative differences in digital blood flow in normal and laminitic horses. There is clearly a need for a satisfactory non-invasive means to examine pedal haemodynamics of the horse's hoof. Indeed, a really effective method would have a major impact in this field. Preliminary investigations into afferent and efferent blood flow, and into the recording of hoof wall temperatures, is outlined in part 4.1 below. Finally, bearing in mind that the *in vivo* study of soft tissue within the hoof is equivalent in difficulty to appraisal of cerebral vascular disturbance, an entirely new method of estimating blood flow is studied in Chapter 4.2 - Near Infra Red Spectroscopy

4.1 Infra Red Thermography

4.1.1 Introduction

Anyone injured in sport will vouch for the heat that accompanies painful soft tissue inflammation and injury. Even before swelling is evident, pain and heat will be present, reflecting the increased blood supply, associated with inflammation, of the injured area (Ganong, 1991). Thermography is based on the infrared part of the electromagnetic spectrum. Infra red light radiates from surface tissues which enables the surface temperature of the skin to be mapped. Thermography has been used in human clinical medicine to detect early signs of illness and sub-clinical injuries (Land, 1987 a,b). Microwave thermography, using a different wavelength, penetrates deeper body tissues and can detect vascular malignancies such as breast cancer (Barret *et al.*, 1980) and appendicitis (Stallard *et al.*, 1987). Microwave thermography wavelengths are more suitable than those of infrared thermography when investigating deep digital tendon injuries and will detect deep seated damage within the tendon (Marr, 1990; Marr, 1992). Unfortunately this technique is not suitable for the soft tissue within the hoof as the light 'beam' will not penetrate the hard exterior of the hoof. Infra red thermography can assess soft tissues injuries in horses, particularly injuries of the limbs (Webbon, 1978). The principle has been of considerable use to racehorse trainers who have assessed superficial tendon conditions in horses prior to competition with a relatively simple infrared thermometer (Turner *et al.*, 1980; Palmer, 1981). Thermography can be used on the vessels of the limb to visualise the peripheral circulation of the coronary band. Anecdotally a 'warm hoof' accompanies acute laminitis (Robinson, 1990), and although infrared thermography will not detect the circulation within the hoof, surface hoof temperatures could be recorded. The present study assesses infrared thermography in recording temperature differences in limbs and hooves of normal and laminitic horses

4.1.2 Materials and Methods

Animals. Two horses and seven ponies were kept under controlled husbandry of feed, exercise and routine care. Of these, 5 were classified as 'chronic laminitic cases' having a history of the disease in previous years. The study was conducted during late spring when ambient temperatures were not too cold, but when the animals were still receiving hay fodder so it was unlikely that any of the animals were in the developmental stages of laminitis. None of the animals was shod but all had received regular foot trimming. Hooves were washed with lukewarm water to remove surface debris and dried with a towel. The horses were left to stand for at least 50 minutes inside a large barn so that normal hoof temperature was restored. Images were obtained in an anterior -posterior direction and in a lateral -medial direction.

Equipment

A thermal imaging system (Land Cyclops TI35 sm, Land Infrared, Dronfield, Derbyshire) was used to record infrared images. This machine is battery operated with a temperature range of -20 - 1500°C. Temperature resolution is 1°C and sensitivity is $\pm 1\%$ at the low range of up to 120°C. Calibration is internal for ambient temperature and for atmospheric absorption - both of which are recorded with data. Thermal images are stored on video (Sony Video Walkman System). Images were processed using a LIPS image processing system (Land Infrared, Dronfield, Derbyshire). This software provides temperature profiles from chosen points in the image, including the range of values, means and ambient temperatures. Thermal images, charts and temperature profiles were printed on a colour printer (HP Paintjet, Hewlett Packard).

Statistics

Means were calculated, and statistical differences between groups assessed with a Student's t-test, using Microsoft Excel software (Microsoft, USA).

4.1.3 Results

A thermal image of a hoof was obtained. The image can be produced in a variety of colours and the specificity / sensitivity of the colour range selected on the software. In other words, the recorded image remains the same but how it is reproduced may be modified. Colours normally range from dark blue or black (coldest) to yellow, orange and red (hottest) but black, grey and red can be selected for increased sensitivity. Differences in colour are shown on the scale beside each image. A thermal image of a normal equine foot showing anatomical landmarks was compared with a diagram of the digit showing superficial major vessels (See Page 6, Figure 4.1).

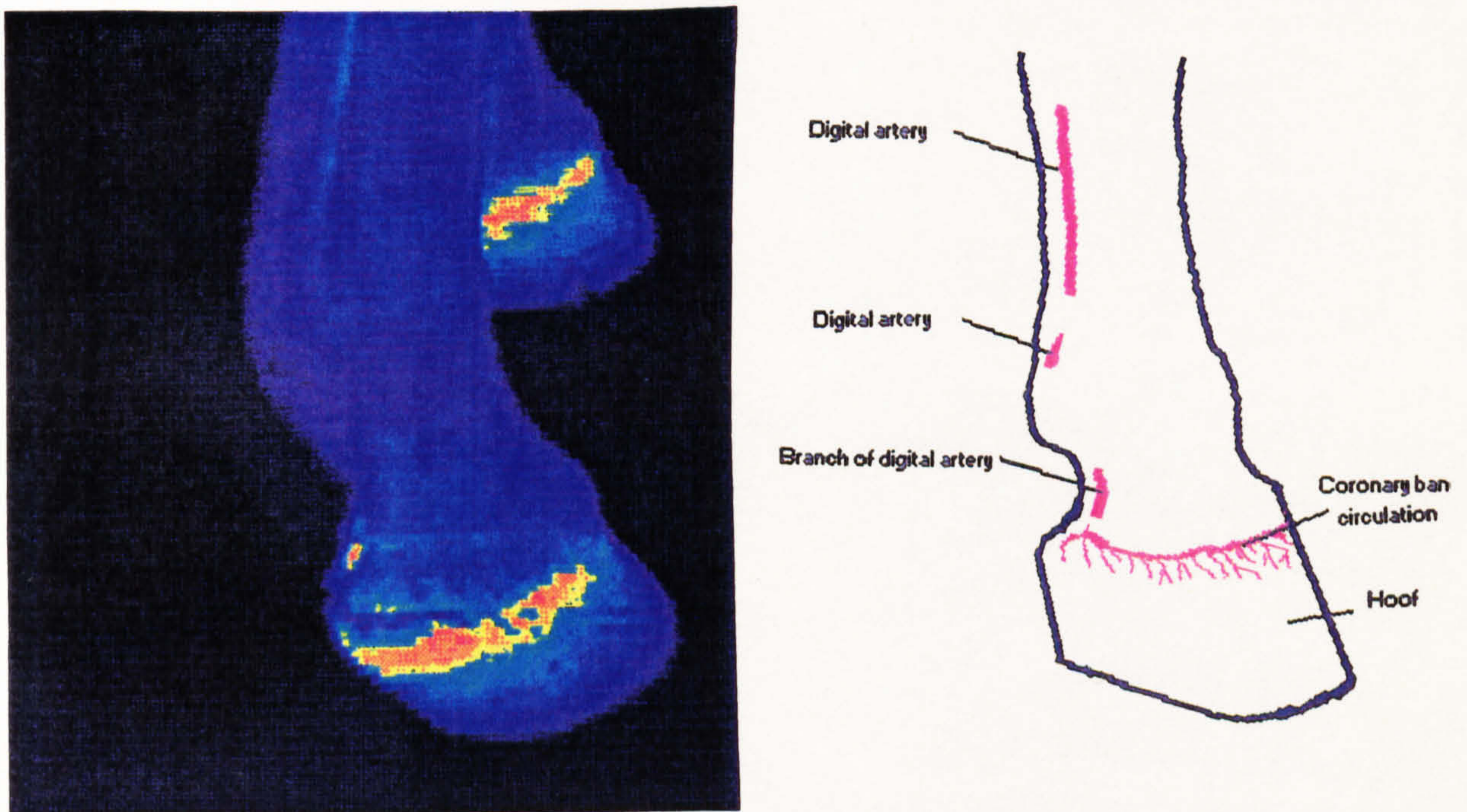


Figure 4.1 Thermal image of a normal horse's hoof (a) compared with a diagram of the external anatomy (b)

Spot temperatures can be identified from the recorded image. This can be done for multiple points on a single limb or two limbs recorded simultaneously (Figure 4.2). The warmest areas on all horses and ponies studied were found on the coronary bands.

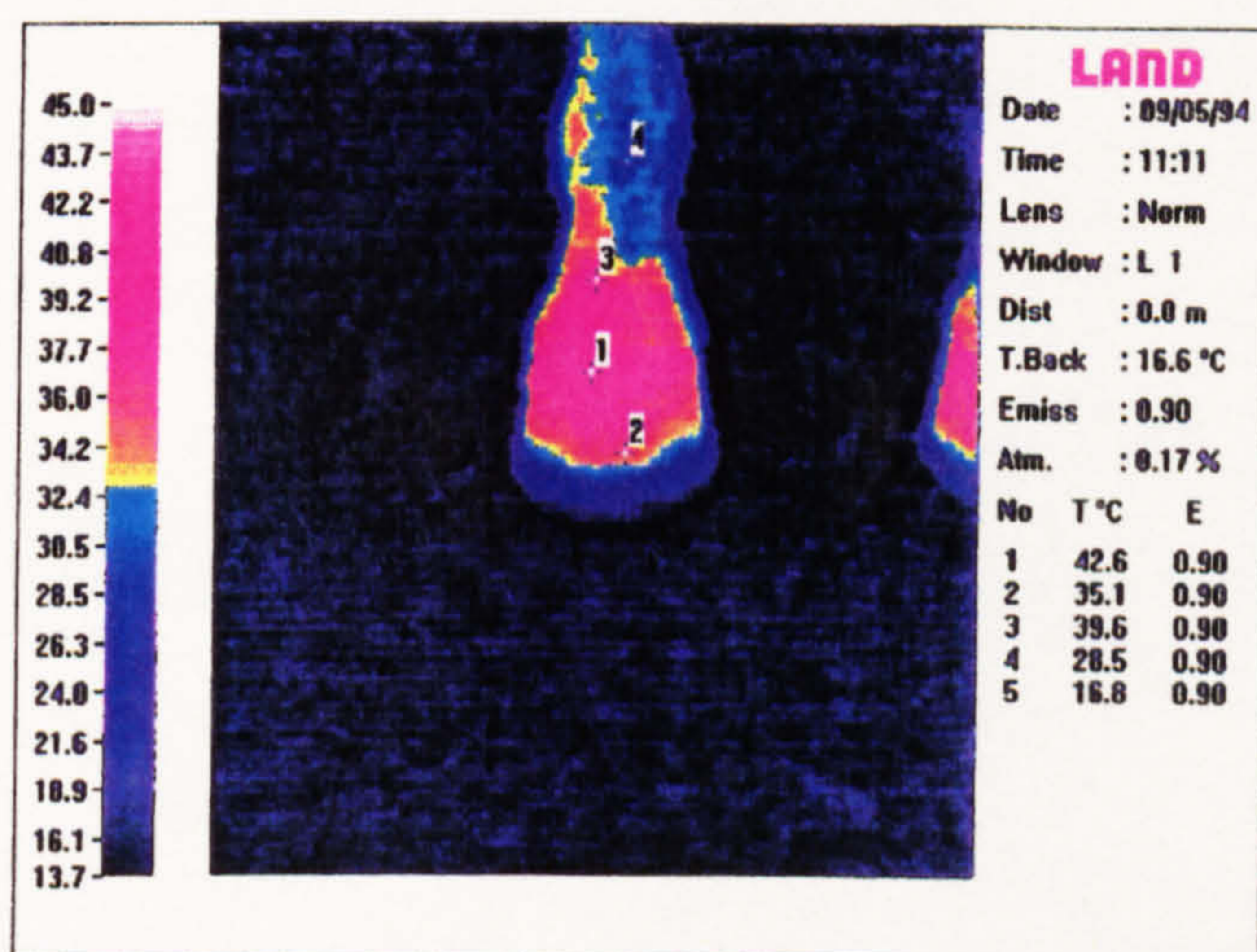
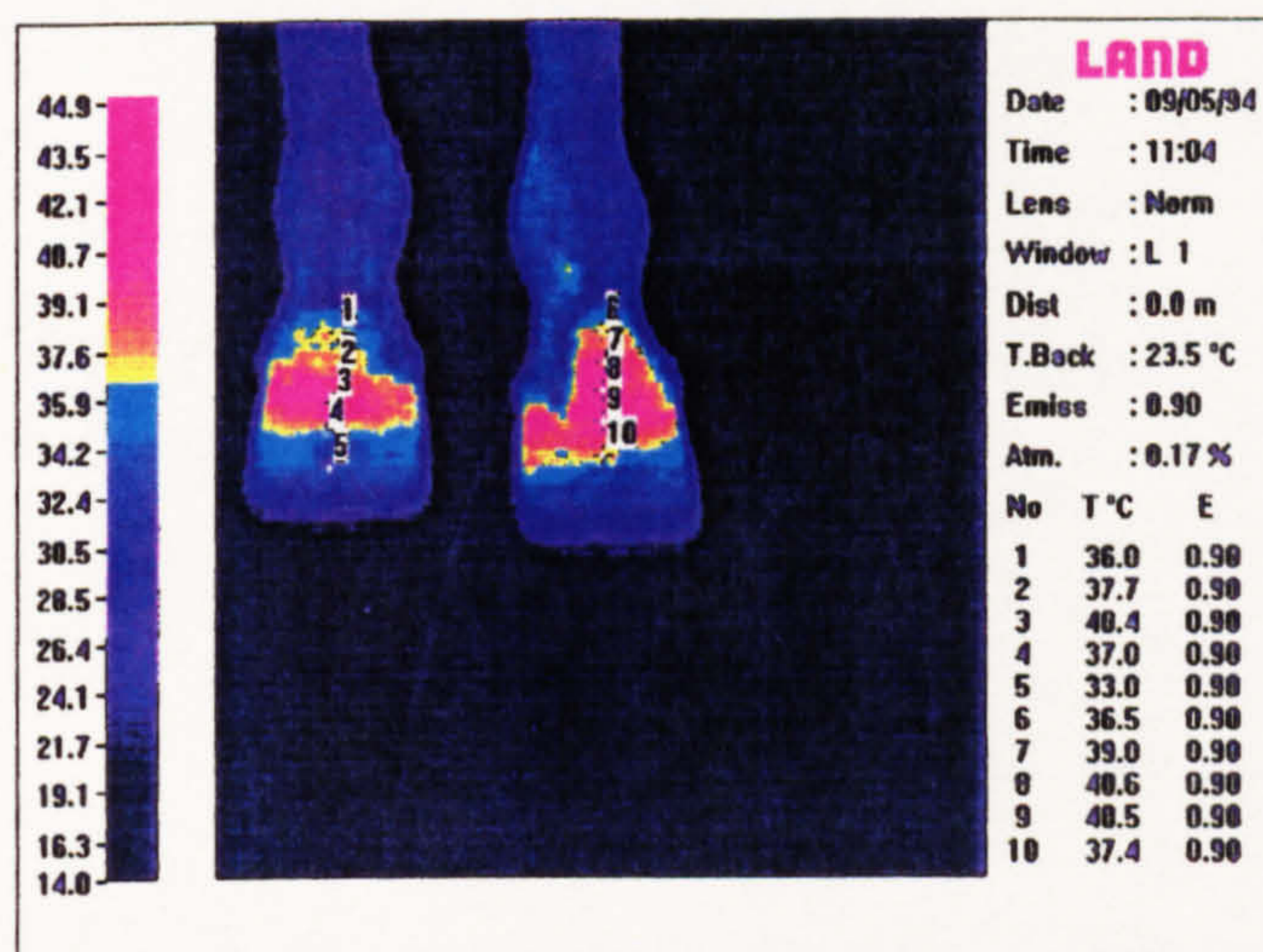


Figure 4.2 'Spot' temperatures taken from recorded data showing different points on one limb(a) or two limbs, recorded simultaneously (b)

The surface temperature gradients across a chosen line can be plotted as a temperature profile chart. These profiles allow maximum, minimum and mean temperatures to be calculated. (Figure 4.3).

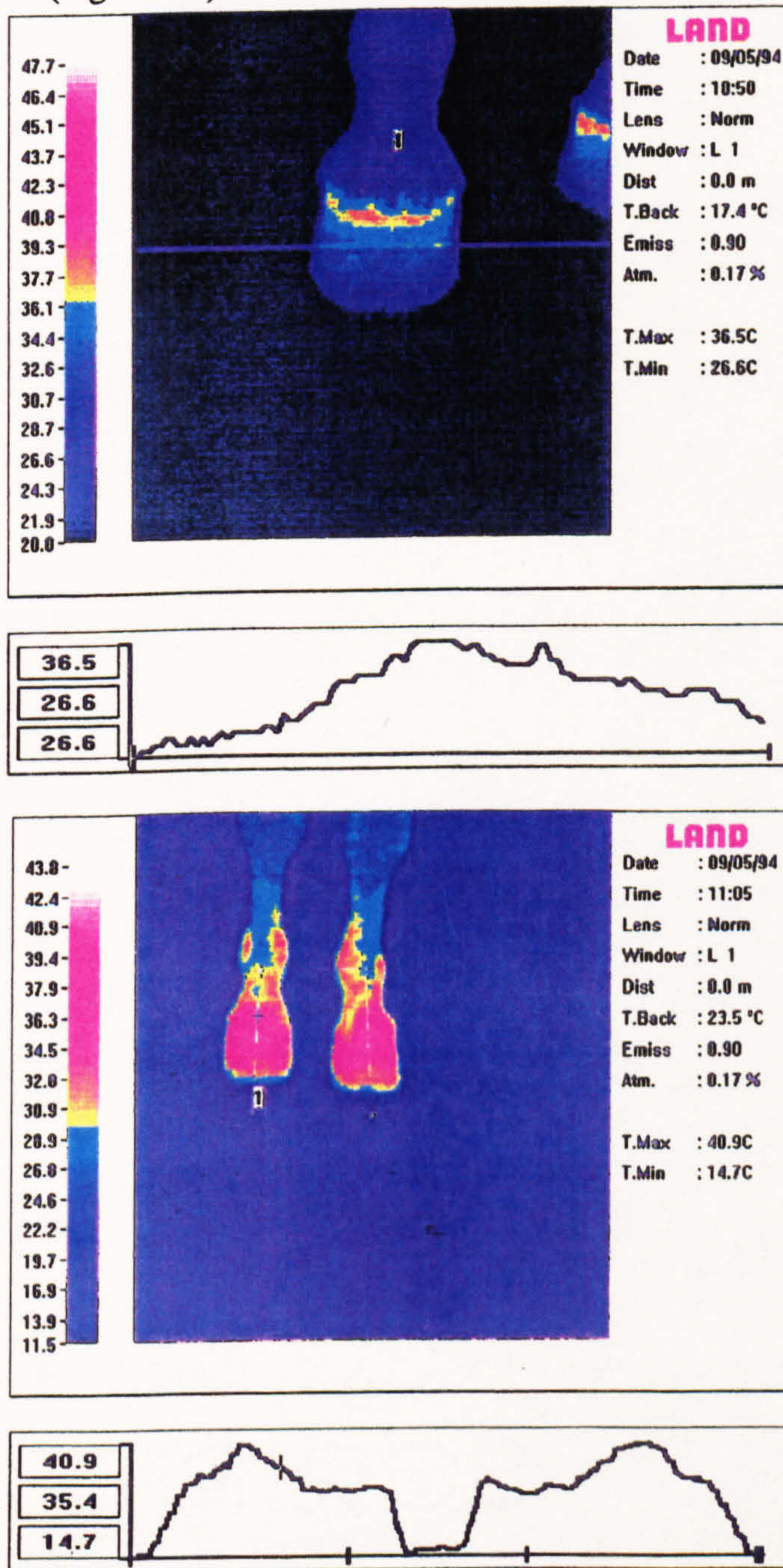


Figure 4.3 Thermography profiles showing surface temperatures along a given line and plotted as a chart. Minimum, maximum and mean temperatures are given.

Thermal images of the front hooves of two normal (a,b), two chronic laminitic (Obel Grade 0) (c,d) and two chronic laminitic ponies (Obel Grade 2) (e,f) were compared subjectively - for differences in the image itself, and for temperature gradient / profile differences on a line from the fetlock joint to the most distal part of the hoof. There were no observable differences between the individuals or the groups (Figure 4.4)

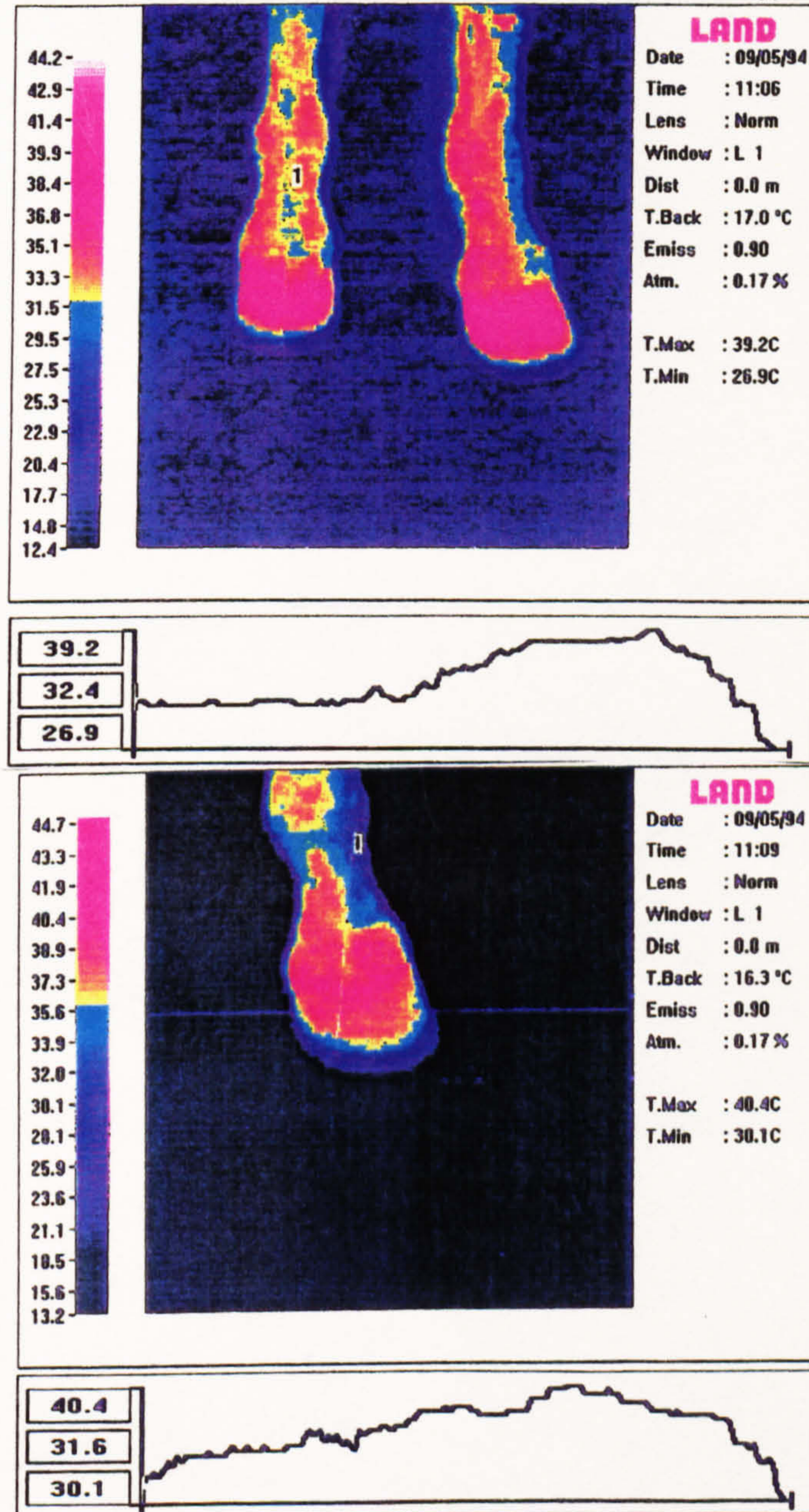


Figure 4.4 Thermal images, and temperature profiles, of front limbs and hooves of normal (a, b) compared with (continued overleaf).

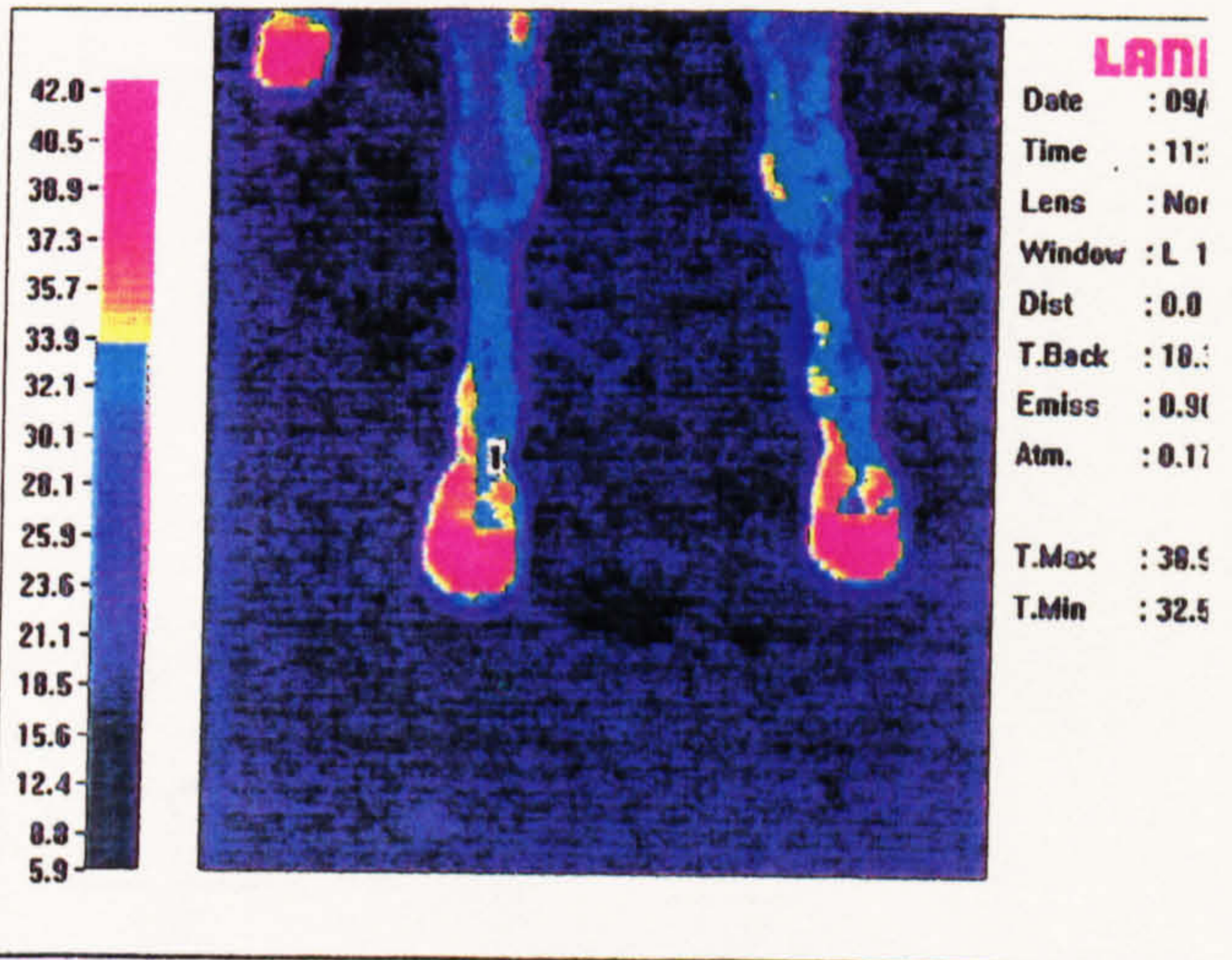
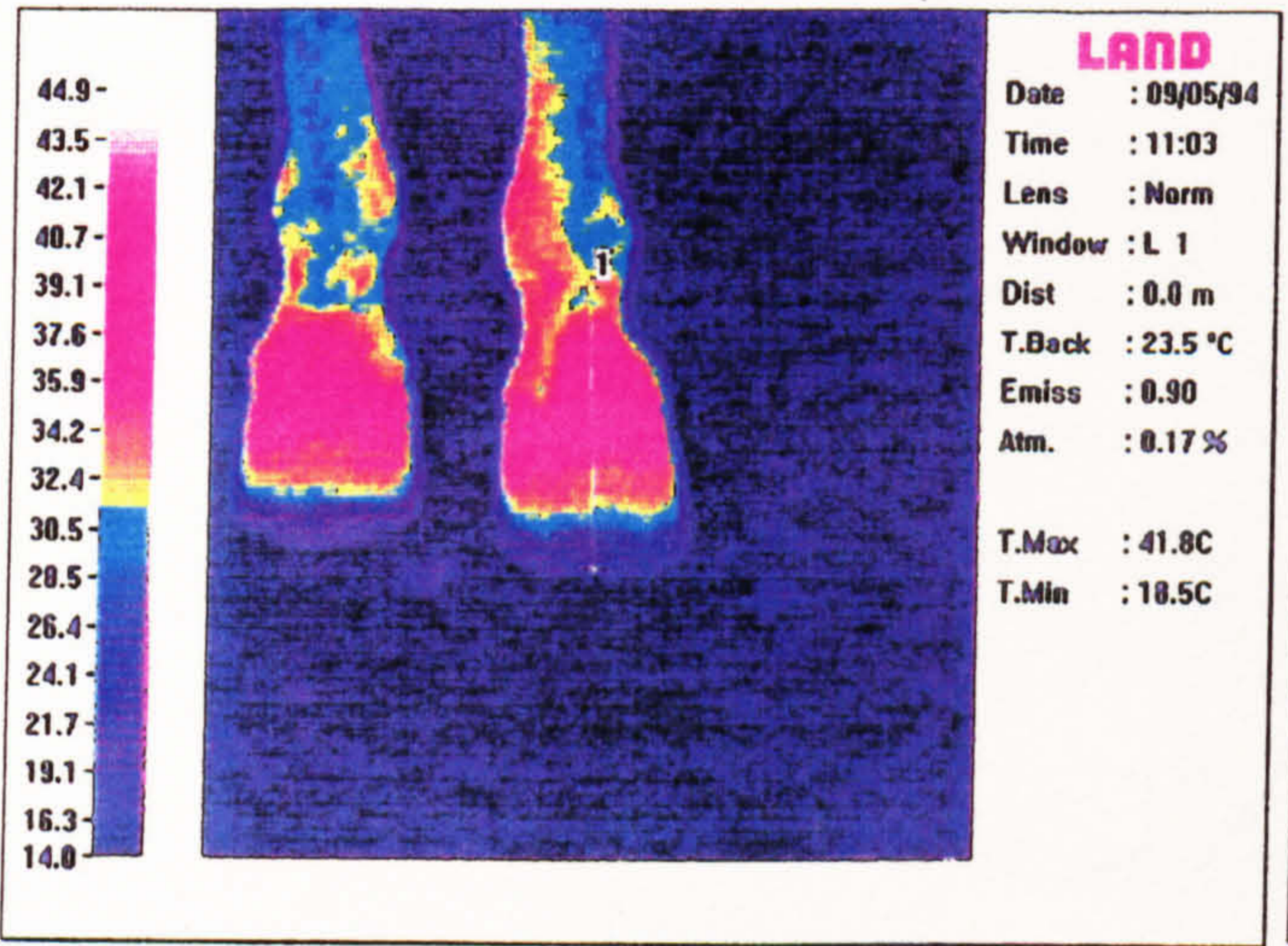
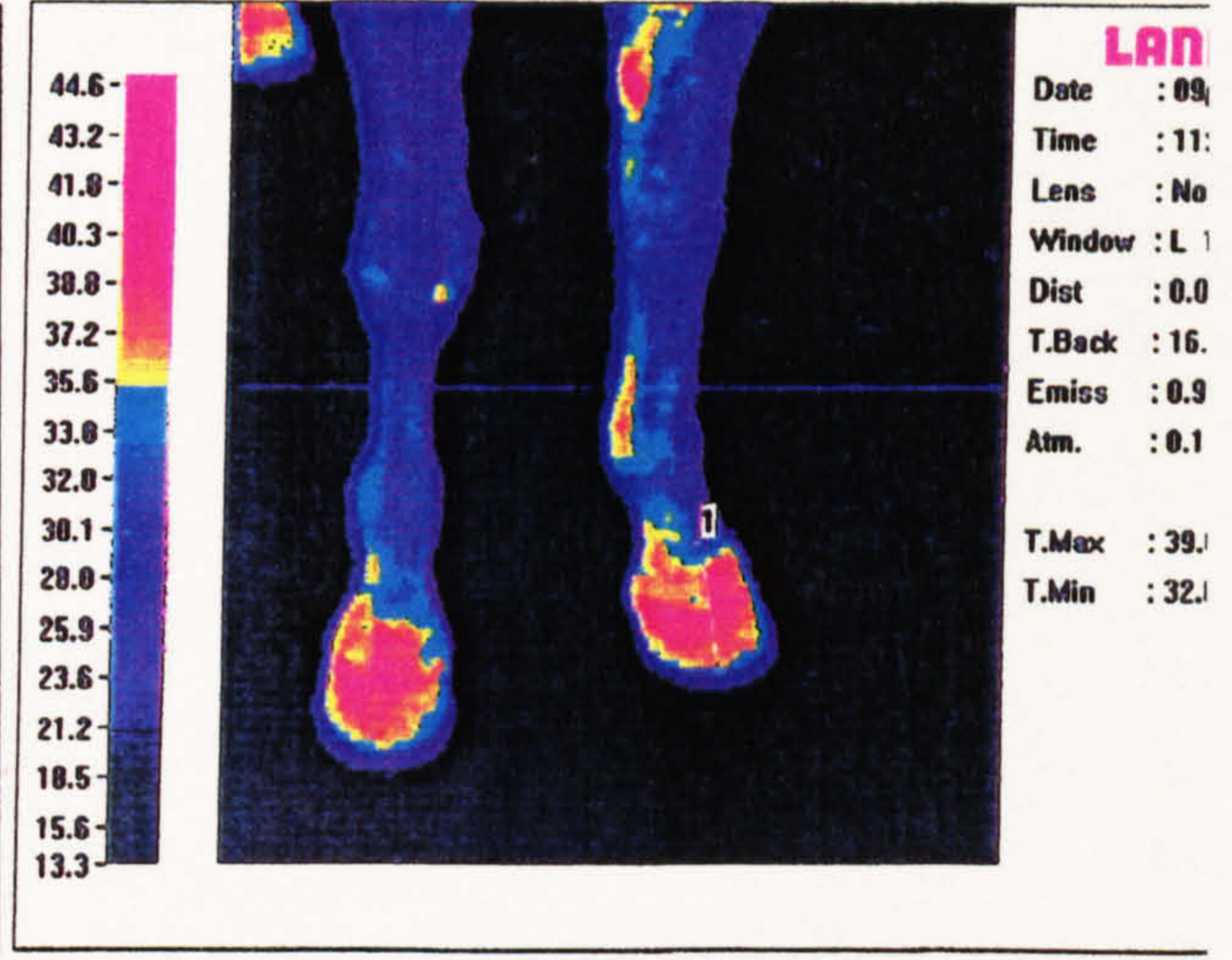
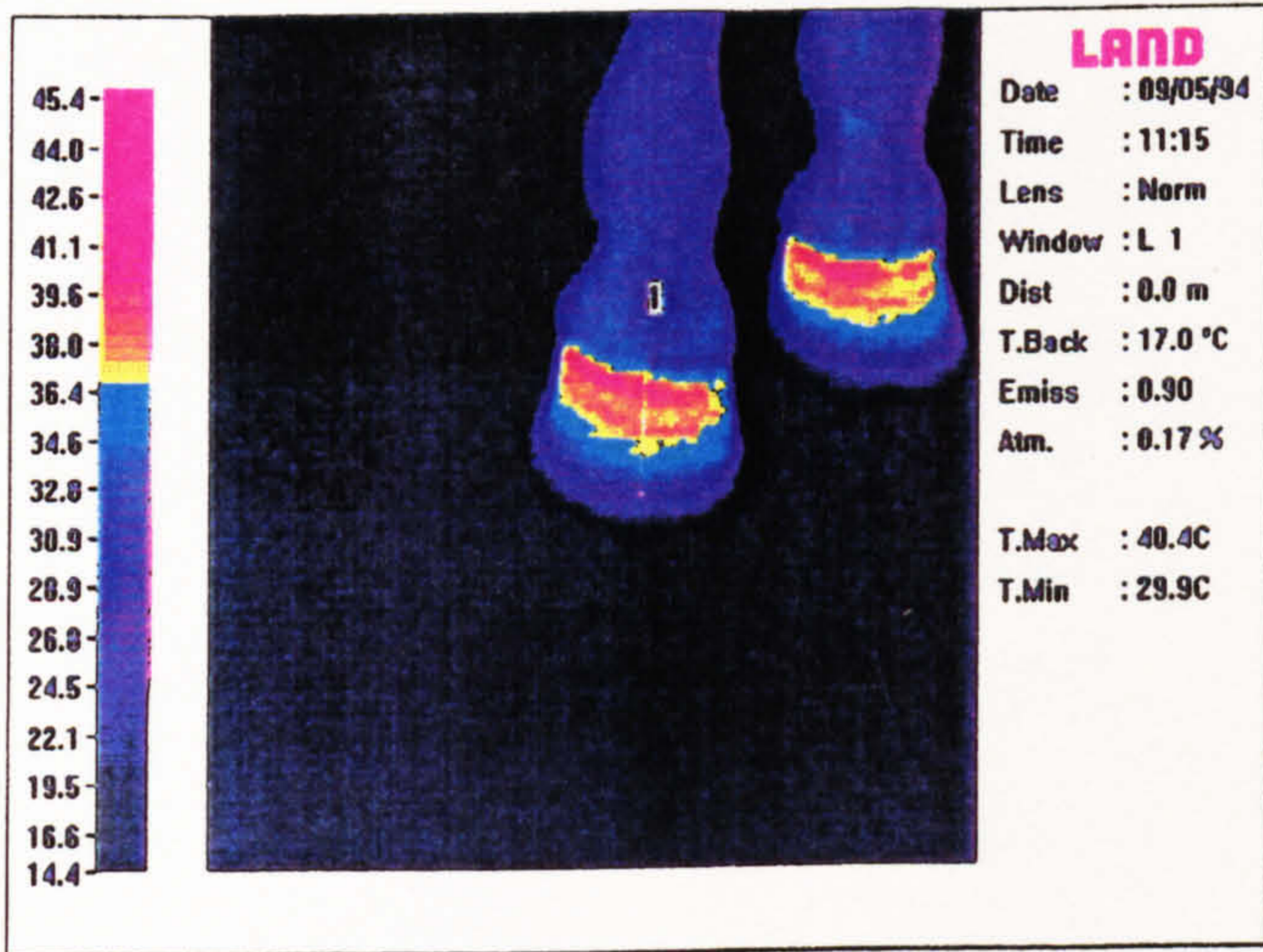


Figure 4.4 (contd) Thermal images, and temperature profiles, of front limbs and hooves of chronic laminitic ponies (c, d, e, f).

Thermal profiles were drawn from a point mid-pastern to the point at which the hoof touched the ground. Minimum and maximum temperatures were calculated from this curve and a group means calculated. (Table 4.1)

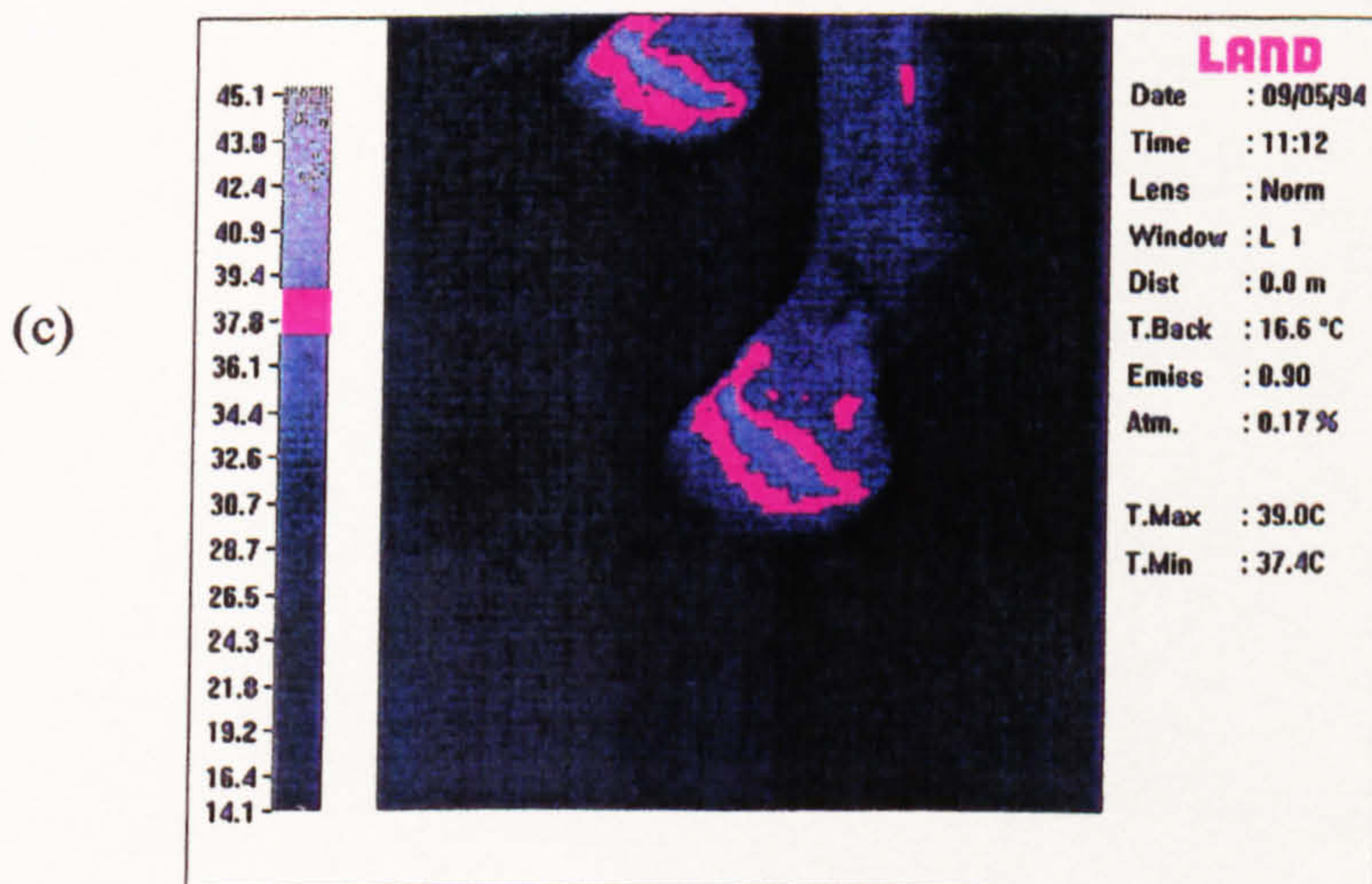
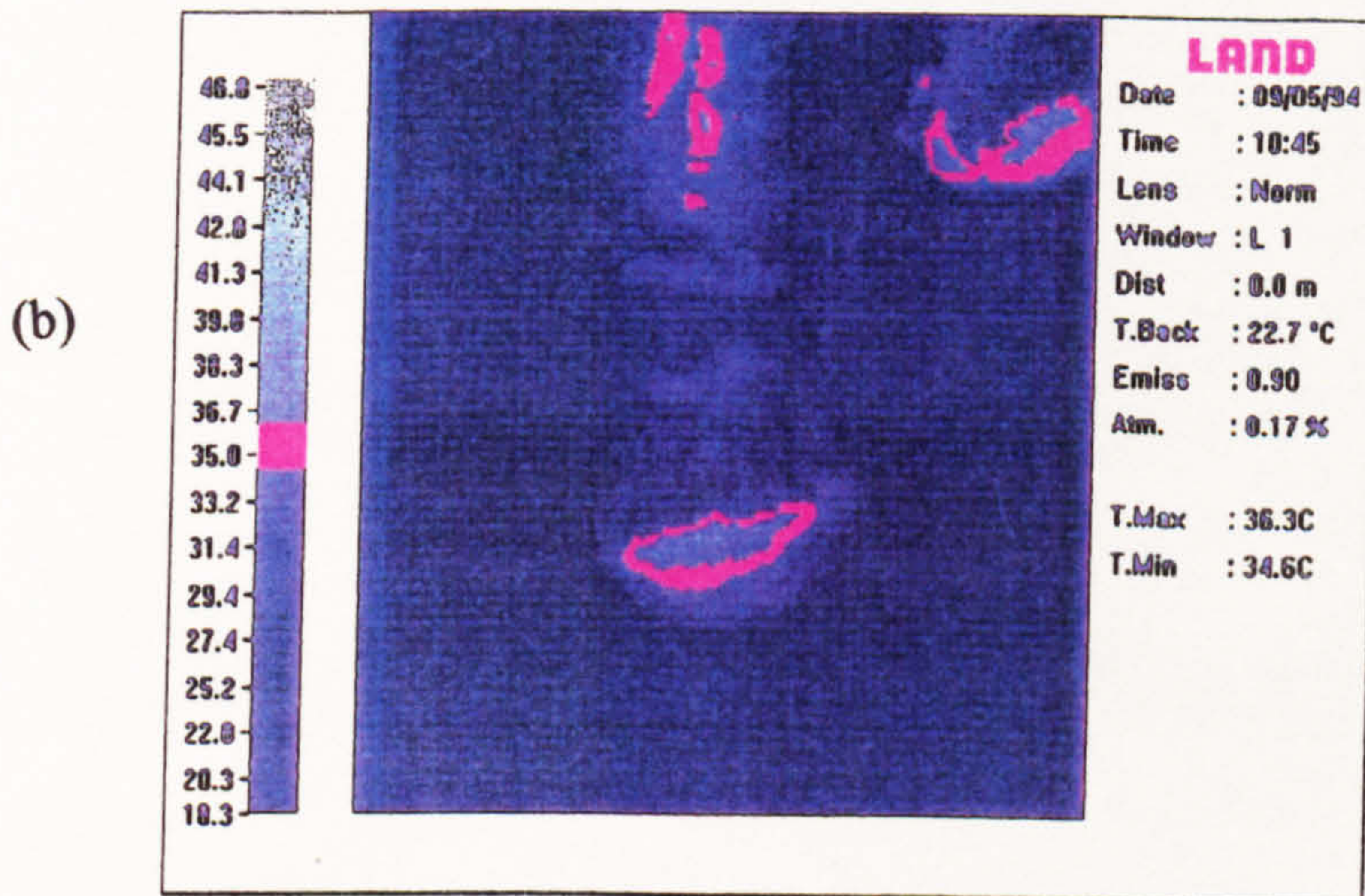
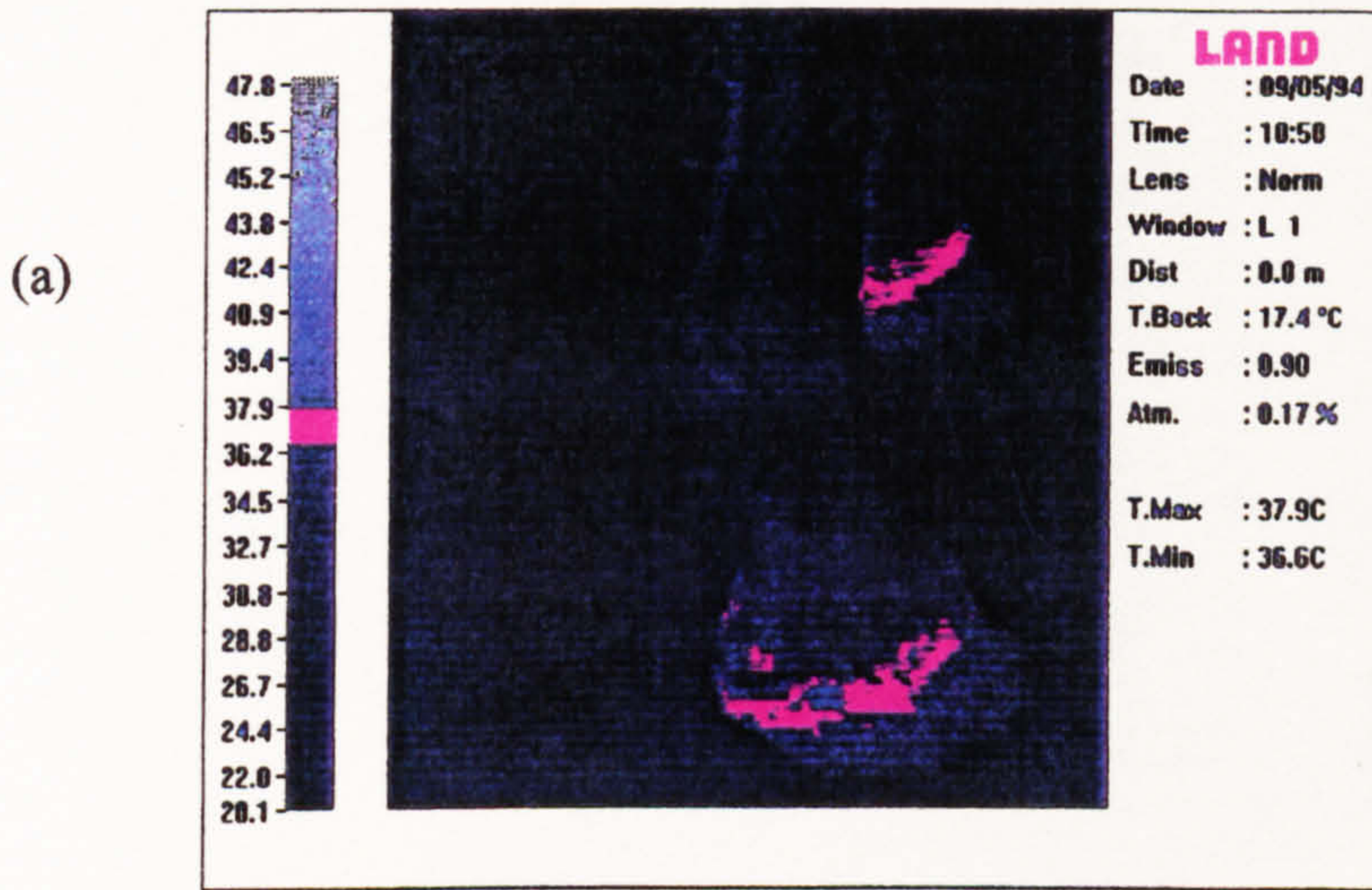
Table 4.1 Infra red thermography of equine hoof temperatures

Name	Type	Temp	Temp	Difference	Hoof Temp	Ambient
		Min.°C	Max.°C	Min-Max°C	Mean °C	Temp °C
<i>Biscuit</i>	Normal horse	20.6*	37.7	17.1*	34.8	17.1
<i>Rose Marie</i>	Chronic Laminitic horse	22.6*	40.5	17.9*	26.1	11.3
<i>Tess</i>	Normal Pony	32.5	38.9	6.4	33.0	18.3
<i>Marron</i>	Normal Pony	30.1	40.4	10.3	31.0	16.3
<i>Jasper</i>	Normal Pony	26.9	39.2	12.3	32.6	17.0
<i>Beau</i>	Chronic Laminitic Pony	26.9	39.2	12.3	32.4	23.5
<i>Domino</i>	Chronic Laminitic Pony	30.0	40.3	10.3	30.5	16.6
<i>Cobweb</i>	Chronic Laminitic Pony	29.9	40.4	10.5	31.9	17.0
<i>Misty</i>	Chronic Laminitic Pony	27.0	39.7	12.7	31.1	16.8
	* p <0.05					

There were no differences between the temperatures of the normal and chronic laminitic ponies. The minimum temperatures of the horses' hooves were significantly lower than minimum temperatures of ponies hooves ($p < 0.05$). The mean temperature differences (that is the range between minimum and maximum) of horses were significantly different to that of ponies ($p < 0.05$).

Thermal images of a normal horse, a chronic laminitic horse and a chronic laminitic pony were compared using black, grey and red colours. These colours show temperature differences of a narrower temperature range than the full range i.e greater sensitivity between 37°C and 38°C. Subjectively, the coronary circulation of the normal horse was shown as a fairly complete red band but the laminitic had a less complete coronary circulation (as shown by an incomplete red area) (Figure 4.5).

Figure 4.5 Comparison of thermographic coronary band circulation of a normal horse (a), chronically laminitic horse (b) and pony (c).



4.1.4 Discussion

These preliminary investigations aimed to assess the suitability, or otherwise, of infra red thermography to examine digital haemodynamics in normal and laminitic horses.

Infra red thermography is an easy method to use under field conditions. The apparatus was light to carry, did not require mains electricity and did not frighten the horses. The video was an ideal method of storing thermal images and the selection of "frames" for later analysis an excellent investigation attribute, allowing all data to be re-examined. The software with which point temperatures were chosen was "user friendly". Infra red thermography has some disadvantages however. First, the intra-pedal microcirculatory haemodynamics are not assessed, only hoof surface temperature and the afferent and efferent blood vessels, alongside the coronary circulation are visualised. Secondly, thermoregulation may alter the blood flow to the areas under study. The importance of ambient temperature during thermographic evaluation has been supported by previous studies (Mogg and Pollitt 1992). Ambient temperature therefore should be maintained within a narrow "hunted " range for scientific and clinical purposes. When the animals to be housed are horses this is not easy. Thirdly, if temperatures of the digital vessels are to be recorded then the hair should be clipped and / or shaved from the site. Tissue conductance of heat clearly depends on the amount of hair covering the skin's surface.

Notwithstanding these disadvantages, infra red thermography is a useful method for measuring hoof and coronary band temperatures and creating an image of coronary and of gross digital circulations.

In this study, there were no observable differences in hoof temperature, or profiles, between normal and laminitic horses or ponies. This suggests that the hoof

temperature is normal, unless the animals are in the acute phase, when anecdotally the hooves are warm (Robinson, 1990). Chronic laminitics that had been allowed to grow excess horn at the toes might indeed show reduced surface hoof temperatures because of the distance between the 'warm' tissues of the dermal laminae and the surface of the hoof. The feet of the chronic laminitic ponies and horses in this project were regularly trimmed; so this could therefore not be assessed. Reduced temperature indicates a greater thickness of horn rather than compromised laminar circulation and if this method was to be used experimentally, then the animals must act as their own controls. Differences between surface hoof temperatures of horses and ponies were recorded; these occurred because of the distance, and thickness of horn, between the source of the infra red emissions and the surface of the hoof where temperature was recorded. Not only were minimum temperatures of horses' hooves significantly less than ponies but the Minimum - Maximum differences were significantly greater, due entirely to the different thickness of horn, hoof size and perhaps density of keratinised horn (the horses used were Registered Irish Draught, a breed characterized by large tough hooves!).

Proximal to the hoof, anatomical landmarks were clearly seen in the infra red thermography images. Notably, the digital vessels could be observed without clipping or trimming at points ranging from the palmar aspect of the pasterns to above the carpal joint; the coronary circulation was also plainly visible. Subjectively, differences in the pattern of coronary band circulation were apparent using the increased definition of a narrow range of temperature. The normal horse had profuse and 'full' circulation in this area but the chronic laminitic horse and pony had a distinct circulatory arrangement - less blood flow in the centre with a warm ring surrounding the coronary area. Angiography of the coronary band shows that there can be permanent vascular changes (Pollit, 1995) akin to the thermographic images;

thermography may reveal those animals with such compromised circulations and lack of normal pattern of horn. Altered blood flow in this area may explain the reluctance of horn to grow following acute laminitis.

Further studies to investigate the alteration of coronary band circulation during manoeuvres to alter blood flow (perhaps in response to hot or cold 'soaks' - often recommended for laminitics) during administration of vasodilators or after exercise are clearly required. Some studies have been carried out and responses to adrenaline and sedative agents examined (Purohit, 1980; Purohit *et al.*, 1981), but progress is slight.

It would be interesting to record hoof changes during induced laminitis. The changes in coronary band circulation and hoof temperature could be recorded for days on video and the exact time of onset perhaps recognised by altered blood flow in the coronary circulation and /or hoof temperature. The shape of temperature profiles may alter during experimental protocols and show regional surface distribution of blood flow in the coronary band.

As digital vessels could be seen so easily, it should be possible to measure the width of these by measuring the image and using a reference marker of known length to calculate the size. Experimentally, the typical bounding pulse could be quantified non-invasively. To aid calculations and ease the comparison of images, experimental horses should be kept in a crush and the camera mounted at 90° and a given distance from the limbs.

One advantage of the infra red thermography system is that two limbs can be monitored at once. If local studies are performed (perhaps using transdermal vasoactive agents proximal to the area studied) then the other limb acts as control.

There is considerable potential for experimental use of infra red thermography especially during administration of vasoactive agents and when laminitis is induced or expected to occur. Under these conditions images of peripheral circulation in other sites such as the horse's ear might be of interest and would compliment studies of the microcirculation within the hoof using other methods, such as those described below.

4.2 Near infrared spectroscopy of pedal haemodynamics.

4.2.1 Introduction

In human clinical medicine, near infrared spectroscopy (NIRS) is used successfully to monitor cerebral haemodynamics following vascular episodes (Jöbsis-Vandervliet, 1987; Bucher *et al.*, 1993). NIRS has an obvious potential for application to the study of laminitis. The present investigation assesses the technique's applicability to the study of pedal microvascular blood flow and oxygenation in laminar tissues to establish a non-invasive method for the determination of pedal haemodynamics under normal and pathological circumstances.

4.2.2. Materials and Methods

Animals. Six adult cross-bred ponies, 5 mares and 1 gelding, age 4 - 18 years, and two normal adult horses, one mare and one gelding aged 18 and 20 years, were kept on identical management systems of diet, exercise and routine veterinary care for 6 months. Six animals were normal but 2 ponies had suffered laminitis at least 12 months before the study and prior to joining the group. These animals exhibited signs of chronic laminitis of Obel Grades of lameness 1 and 2, and divergent growth rings although no special shoeing was necessary. One of the chronic laminitic animals developed acute laminitis and Obel Grade 4 lameness spontaneously while at grass.

Medication Animals were restrained in a standard crush unless sedated. Sedation involved i.v. administration of 0.006 mg/kg b.wt. detomidine hydrochloride (Demosedan, Smith Kline Animal Health Ltd., Stevenage Herts, UK) and 0.012 mg/kg bwt butorphanol (Torbugesic, C-Vet, Bury St. Edmunds, Herts, UK) Five animals were studied sedated and unsedated. All procedures were carried out under the Animals (Scientific Procedures) Act 1986.

Basis of near infrared spectroscopy (NIRS)

The basis of NIRS relies on the transparency of bone and other tissues to electromagnetic radiation in the near infra-red spectrum, allowing transmission of these wavelengths through several centimetres of tissue. Changes in light absorption at selected NIR wavelengths allow inferences to be drawn regarding changes in haemodynamics, oxygen delivery and intracellular oxygen utilisation within the optical field. Changes in cytochrome aa3 (cyt aa3) reduction-oxidation (redox) status, together with changes in amounts of oxyhaemoglobin (O₂Hb), deoxyhaemoglobin (HHb) and total haemoglobin (tHb) are monitored as deflections of a trend waveform from a baseline. Total haemoglobin is obtained by summing the O₂Hb and HHb signals and reflects local changes in blood volume. Cytochrome aa3, the terminal enzyme in the mitochondrial electron transport chain, accounts for more than 95% of oxygen utilisation in aerobic metabolism and indicates intracellular oxygen sufficiency. The factors causing signal changes are outlined by Brazy (1991) and are summarised in Table 4.2.

Both O₂Hb and HHb exhibit weak absorption throughout the NIR range of 700-1300nm. HHb has a peak at 760nm which disappears on oxygenation, while O₂Hb has a broad band around 900nm which is not present in the HHb spectrum. Oxidised cyt aa3 has a weak absorption band between 780-870nm which disappears upon

reduction, hence cyt aa3 absorption in this range is due to the presence of the oxidised form. This enzyme may indicate mitochondrial oxygen availability as its absorption characteristics change in parallel with the availability of oxygen for its function. A downward deflection of the cyt aa3 trace may be caused by an increased amount of electrons on the respiratory chain, due to a decreased oxygen availability, decreased metabolic activity or increased cellular activity (Table 4.2)

Table 4.2 Factors causing signal changes in Near Infrared Spectroscopy (after Brazy 1991)

Increased deoxyhaemoglobin

- Decrease in oxygen saturation
- Obstruction to venous return
- Increase inflow of desaturated blood
- Increase in concentration of deoxyhaemoglobin

Increased oxyhaemoglobin

- Increase in oxygen saturation
- Increase in blood flow
- Increase in concentration of oxyhaemoglobin

Increased total haemoglobin

- Increase in blood flow
- Obstruction to venous return
- Increase in concentration of haemoglobin

Increased cytochrome aa3 oxidation

- Increase in oxygen delivery to cells
- Increase in metabolic activity of cell with oxygen sufficiency
- Decrease in supply of electrons to respiratory chain

Real time monitoring of these parameters provides information on arterial delivery, venous return and cellular utilisation of oxygen, as well as an insight into potential venous pooling. Hampson and Piantadosi (1988) have successfully applied and validated NIRS in a study of the human forearm undergoing ischaemia and reperfusion. The same study showed venous occlusion to be characterised by increases in HHb and tHb while O₂Hb and cyt aa3 remained constant. In contrast, total arterial and venous occlusion was associated with a decrease in oxygenated haemoglobin and

a downward shift in the cyt aa3 trace. These changes were reversed during reperfusion, with an overshoot from the baselines indicating a period of hyperaemia (Fig. 4.6).

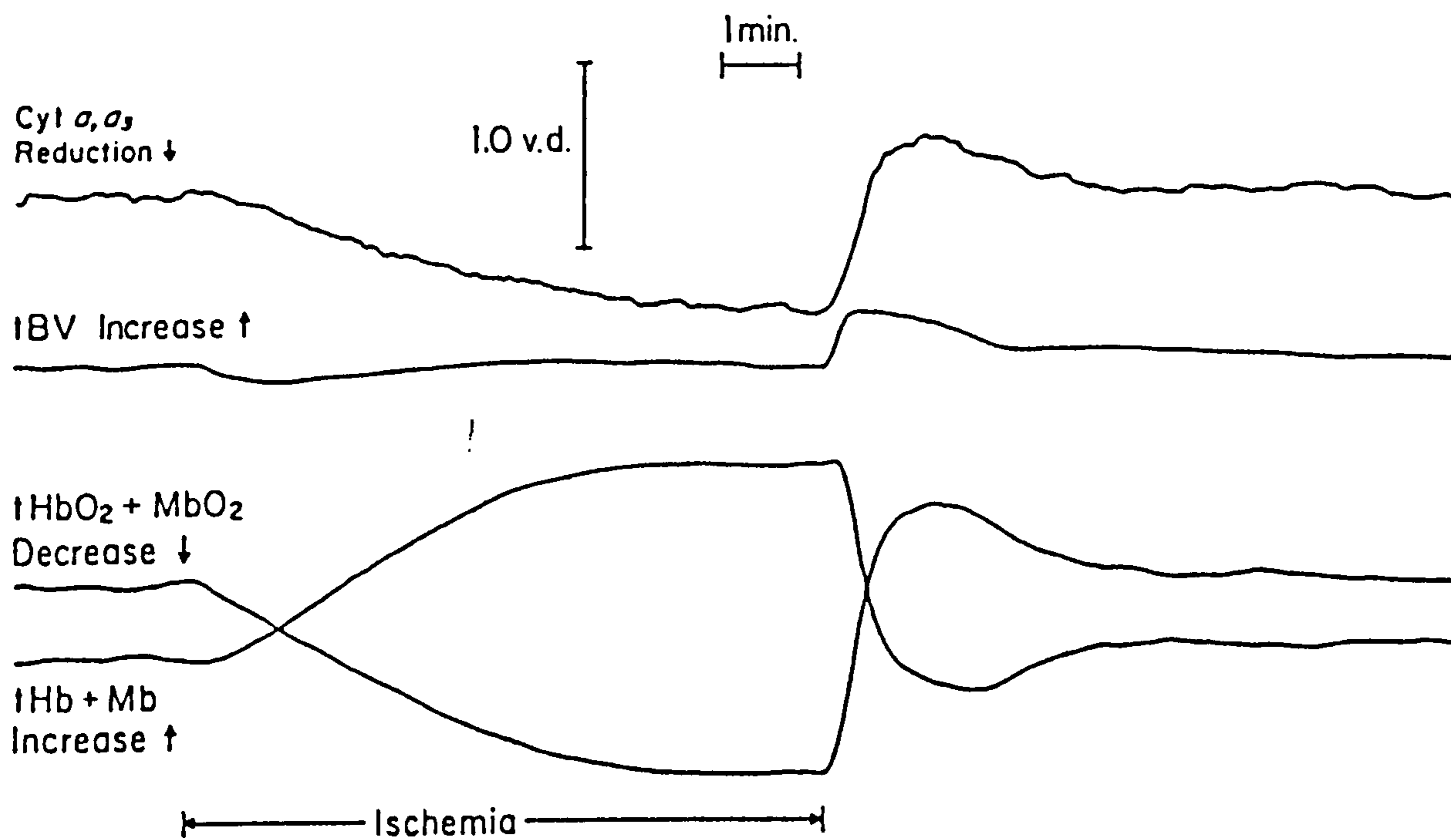


Figure 4.6 Near Infrared spectroscopy (NIRS) traces of human forearm skeletal muscle during 8 min of tourniquet induced ischaemia (Hampson and Piantadosi, 1988)

Equipment

A Critikon Cerebral RedOx Monitor Model 2000 (Critikon, Johnson and Johnson, Ascot, UK.) was used to assess vascular function within the hoof. An electro-optic cable connected the monitor to the sensor which was placed on the dorsal surface of the hoof. Adult or neonatal sensors were chosen according to hoof size to give an optimal strength of signal. The placement of sensors on the hooves, are shown in Figure 4.7. A 2 cm square area of the coronary band was clipped dorsally mid-line to ensure good surface contact of the adult sensor. The emitter section of the neonatal sensor was placed on the dorsal surface of the hoof wall of one front foot. On horses with an uneven hoof wall, contact was aided by placement of a small amount of cotton wool under the emitter section of the sensor. Exclusion of ambient light and constant sensor to hoof coupling were achieved by the application of adhesive tape (Treatplast, Animalcare Ltd., York, UK) or polythene adhesive tape (Gaffatape).

In later procedures, an improved version of the NIRS instrumentation, the Critikon Cerebral Redox Monitor Model 2001, was used to quantify changes (in $\mu\text{mol l}^{-1}$, μM). The biochemical and biophysical phenomena underlying this methodology are outlined by Essenpries *et al.* (1993) and are beyond the scope of this thesis; the benefits of the ability to compare data between animals are however clear. It should be recognised that quantification of the signals will result in some differences in the traces of the two instruments. These differences show particularly on the cyt aa3 data, where changes will not be as great as those displayed on the trend device. This arises mainly from the fact that as the tissue concentrations of cyt aa3 in tissue are low, concentration changes will be small. A further experimental difference from the previous study was the use of adhesive pads (supplied by the manufacturer) which

improve sensor function and prevent the detection of light which has not passed through the hoof.

Sensor positioning was broadly the same as with the previous method, but a new instrument feature, signal status , allowed precise placement of the sensor to maximise signal strength. Real time changes in deoxyhaemoglobin (HHb), oxyhaemoglobin (O₂Hb), cytochrome aa3 (cyt aa3) and total haemoglobin (tHb) were displayed on the monitor cathode ray screen as coloured traces and data were recorded directly from the monitor RS232 port on a portable computer (Thinkpad, IBM, UK). Data from the Redox Monitor 2001 were again recorded on a PC and charts produced in a spreadsheet using Microsoft Excel.

Figure 4.7 NIRS emitters /sensors on the horses hoof (a) diagrammatically to show relationship to internal anatomy and (continued on next page)

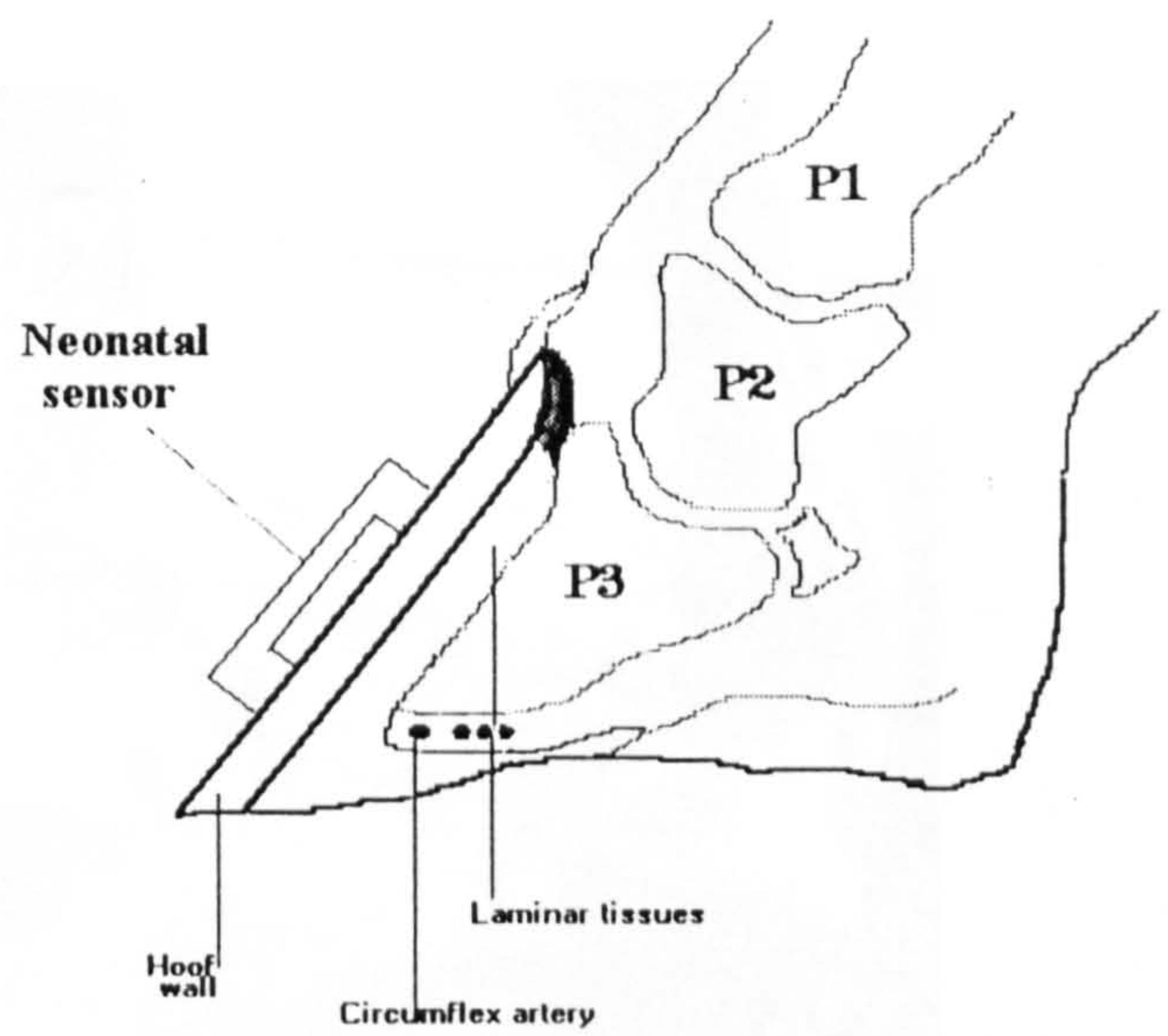
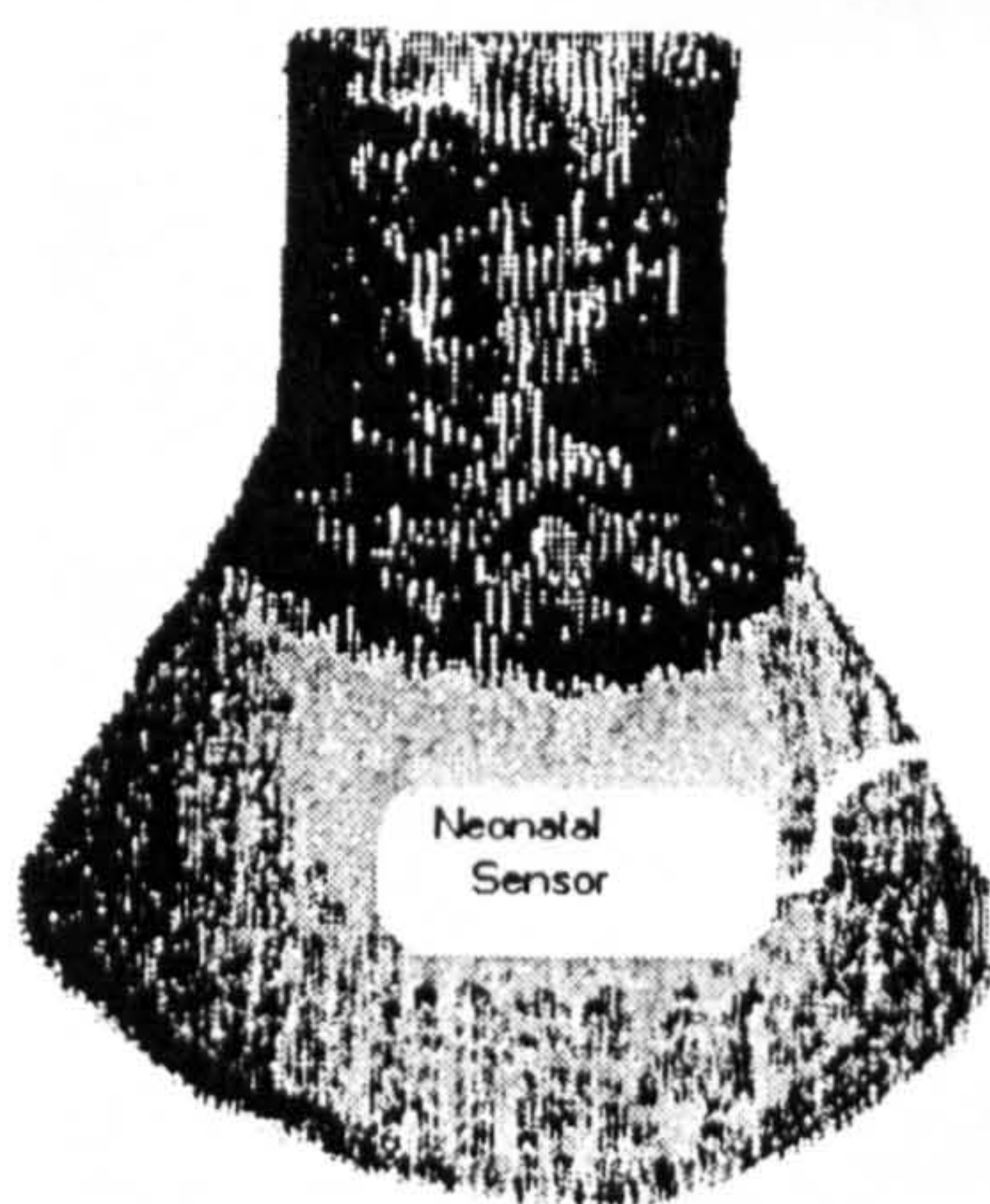
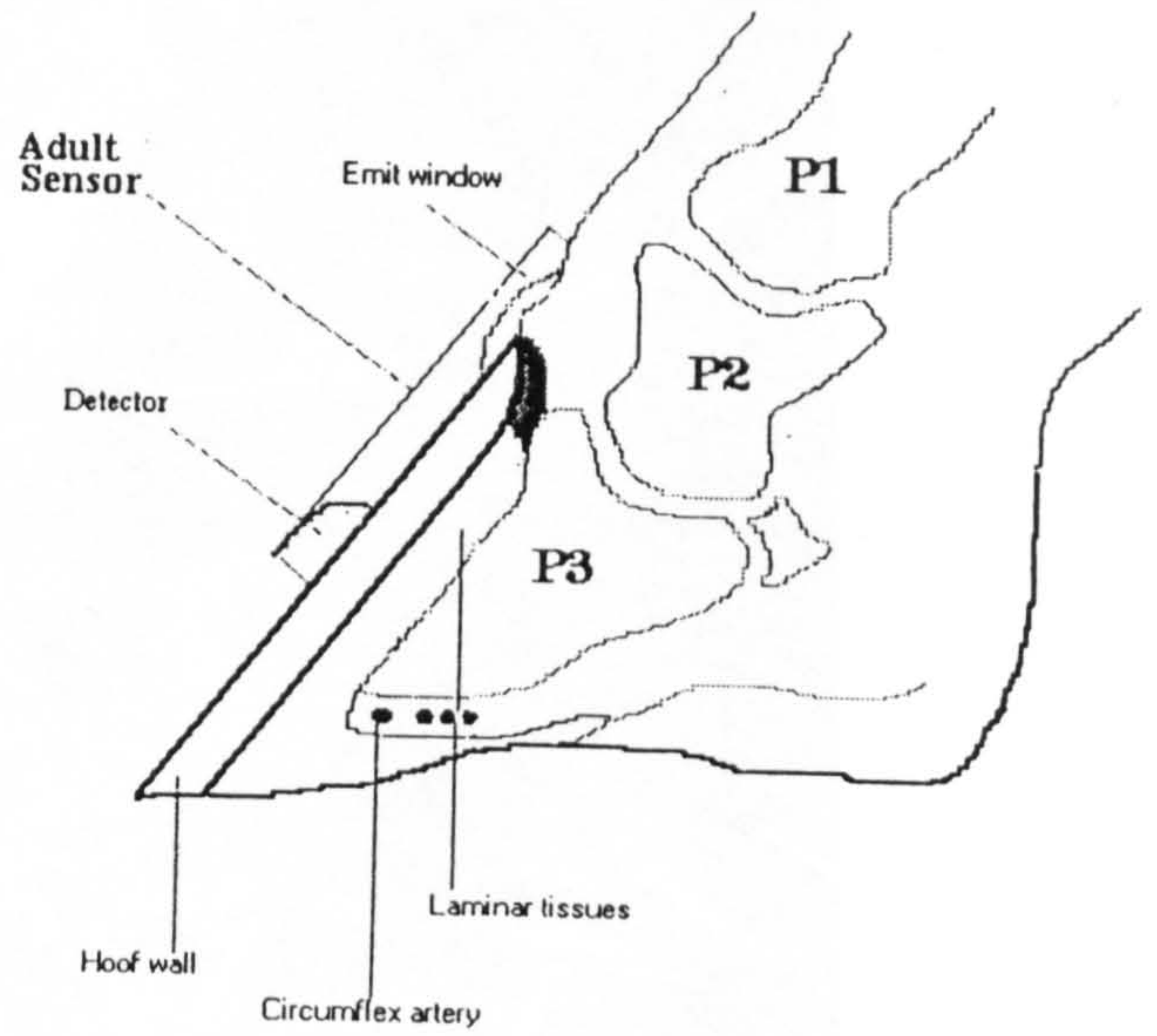
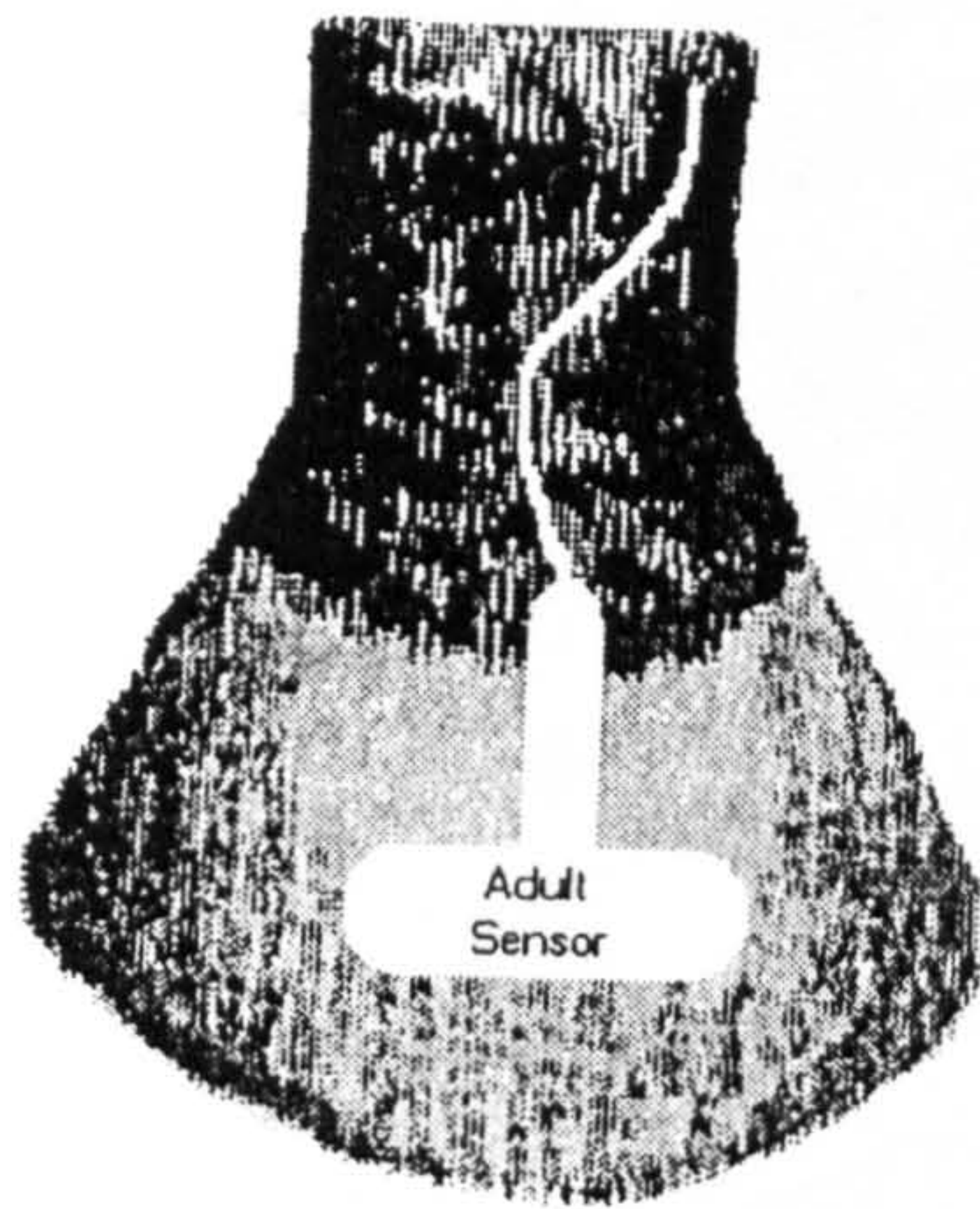


Figure 4.7 (b) NIRS emitters /sensors in place and taped on the hoof.



Assessment of pedal haemodynamics

Cuff Inflation: A human adult blood pressure cuff attached to an aneroid manometer (Perimed, Norfolk) was placed over a cotton wool pad placed medially on the animals left foreleg, proximal to the carpal joint. Points were marked on the monitor traces when the cuff was inflated to a pressure of 280 mmHg to occlude the brachial artery and vein for 1 min., and marked again when deflated rapidly. This method was performed only on unsedated animals. The start of cuff inflation for a given animal was marked on the monitor trace with the built in 'event mark' function. Release of the cuff and movement artefacts were similarly identified, each event mark being automatically assigned a unique reference number. The event reference and related intervention were recorded by a separate observer.

Manual occlusion of digital vessels: Branches of the digital arteries and veins were palpated and occluded manually for periods of up to one minute. Moments of occlusion of digital vessels and subsequent release were recorded on the traces. This procedure was performed on sedated and unsedated animals.

Lifting of contralateral limb: One forelimb was raised and held up and changes in blood flow during a period of extra weight bearing were assessed. Traces were marked when the contralateral forelimb was raised and returned to the ground. This procedure was performed on unsedated and sedated animals.

Application of latex rubber bandage (Vetwrap): A latex rubber bandage was applied with sufficient pressure to the fetlock joint for a period of one minute. The times of application, and removal, were recorded as event marks on the traces.

4.2.3. Results

The Critikon Cerebral RedOx Monitor 2000 and the Critikon Cerebral RedOx Monitor 2001 both identified changes in oxyhaemoglobin (O₂Hb), deoxyhaemoglobin (HHb) cytochrome aa3 (cyt aa3) and total haemoglobin (tHb) within the hooves of normal chronic and acute laminitic horses (unsedated or sedated) following manipulations in field conditions. Therefore, results were obtained from both monitors on sedated and unsedated horses during the manoeuvres designed to alter blood flow. Table 4.3 summarises qualitative changes in parameters monitored under the various protocols.

Key to Table 4.3 (on next page):

↑↑	increase above baseline
↓↓	decrease below baseline
=	no change
(↑↑)(↓↓)	indicate slow changes in oxygenation over a period of time which are basal
O ₂ Hb	oxyhaemoglobin,
HHb	deoxyhaemoglobin,
cyt aa3	cytochrome oxidase,
tHb	total haemoglobin.

U S N - unsedated normal horse/pony;

S N- sedated normal horse/pony

S N (sal)- sedated normal pony during infusion of 0.9% saline

S Ac Lam - Sedated acute laminitic pony

S C Lam -Sedated chronic laminitic pony

Table 4.3 NIRS changes in equine pedal haemodynamics during manoeuvres to alter blood flow.

<u><i>NORMAL</i></u> <i>C.R.M.2000</i>		HHb	O ₂ Hb	cyt aa3	tHb
	Manual occlusion of vessels				
US N	Albert	↓	=	↓	↓
US N	Marron	↓	=	↓	↓
US N	Selluci	↓	=	↓	↓
S N	Despina	↑	↓	↓	↑
S N	Marron	↑	↓	↓	↑
S N	Selluci	↑	↓	↑	↓
	Lifting contralateral limb				
US N	Albert	↑	↓	=	=
S N	Marron	↑	↓	↑	↓
S N	Selluci	=	↓	↑	↓
<i>C.R.M.2001</i>					
	Continuous recording >40 mins, no manoeuvres to alter blood flow				
US N	Albert	(↓)	(↓)	=	(↓)
S N		(↓)	(↓)	=	(↓)
S N (sal)		(↓)	(↓)	=	(↓)
	Manual occlusion of vessels				
S N (sal)		↑	↓	(↓)	↑
	Lifting contralateral limb				
S N		↑	↓	=	↓
S N (sal)		↑	↑	↑	↑
	Wrap appl				
S N		↑	↓	=	↑
<u><i>LAMINITIS</i></u> <i>C.R.M. 2000</i>					
	Manual occlusion of vessels				
US C. Lam	Beau	↑	↓	↓	=
US C. Lam	Domino	↑	↓	↓	=
	Manual occlusion of vessels				
S Ac Lam	Beau	=	=	=	=
	Lifting contralateral limb				
S Ac Lam	Beau	=	=	=	=
<i>C.R.M 2000</i>	Critikon Redox Monitor Model 2000				
<i>C.R.M.2001</i>	Critikon Redox Monitor Model 2001				

Responses to cuff inflation Only small changes were seen when the cuff was inflated. When the blood pressure cuff was inflated there was a slight increase in the deoxyhaemoglobin (HHb), an increase in total haemoglobin (tHb) together with a slight decrease in oxyhaemoglobin (O₂Hb). Cytochrome aa3 (cyt aa3) decreased. After release of the cuff, cyt aa3 and O₂Hb increased alongside concurrent decreases in HHb and tHb. Those animals with well developed forearm musculature and/or deep set vessels failed to show any significant responses to cuff inflation, suggesting that occlusion of the vessels was incomplete at this site. This method was not continued.

Manual occlusion of digital vessels Unsedated normal horses, using Critikon RedOx Monitor 2000, showed a decrease in HHb, tHb and oxidised cyt aa3, but little change in O₂Hb. Movement artefacts sometimes interfered with the changes shown in trace values (Fig. 4.8) but changes in values were nevertheless apparent. It was rather difficult to occlude the vessels on unsedated horses and frequent restoration of baseline values was required. Light sedation prevented movement artefacts and enabled smooth baseline values to be observed, therefore improving the quality of the traces. Sedated normal horses showed classic responses to ischaemia and to reperfusion (Fig. 4.9) - namely divergence of O₂Hb and HHb and downward deflection of the cyt aa3 trace. Another sedated horse showed a different response to manual occlusion of digital vessels having an increased cyt aa3 and decreased tHb (Fig. 4.10). Release of occlusion, and hence reperfusion, resulted in HHb and tHb returning to baseline values while cyt aa3 not only returned to baseline but also 'overshot' previous baseline values.

Weight bearing Movement artefacts produced rather erratic traces of unsedated horses on the 2000 Monitor; nevertheless a divergence of the O₂Hb and HHb traces

was clearly seen, although there was little change in cyt aa3 and tHb following lifting of the contralateral limb. The divergent pattern for O₂Hb and HHb was reproduced in a sedated normal horse, but unusually there was an increase rather than the usual decrease in cyt aa3 for this particular horse and little change was seen in the value for HHb until after release of the vessels (Fig 4.10).

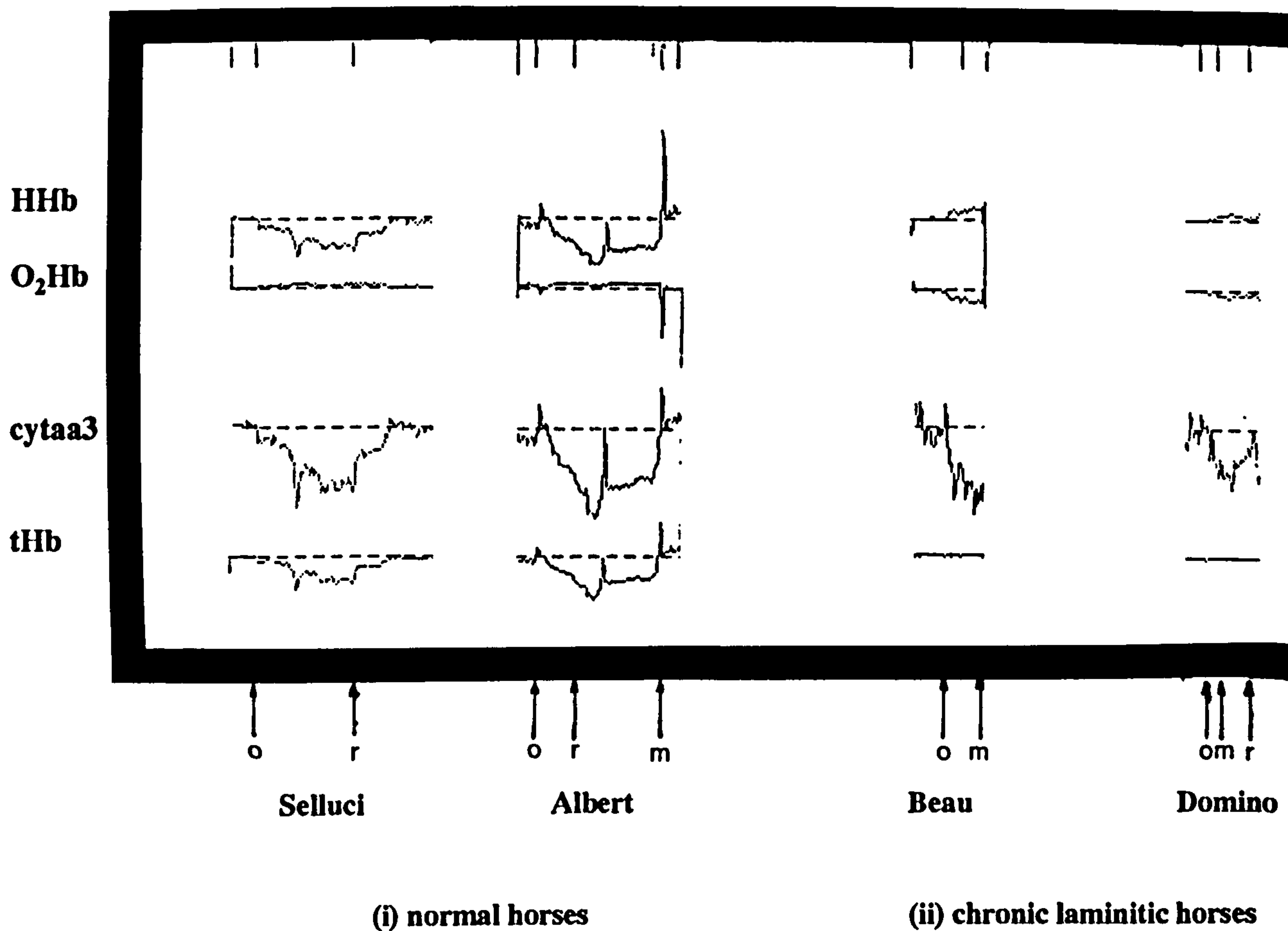
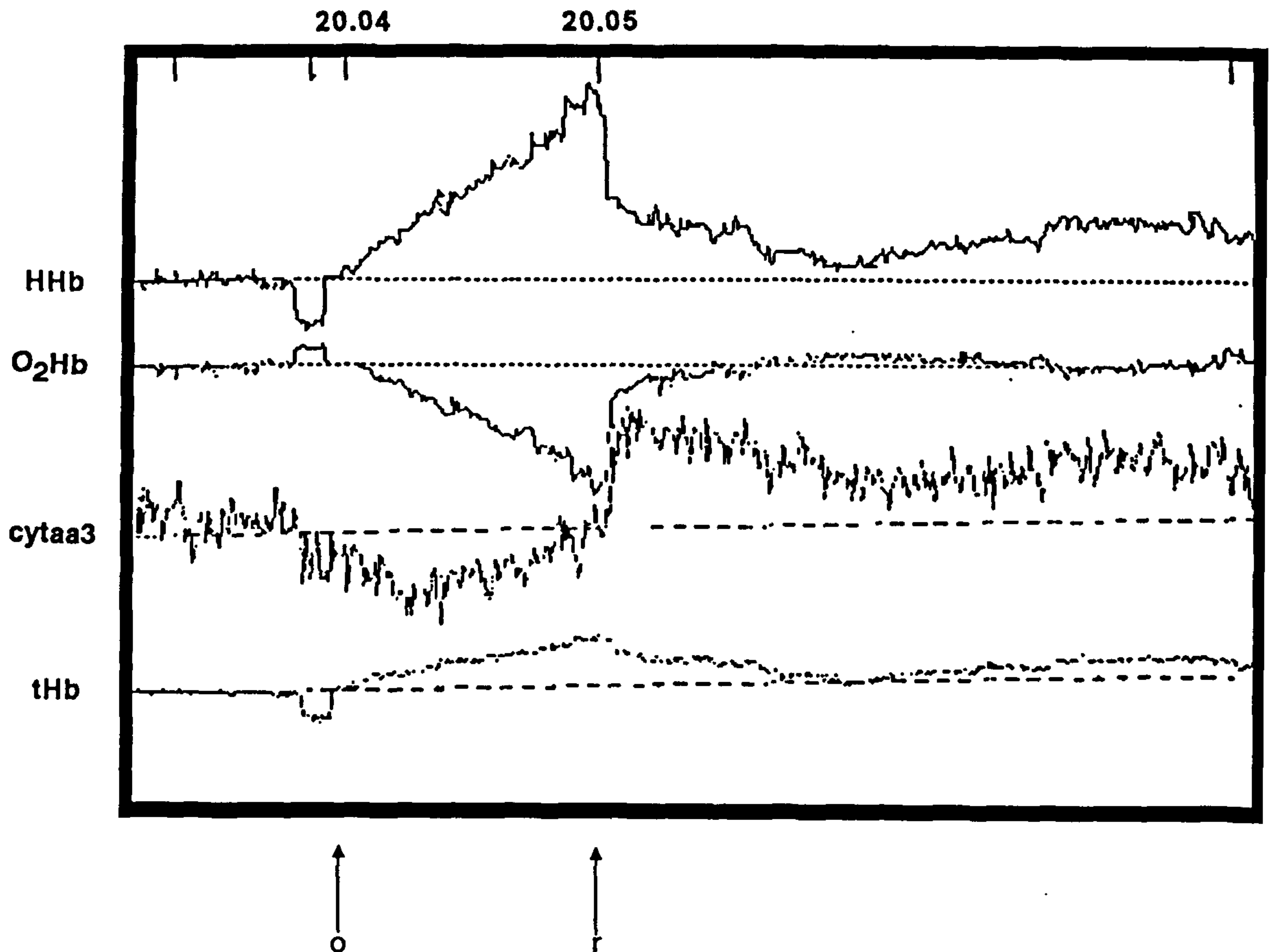


Figure 4.8 Near infrared spectroscopy (NIRS) traces of unsedated normal horses (i) and unsedated chronic laminitic horses (ii) during 1 min manual occlusion (o) and release (r) of digital vessels. Movement artefacts (m) are also shown.

Figure 4.9 Changes in near infrared spectroscopy (NIRS) traces of a sedated normal horse upon manual occlusion (o) of digital vessels for 1 min and release (r).



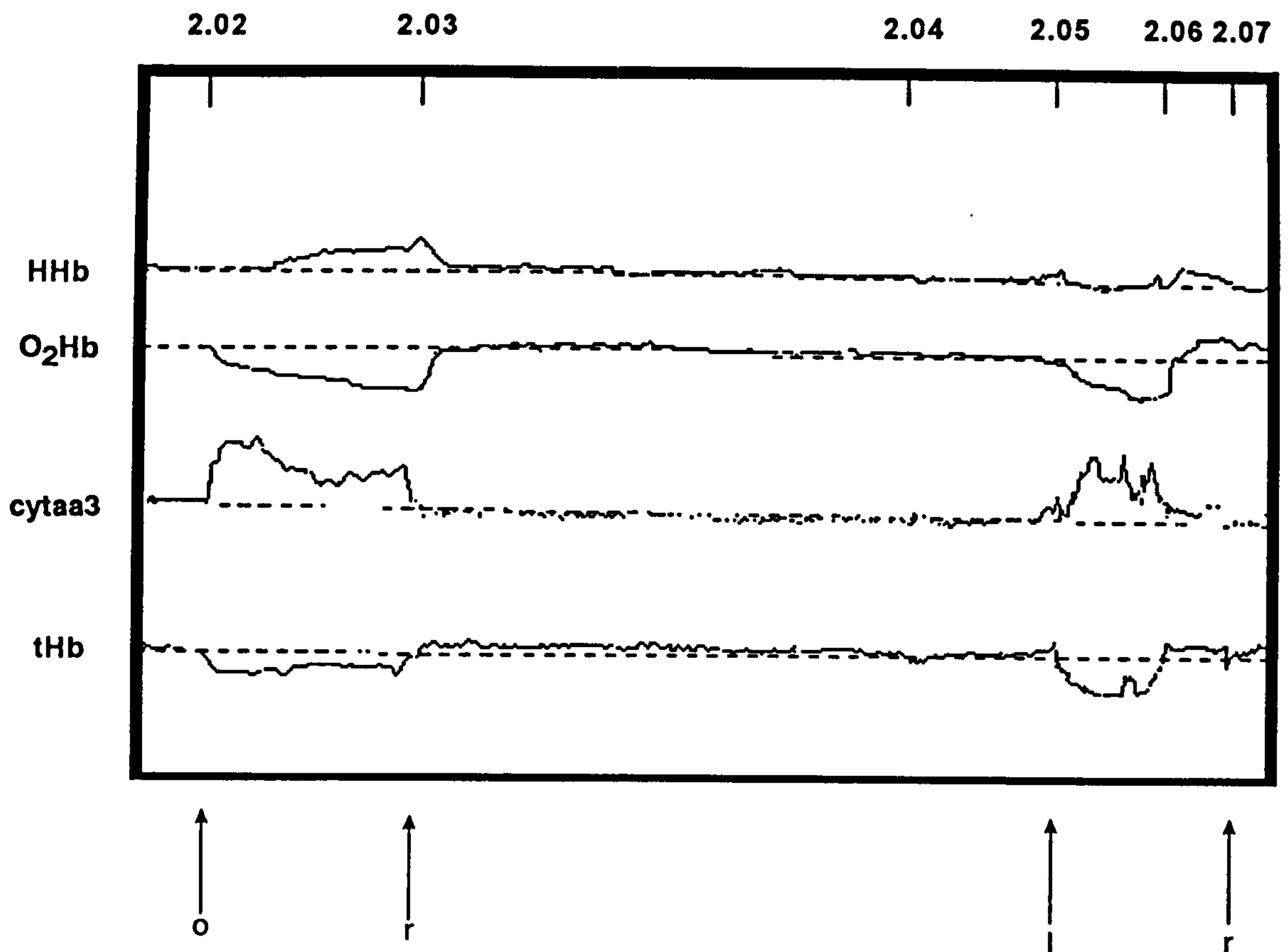


Figure 4.10 Changes in near infrared spectroscopy (NIRS) traces of a sedated normal horse showing abnormal responses of cyt aa3 and tHb during 1 min manual occlusion (o) of digital vessels; and lifting (l) of the contralateral limb; (r) release of occlusion or return of hoof to ground.

Application of Critikon RedOx Monitor 2001 to record haemodynamic changes with time

The later version of the Monitor allowed continuous recording of haemodynamics as the animals stood in the procedure box. As baselines were not reset for protocols, haemodynamic changes with time could be assessed. All horses showed haemodynamic settling after walking into the box, evidenced by a slight downward trend of all traces over a period of more than 40 minutes. This pattern was evident in unsedated and sedated normal horses and in a normal sedated horse given i.v. 0.9% saline (Figures 4.11 - 4.13). The slight 'settling' did not affect responses to various manoeuvres designed to alter blood flow (Figures 4.14).

Manual occlusion of vessels using Critikon RedOx Monitor 2001

Traces showed decreased O₂Hb and a downward change in cyt aa3. traces. HHb and tHb increased in line with the previous studies with the 2000 Monitor.

Increased weight bearing by lifting of the contralateral limb assessed by Critikon RedOx Monitor 2001 Lifting the contralateral limb produced similar responses to this procedure using the Critikon RedOx Monitor 2000.(Figure 4.14)

Effect of a latex wrap applied to the fetlock joint assessed by Critikon RedOx Monitor 2001 This protocol revealed similar changes on the trace on inhibition of blood flow, namely ischaemia and reperfusion.(Figure 4.14)

In conclusion, the changes in traces on the 2001 machine were essentially similar to the 2000 monitor except for individual responses of cyt aa3 and for the unsedated horses during manual occlusion.



Figure 4.11 NIRS assessment of pedal haemodynamics of an unsedated normal horse; traces were recorded over a period of 30 min.

Key : O₂Hb - oxyhaemoglobin; HHb - deoxyhaemoglobin;
cyt aa3 -cytochrome aa3; tHb - total haemoglobin.

NIRS of pedal haemodynamics of a normal unsedated horse.

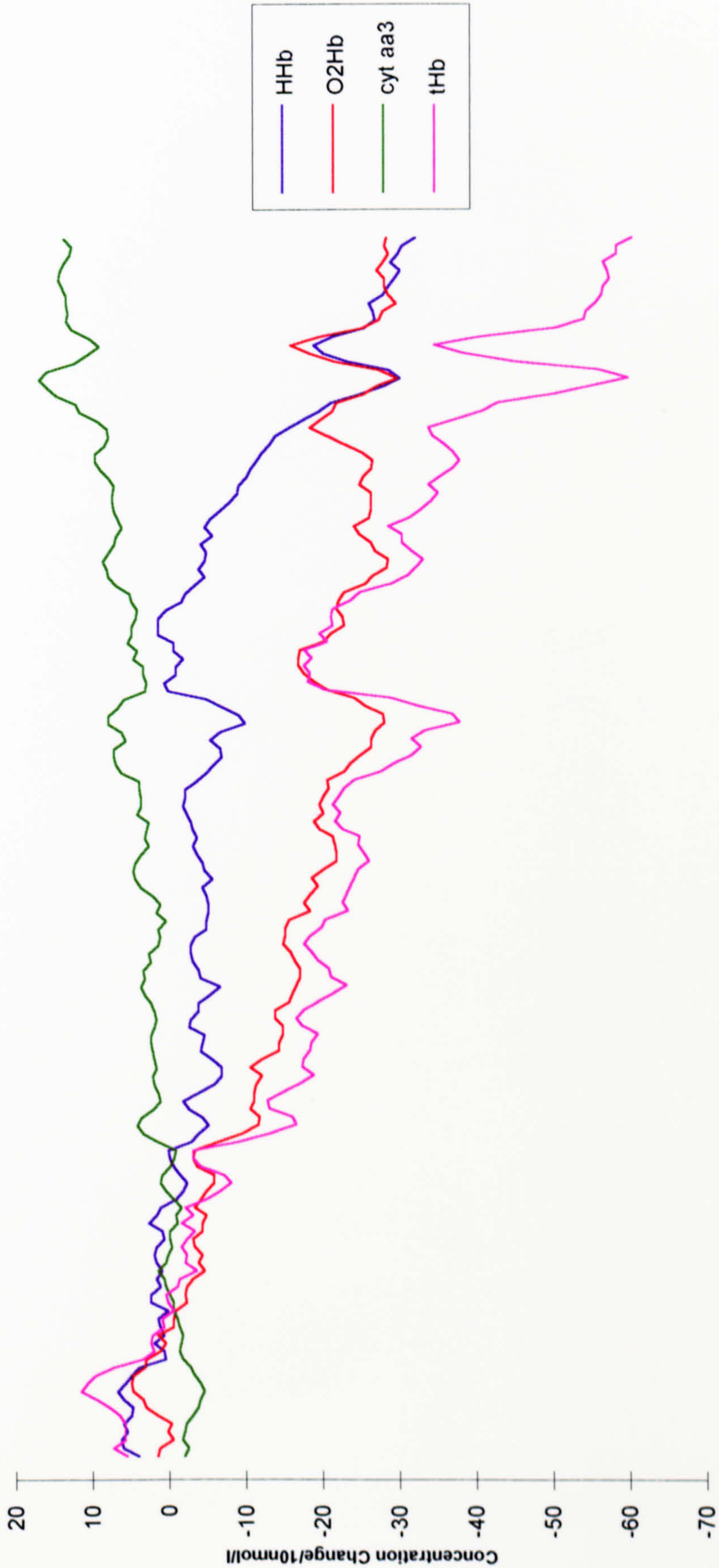


Figure 4.12 NIRS assessment of pedal haemodynamics of a sedated normal horse; traces were recorded over a period of 30 min.

Key : O₂Hb - oxyhaemoglobin; HHb - deoxyhaemoglobin;
cyt aa3 -cytochrome aa3; tHb - total haemoglobin.

NIRS of pedal haemodynamics of a sedated normal horse.

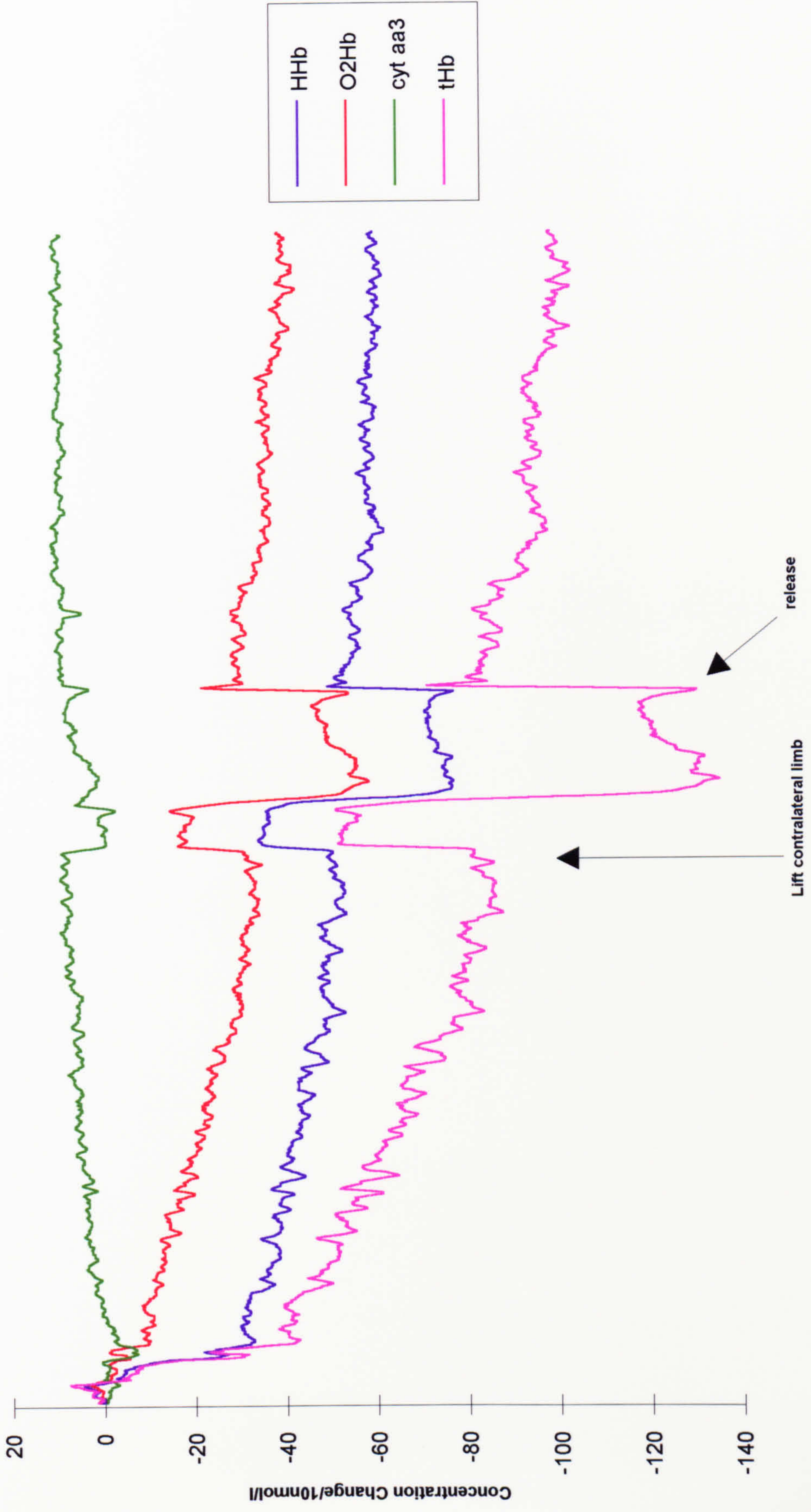


Figure 4.13 NIRS assessment of pedal haemodynamics of a sedated normal horse during infusion of 0.9% saline; traces were recorded over a period of 30 min..
Key : O₂Hb - oxyhaemoglobin; HHb - deoxyhaemoglobin;
cyt aa3 -cytochrome aa3; tHb - total haemoglobin.

NIRS of pedal haemodynamics of a normal sedated pony during 0.9% saline infusion.



Chronic cases of laminitis. Using the original monitor (2000), unsedated chronic laminitis cases showed a decrease in O₂Hb and oxidised cyt aa3 on occlusion of digital vessels, with a corresponding increase in HHb. tHb remained constant. The rates of response differed from normal horses. Laminitis cases showed a more rapid change in cyt aa3 on occlusion. Laminitic individuals also showed a slower change in HHb upon reperfusion. Subjectively, unsedated laminitic ponies seemed to find occlusion of digital vessels uncomfortable and frequently lifted their feet, although it is usual for laminitic animals to more frequently shift their weight from one foot to the other to some extent. Laminitics differed from normal horses: return of HHb to baseline was slower and the change in cyt aa3 more rapid than normal in cases of chronic laminitis, taken to indicate reduced O₂ stores as a result of compromised basal perfusion.

Acute laminitis. The pony suffering acute laminitis gave clear baseline signals but manual occlusion produced little or no deviations. Lifting of the contralateral limb (with difficulty) had no effect on the traces, nor was any change seen when the limb was returned to the ground. (Figure 4.15) There were no movement artefacts despite the pony initially moving his weight from limb to limb, which is typical of laminitis. The inability of manoeuvres to alter blood flow suggested haemostasis within the foot.

Figure 4.14 NIRS assessment of pedal haemodynamics of a sedated normal horse during infusion of 0.9% saline; traces were recorded over a period of 10 min. Each manoeuvre to alter blood flow lasted for 1 min.

Key : O₂Hb - oxyhaemoglobin; HHb - deoxyhaemoglobin;
cyt aa3 -cytochrome aa3; tHb - total haemoglobin.

NIRS of pedal haemodynamics of a sedated normal pony during 0.9% saline infusion and occlusion tests

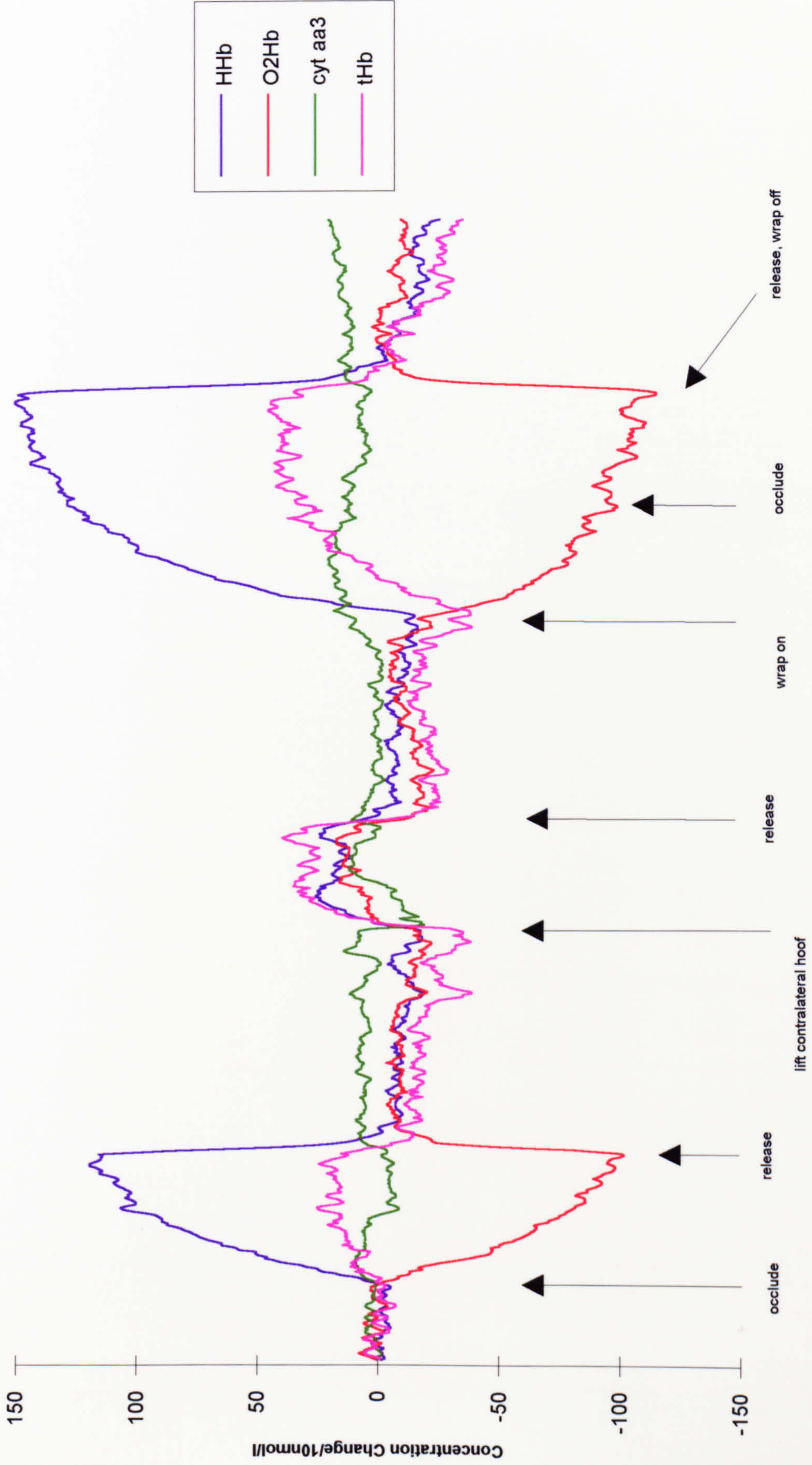
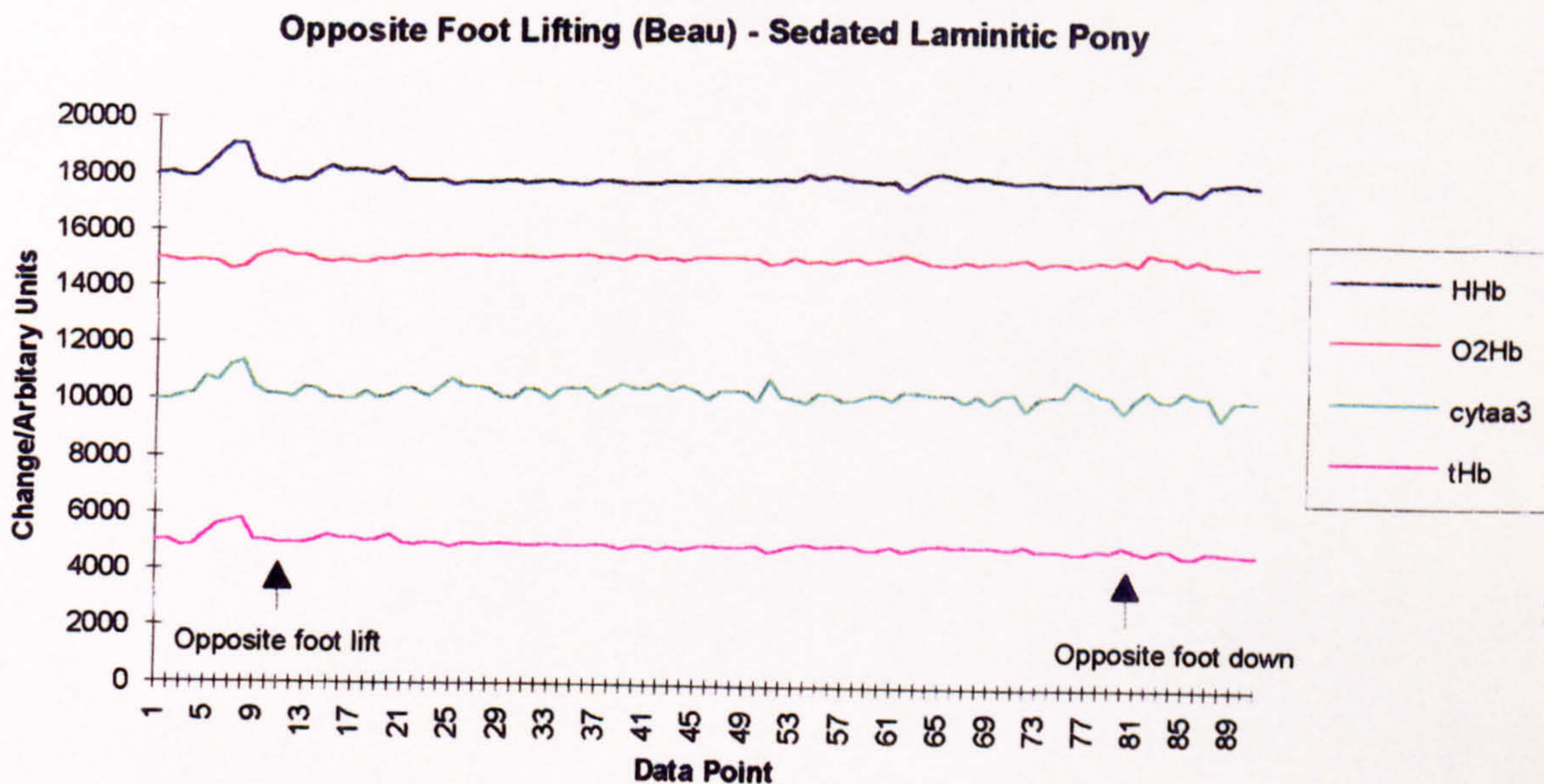
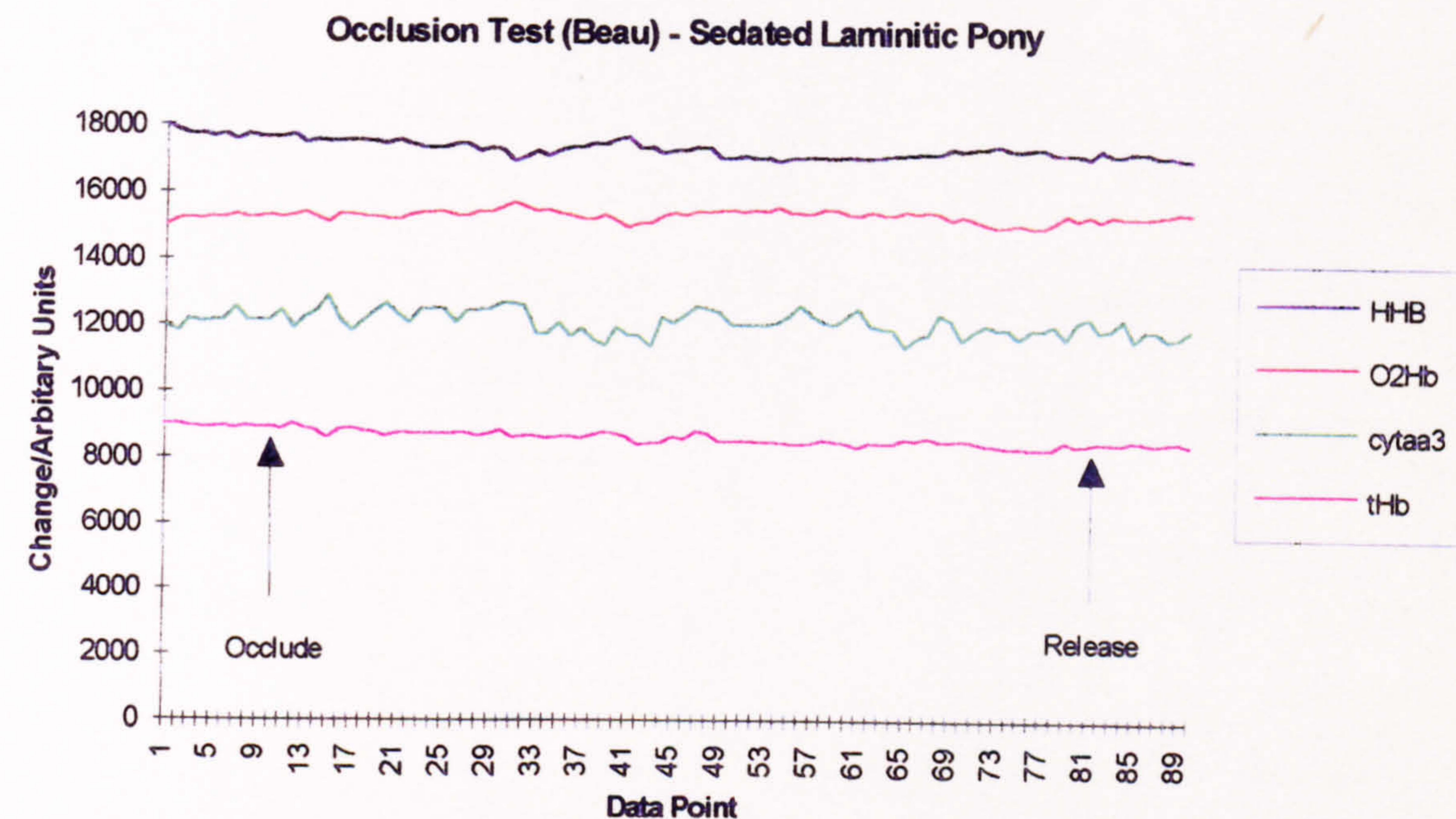


Figure 4.15 NIRS assessment of pedal haemodynamics of a case of acute laminitis (sedated) - no changes are seen in response to manoeuvres designed to alter blood flow; traces were recorded over a period of approximately 2 min. Each manoeuvre to alter blood flow lasted for 1 min.

O₂Hb - oxyhaemoglobin; HHb - deoxyhaemoglobin; cyt aa3 -cytochrome aa3; tHb - total haemoglobin.



4.2.4 Discussion

Near infrared spectroscopy is a rapidly developing technique used to monitor non-invasively changes in haemodynamics and oxygen utilisation in tissues otherwise inaccessible without surgery. It has been applied to various human and animal systems (Jöbsis-Vandervliet 1987; Hampson & Piantadosi 1988; Brazy 1991; Bucher *et al* 1993) but these are the first observations to be made on the equine hoof. It is therefore apt to consider the basic features of the monitoring system and to assess in general terms the suitability of the technique for the study of normal and diseased pedal haemodynamics of horses.

The neonatal sensor has one emitter which directs the near infrared light into the tissue and one detector 3.5cm from the centre of the emit window. The adult sensor consists of one emitter and an array of seven equally spaced detectors on an arc 5.5cm from the centre of the emit window. It is not possible to describe exactly the path of the photons through the equine foot, but the basic principle of light transmission through the human skull may be used for reference. When NIR light strikes biological tissue it is randomly scattered so that it is not possible to describe with exactitude the path of any one photon between emitter and detector. A mean path length can however be determined. The mean path length is the distance travelled by the 'average' photon and is calculated from the 'Time of Flight' (TOF) - a calculation based on the measurement of the length of time it takes a photon to travel between emitter and detector. Based on TOF, it can be shown that NIR light penetrates up to 4 cm into the human head for the neonatal sensor and 5 cm for the adult sensor.

These values cannot be extrapolated directly to the equine hoof but they give an empirical idea of the tissues that are being monitored - the laminar tissues and to an

extent the tissue of the coronary band. Figure 4.7 diagrammatically shows the arrangement of the sensors on the dorsal surface of the equine hoof. Exclusion of ambient light, and constant sensor to hoof coupling, were achieved by the application of opaque tape.

Normal and laminitic animals (acute and chronic) were subjected to manoeuvres (cuff tourniquet; digital vessel occlusion at the palmar surface of the pastern; lifting of contralateral limb) predicted to change pedal haemodynamics. The procedures produced changes in pedal haemodynamics and oxygenation, predicted on the basis of similar studies on the ischaemic / reperfused human forearm.

Base line traces for the haemodynamic and tissue perfusion indices were obtained in all horses which could, for the most part be predictably modified by experimentally occluding and reperfusing the hoof. In terms of the general methodology applied, it is worthwhile to compare the present data with studies of the human forearm during compromised circulation (Hampson and Piantadosi, 1988), (Figure 4.6) The traces produced by the sedated normal horse in response to deliberate ischaemia and reperfusion are equivalent to the traces produced on the human forearm under similar circumstances. Figure 4.6 and Figure 4.8.- should be compared. The divergence of O₂Hb and HHb upon vessel occlusion indicates a reduction in arterial input of O₂Hb. The initial fall in the cyt aa3 trace (shift of more cyt aa3 into a chemically reduced state) follows the known response of this enzyme to decreased oxygen availability (Jöbsis-Vandervliet, 1987).

The divergence of O₂Hb and HHb may also indicate an increased oxygen extraction to maintain aerobic respiration. The increase in tHb during ischaemia contrasts with previous observations (Hampson and Piantadosi, 1988) and suggests venous

occlusion with incomplete arterial occlusion and/or collateral input. The greater deflection of HHb than O₂Hb supports this hypothesis. Hampson and Piantadosi (1988) characterised venous occlusion as an increase in tHb and HHb with little change in O₂Hb and cyt aa3. The rise in cyt aa3 after an initial fall also supports the incomplete arrest of arterial input hypothesis.

Upon release of the occlusion, the return of O₂Hb and HHb towards baseline is consistent with reperfusion with oxygenated blood and washout of deoxygenated blood. Hampson and Piantadosi (1988) noted a phase of hyperaemia during reperfusion in which O₂Hb, tHb and cyt aa3 overshoot their baseline values. In the present investigation, O₂Hb and tHb did not behave in this fashion, but the cyt aa3 did, indicating the sensitivity of the cyt aa3 signal to changes in oxygenation. In horses, the cyt aa3 recordings following reperfusion are typical of hyperaemia seen in man (Hampson and Piantadosi, 1988; Thorniley *et al.*, 1988). The slower return of HHb and tHb to baseline may indicate compromised venous return from the pedal circulation.

The response to arterial occlusion in this animal is particularly clear and of note is the decrease in O₂Hb preceding the increased HHb. This is consistent with the physiological model of increased oxygen extraction beginning after, and as a response to, decreased perfusion. It also perhaps indicates the great sensitivity of the Critikon Cerebral RedOx Monitor.

Increased weight bearing by the hoof (following raising of the contralateral foot; Fig. 5) resulted in an increased cyt aa3 alongside a decreased O₂Hb, i.e. these two indices

need not change in the same direction. This suggests that changes in cyt aa3 reflect rather more than simple oxygen dependence.

Several interesting features are apparent when the vascular responses to manual digital arterial occlusion of normal and laminitic vessels are compared. The more rapid response of the laminitic hoof in terms of cyt aa3 may indicate a reduced oxygen store, as a result of compromised perfusion and, together with changes in haemoglobin, are consistent with decreased oxygenation of tissues. The more sluggish haemodynamic responses of the laminitic horses may be taken to indicate poor vascular tone. The digital vessels of the acute laminitic had a bounding pulse and could be palpated easily. Despite repeated manual occlusion of the vessels, no responses on the NIRS traces were seen. No responses to lifting the contralateral limb, nor any movement artefacts were seen prior to sedation. Together these observations are indicative of haemostasis in the acutely laminitic hoof.

Basal values. Animals were monitored continually by NIRS during the period when they stood in the examination box. The traces of all but one of these animals (n = 6) displayed a gradual reduction in concentration of O₂ Hb, HHb, tHb and an increase in cyt aa3. to stabilise after approximately 30 minutes. The changes were similar in unsedated and sedated animals (Figures 4.11 and 4.12 respectively). Sedated animals showed normal responses to manoeuvres designed to alter blood flow to the hoof such as lifting of the contralateral limb and manual occlusion of the digital vessels (Figure 4.12)

Responses of a normal pony to intravenous 0.9% saline Traces showed the typical pattern during the iv infusion of 0.9% saline to a normal sedated pony (Figure 4.13). Saline infusion had no obvious effect on pedal haemodynamics.

The studies with unsedated normal horses in some cases gave major 'movement artefacts'. Following the occlusion cyt aa3 fell from baseline, but O_2Hb did not change and the fall in tHb was due to a fall in HHb . This may be accounted for by the fact that in the unsedated condition full occlusion was not achieved, such that arterial collaterals sustained the O_2Hb while only venous drainage occurred. It must be said that these data are the least reliable as they were the first of the group to be tried.

The sedatives employed are known to have (minor) effects upon respiration and blood gases (Clarke and Paton, 1988) but it is considered that the magnitude of such actions are unlikely to be major reasons for the discrepancies.

The results obtained from the Critikon RedOx Monitor 2001 were of much better quality than those from the 2000 Monitor. This coupled with the fact that the emitter sensor stays in place, and excludes light, makes the later version preferable. Animals were monitored continually by NIRS during the period when they stood in the examination box. The traces of all but one of these animals ($n = 6$) displayed a gradual reduction in concentration of O_2Hb , HHb , tHb and an increase in cyt aa3 . to stabilise after approximately 30 minutes. The changes were similar in unsedated and sedated animals (Figures 4.11 and 4.12 respectively). Sedated animals showed normal responses to usual manoeuvres designed to alter blood flow to the hoof. This haemodynamic settling is acceptable after the horse has walked into the box and begins to doze. This gradual 'settling' did not affect manipulations to alter blood flow and responses were seen to manoeuvres to alter blood flow in sedated and unsedated normal horses / ponies and during a 0.9% saline infusion.

The Critikon RedOx Monitor 2001 was superior to the 2000 version in continuously recording changes over a period of time and the output of recorded traces were of better quality and resolution than those on the 2000 model. The only disadvantage of the 2001 Monitor was that the changes could not be seen immediately on the screen as the scale of change is so small that only a very slight change is visualised. However, this disadvantage is offset by the semi quantitative nature of the data and the ability to make comparisons between animals. The new design of emitter sensor is flexible and self adhesive; this removed many of the problems that were initially encountered regarding fitting to the hoof shape and avoidance of daylight shining on the sensor whilst in use.

In conclusion, NIRS applied to the equine hoof gives important insights into changes in basal haemodynamics and tissue oxygenation under field conditions. The responses seen in the hoof following manoeuvres to interfere with vascular perfusion are broadly similar to those of, for example, the human forearm submitted to ischaemia/reperfusion. Clear differences between laminitic and normal hooves were apparent when arterial flows are interrupted and it has also been demonstrated that oxidation of cyt aa3 is not maximal in the equine hoof, at least at rest. Central to an understanding of laminitis is an insight into blood flow and distribution within the hoof. Methods used to assess pedal haemodynamics during the development of the disease or to ascertain the efficacy of treatments inevitably introduced artefacts in measurement (Robinson, 1990). Near Infrared Spectroscopy (NIRS) overcomes these difficulties since it non-invasively determines changes in pedal haemodynamics and oxygen utilisation. Although sedation with detomidine and/or butorphanol has some effect on blood pressure and heart rate, there were no apparent differences between unsedated and sedated normal controls. NIRS revealed a number of important

differences in the pedal haemodynamic responses to occlusion of the digital vessels of normal and laminitic ponies.

The method described is currently being applied to assess efficacies of various actual and putative therapies for laminitis and its use is described in the next Chapter. It is also possible that NIRS may be used a predictor of laminitis in the pre-laminitic condition as haemostasis occurs before the appearance of clinical signs of lameness (Hood, 1993). This method would be ideal for assessment of clinical cases that were at risk of acute laminitis and would enable urgent prophylactic measures to be taken. This method is also ideal for the testing of the efficacy of treatments given. It is the first really accurate, non-invasive method to assess the haemodynamics, and metabolism, within the hoof and as such should be the basis for much progress in laminitis research.

4.4 General Discussion

Infrared and Near Infrared light form the basis for the thermographic and spectroscopic analysis of pedal haemodynamics. Infra red thermography relies on thermal emissions from the subject whilst NIRS emits NIR light from the probe and measures how much of this source is reflected or absorbed. The first therefore measures the gross thermal status on a large scale of measurement ($\pm 1^{\circ}\text{C}$) whilst the latter measures tiny differences in the oxygen status of the tissues studied. Although both techniques utilise infra red light and are non invasive, they have little else in common. The thermographic study provided information on coronary band and digital arterial flows but details of flow within the hoof were not accurately observed. It follows therefore that this is a useful technique to measure sizes of larger vessels and gross estimates of blood to and from, but not within, the hoof.

The present study applies near infrared spectroscopy (NIRS) to the haemodynamics of the pedal circulation in normal and laminitic horses. NIRS is a non-invasive technique which uses changes in light absorption at 4 wavelengths to provide information on the changes in cytochrome aa3 (cyt aa3) reduction-oxidation (redox) status, and changes in the tissue concentration of oxyhaemoglobin (O₂Hb), deoxyhaemoglobin (HHb) and therefore total haemoglobin (tHb). Other studies have shown NIRS to be sensitive to changes in tissue oxygenation and perfusion in human cerebral and limb circulation. In this study, the NIRS sensor was applied to the dorsal surface of horses hooves.

Infra red thermography is potentially useful for non-invasive measurement of temperature changes in the hoof and coronary band to indicate gross changes in vasculature. Similarly, changes in peripheral circulation at other sites, such as the ear, could be recorded successfully with this method. It is possible that the clipped ears of

horses at risk of laminitis clinically (eg after abdominal surgery) could be surveyed by the 24 hour infra red thermography equivalent to closed circuit television.

Near Infra red spectroscopy is an ideal method for detailed investigation of the laminar tissues. Entirely non-invasive, the site of vascular changes is investigated rather than an equivalent site elsewhere (which may not be identical). It enables the study of the microcirculation itself. There is no risk of damage or interference to that delicate area. As tissue utilisation of oxygen is measured by cyt aa3 changes, this method provides information on the demands and "integral health" of the tissues concerned in addition to data on blood supply.

Clinical use of NIRS is predicted. Developmental laminitis can be diagnosed when haemostasis is noticed, treatments given can be appraised for their efficacy. All therapeutics for laminitis can be tested for their haemodynamic effects, which until now have effectively been conjecture. This method particularly will enable scientists and clinicians to gain understanding of the haemodynamics of the horse's foot.

Chapter 5 Nitric Oxide and equine laminitis

		Page
5.0	General Introduction	184
5.1	Nitric Oxide donors as treatment for acute laminitis	190
5.2	Plasma arginine and endogenous inhibitors of nitric oxide synthesis	239
5.3	Synthetic inhibitors of nitric oxide synthesis <i>in vivo</i>	255
5.4	General Discussion	261

The endothelium... "Its presence in arteries, including the aorta, provides a leakproof lining that retains blood within the vessels."

(Wilson, 1979)

5.0 Introduction

As noted in Chapter 1, equine laminitis features as a commonplace disease in the earliest books of veterinary medicine and farriery (Hodson *et al.*, 1672). The syndrome remains incompletely understood and the many potential aetiological factors include carbohydrate overload, endometritis and surgical complications (Hood *et al.*, 1993). The initiation of acute laminitis is certainly multifactorial. Attempted inductions of acute laminitis have given inconsistent and equivocal results. For example, injected endotoxins fail to induce laminitis (Fessler *et al.*, 1982) and an oral carbohydrate overload to induce the disease experimentally produced the disease in some animals, killed others from shock and had no effect on the remainder.

Fundamentally, laminitis is a vascular disease associated with areas of ischaemia or haemostasis within the hoof (Coffman *et al.*, 1970; Hood *et al.*, 1978). In the overt stages, total blood flow to the hoof may increase (Robinson, 1990), but arterio-venous shunts open (Hood *et al.*, 1978; Pollitt, 1990), to decrease perfusion of dermal laminal tissues (Galey *et al.*, 1990) and cause local distal ischaemia. However the exact mechanisms that compromise pedal circulation within the hoof are arguable (Hunt 1991; Hood *et al.*, 1993). The various hypotheses have been reviewed by Hood *et al.*, (1993). Laminitis may be a sequel to disturbed keratin metabolism within the hoof or endotoxaemia may instigate the acute phase. Circulating endotoxins, lipopolysaccharides (LPS) derived from the cell wall of gram negative bacteria, damage the endothelium by attaching themselves to components of blood vessels and initiate a cascade of events to release inflammatory mediators, including local hormones; normal circulation is then compromised.

In other aetiologies, the mechanisms responsible for disturbing haemodynamics are unclear but plausible features include:

- pooling as a result of venoconstriction to give increased post capillary resistance and secondary physical occlusion of capillaries by microthrombi (Weiss *et al.*, 1994);

- inappropriate function of arteriovenous anastomoses resulting from the actions of vasoactive mediators such as vasoactive intestinal peptide (VIP), calcitonin gene related peptide (CGRP), Substance P or CNS activated hormones (Molyneux *et al.*, 1994) to modify the distribution of blood within the hoof;

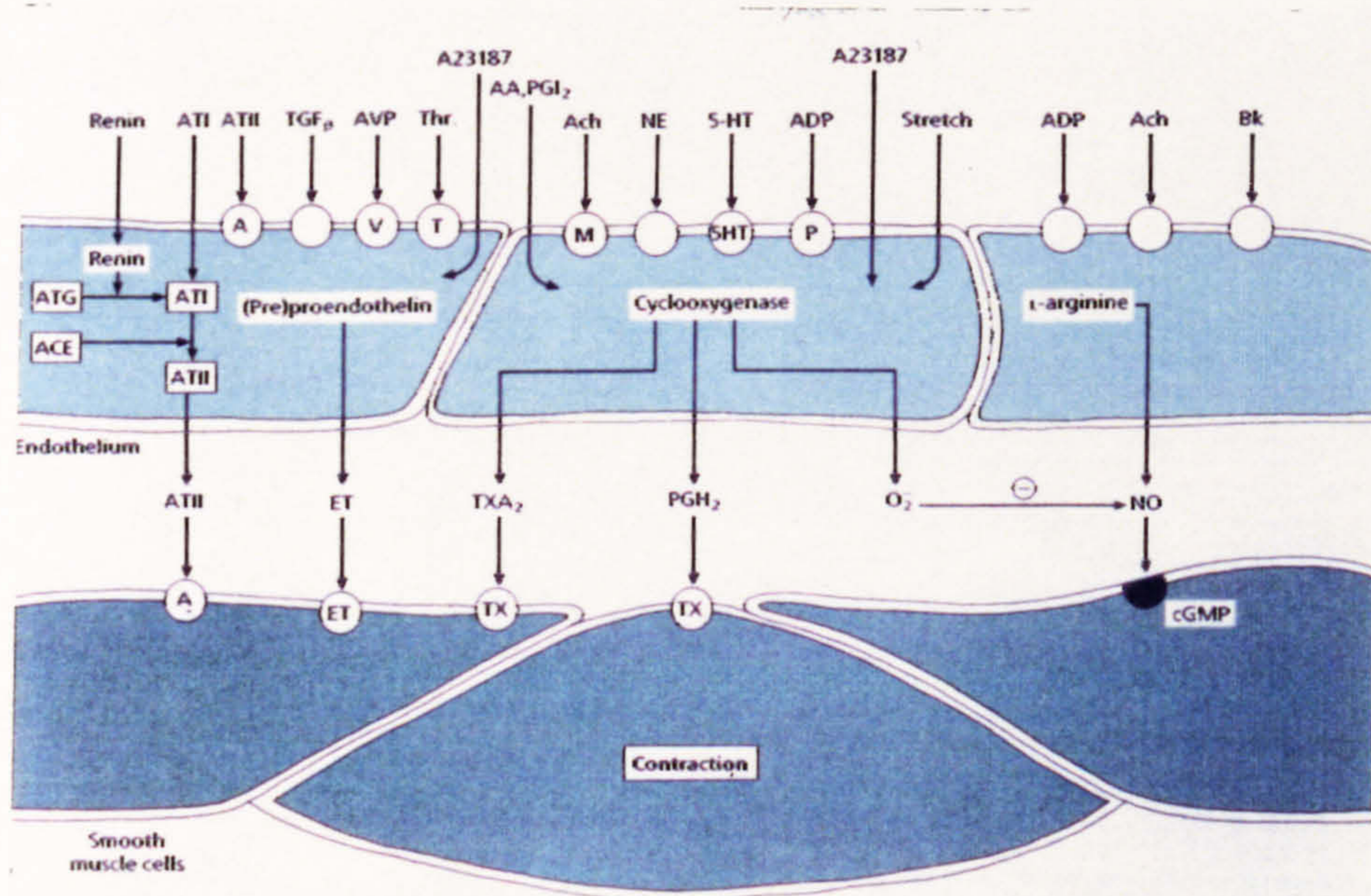
and generalised vasoconstriction as a result of primary vascular events (Hunt, 1991; Hood *et al.*, 1993).

Whatever the exact pathogenesis of laminitis, it is clear that endocrine and paracrine factors regulating digital vascular tone are of importance.

As seen in Chapters 1 and 3, circulation and blood pressure are controlled by many factors, some active, others passive; others are systemic and others are local (see Liu and Barnes, 1994). Passive factors include cardiac output, gravitational force and other aspects of fluid dynamics (see Chapter 3). “Passive” factors in the pathophysiologies include physical obstructions such as thromboses or vessel damage. Active control of the circulation and vascular tone at systemic and paracrine levels involves nervous control, systemic hormones and local paracrine agents.

Nervous control of blood pressure was considered in Chapter 3 and other systemic hormones, Angiotensin II and Atrial Natriuretic Peptide are further discussed in Chapters 6 and 7 respectively. Many systemic and paracrine hormones, and other factors such as prostaglandins, prostacyclin, other arachidonic acid derivatives, and hyperpolarising factors contribute to vascular homeostasis. Their interactions are extremely complex. Endothelial dependent relaxation can be produced by the action of many agents including acetylcholine, bradykinin, histamine, thrombin, 5-HT, Substance P and ADP and ATP (Furchgott and Vanhoutte, 1989). Figure 5.1 shows the complex interactions at endothelial level and the relationships between some vasoactive systemic/ paracrine hormones.

Figure 5.1 Complex interactions between some systemic and endothelial hormones (Lüscher 1994)



Endothelium derived contracting factors. Endothelial cells are a source of numerous substances causing contracting of the underlying smooth muscle. ATII, angiotensin II, ATG, angiotensinogen, ACE, angiotensin converting enzyme;

ET, endothelin; NO, nitric oxide; TXA $_2$, thromboxane A $_2$; PGH $_2$, prostaglandin H $_2$; O $_2$, superoxide; open symbols, receptors.

Obviously, such complex interactions between vascular regulators are difficult to assess *in vivo* but much can be learned by the manipulation of some hormones. This can be done by increasing the circulating levels of the hormone itself by infusion or provision of the substrate, or by inhibition of the hormone using competitive analogues or antagonists or agonists of the receptor.

This chapter considers the role of nitric oxide in equine laminitis. As described in Chapter 1 and below in Chapter 5.2, nitric oxide is synthesised from its substrate l-arginine by the action of nitric oxide synthase (NOS) to cause endothelium dependent relaxation of vascular smooth muscle (Lüscher, 1994). A synthetic source of nitric oxide which does not depend on NOS or the intact endothelium, is glyceryl trinitrate (GTN). To assess the possible involvement of nitric oxide in pathological vascular disease - acute laminitis - nitric oxide donors were given to cases of acute laminitis of different aetiologies and the effect assessed by Near Infrared Spectroscopy and/or blood pressure and clinical observation.

It follows that infusion of inhibitors of nitric oxide production, may induce the disease or produce some of the clinical signs when given to normal animals. A synthetic analogue of l-arginine, L^o-nitro-L-arginine-methyl ester hydrochloride (L-NAME) was given to two normal pony mares with no history of laminitis and blood pressure and clinical signs noted.

Endogenous inhibitors of the l-arginine nitric oxide pathway have been recently identified and investigated (Vallance *et al.*, 1992; Fickling *et al.*, 1993; MacAllister *et al.*, 1993; MacAllister *et al.*, 1995). One such endogenous inhibitor, produced by endothelial cells, is asymmetric dimethyl arginine (ADMA), which is considered in section 5.3. These endogenous inhibitors of nitric oxide are considered to be important regulators of vascular function both locally and systemically (Lüscher, 1994). The production or accumulation of ADMA may provoke, or result from, renal disease and chronic vascular

diseases (Vallance *et al.*, 1992). It was considered possible that the chronic laminitics' compromised vasodilatation (seen with NIRS in Chapter 4) and the supposed predisposition to further attacks of acute laminitis from any cause (Colles, 1991) might reflect increased levels of ADMA compared with normal ponies. Plasma concentrations of ADMA in both normal and chronically laminitic animals were determined over a six month period. In addition, plasma ADMA were measured in one case of acute laminitis when treated with l-arginine and GTN transdermally.

This study of nitric oxide substrates/donors and endogenous and synthetic inhibitors of nitric oxide was designed to clarify the putative involvement of this paracrine hormone in the homeostasis of the equine pedal vasculature.

5.1 Nitric Oxide donors as treatment for acute laminitis

5.1.1. Introduction

It is now recognised that the endothelium of the vasculature represents a major source of materials that regulate, primarily or secondarily, blood pressure and the distribution of flow within tissues and organs (Änggård, 1990; Vane *et al.*, 1990; Griffith, 1994). Endothelial derived relaxing factor (EDRF) is a potent vasodilator, discovered by Furchgott and Zawadzki (1980) and later identified as nitric oxide (Palmer *et al.*, 1987). Nitric oxide (NO), produced by vascular endothelial cells maintains circulatory tone in a paracrine fashion (Moncada *et al.*, 1991, Rees *et al.*, 1989). Moncada *et al.*, (1991) notes that nitric oxide "maintains the vascular system in a state of constant vasodilatation". The relaxation of blood vessels by NO can be observed *in vitro* and *in vivo* (Lüscher, 1994).

NO, synthesised from the guanidino group of l-arginine (Palmer *et al.*, 1988; Moncada and Higgs, 1993), is responsible for cGMP mediated relaxation of vascular smooth muscle. L-arginine is therefore the substrate from which nitric oxide originates. It is a strongly basic amino acid with a formula of $C_6H_{14}N_4O_2$ and a molecular weight of 174 Daltons (Figure 5.2).

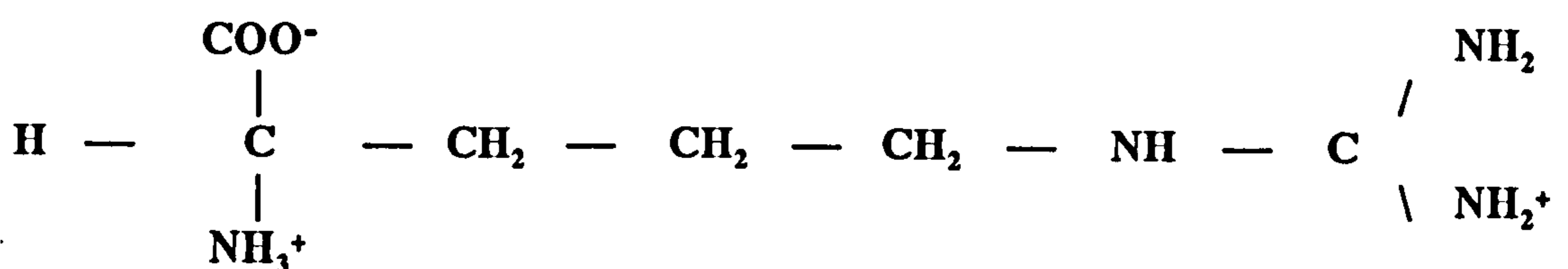


Figure 5.2 Molecular formula of l-arginine (Voet and Voet, 1990)

The amino acid is converted to nitric oxide and citrulline by nitric oxide synthase (NOS). There are at least three types of NOS : endothelial or constitutive (eNOS), inducible NOS (iNOS) and brain NOS (bNOS), each encoded by separate distinct genes. These enzymes bear a close homology (50 %) with each other and with cytochrome P450 reductase (Moncada and Higgs, 1995; Garthwaite, 1992; Schini-Kerth and Vanhoutte, 1995). The NOS family of enzymes can be divided into two types - those that are calcium dependent and those that are calcium independent (Bryant and Elliot, 1994). The constitutive enzymes in endothelial cells are calcium dependent and rely on the intracellular calcium concentration, requiring calcium ions, calmodulin and NADPH as cofactors (Palmer and Moncada, 1989; Lüscher, 1994; Bryant and Elliot, 1994). L-arginine is transported across the cell membrane by an active transport system which is sodium independent and sensitive to other amino acid concentrations (Bogle *et al.*, 1992; Cunningham *et al.*, 1992). It has been suggested that the transport system is linked to the regulation of NOS (Bogle *et al.*, 1992). In addition, receptors on the cell membrane cause calcium mobilisation and activate NOS - *vide supra* (Vanhoutte, 1992). L-arginine is converted to NO and l-citrulline via intermediate compounds (Figure 5.3); NO crosses plasma membranes where it activates cyclic guanosine monophosphate (cGMP) in vascular smooth muscle, which in turn causes relaxation (Figure 5.4).

Figure 5.3 Conversion of l-arginine to nitric oxide and l-citrulline (Curzon *et al.*, 1994)

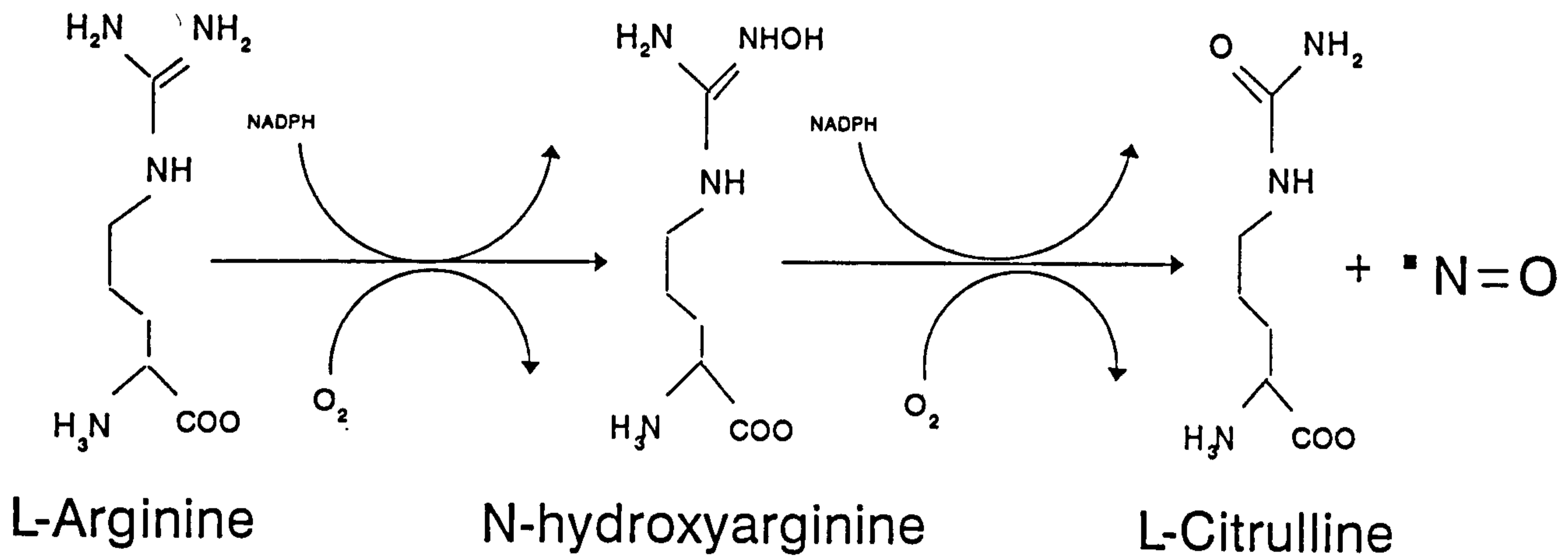
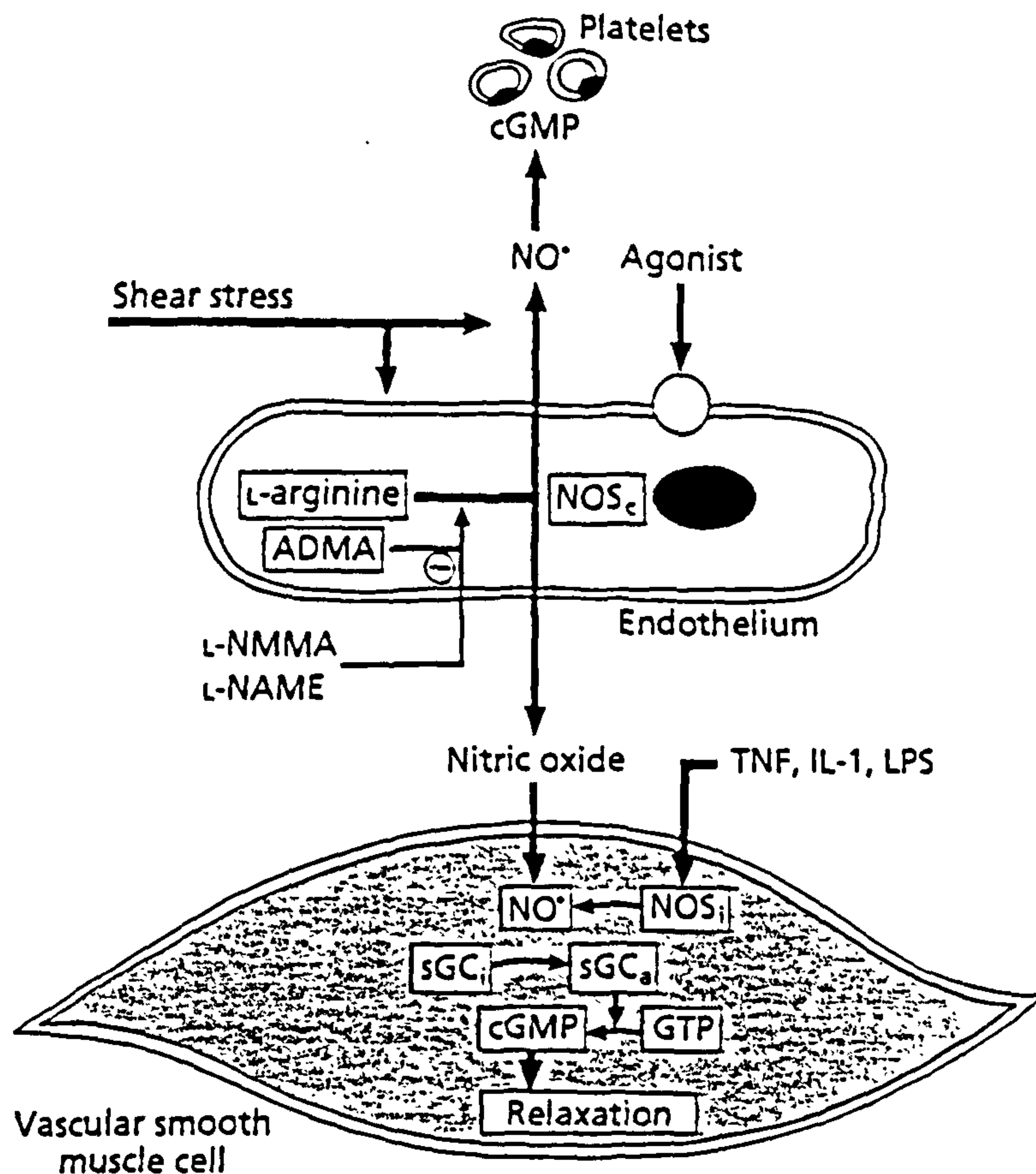


Figure 5.4 Relaxation of vascular smooth muscle by the L-arginine-nitric oxide pathway (Lüscher, 1994)



The L-arginine pathway in the blood vessel wall. Endothelial cells form nitric oxide (NO) from L-arginine via the activity of the constitutive nitric oxide synthase (NOS_c), which can be inhibited by analogues of the amino acid such as asymmetrical dimethyl arginine (ADMA), L-N^G-monomethyl arginine (L-NMMA) or L-nitro arginine methylester (L-NAME). Nitric oxide activates soluble guanylyl cyclase (sGC) in vascular smooth muscle and platelets, and it causes increases in cyclic 3'5'-guanosine monophosphate (cGMP), which mediates relaxation and platelet inhibition, respectively. Shear stress and receptor-operated agonists stimulate the release of nitric oxide. In addition, vascular smooth muscle cells can form nitric oxide via the activity of an inducible (by tumour necrosis factor, TNF; interleukin-1, IL-1; and lipopolysaccharide, LPS) form of nitric oxide synthase (NOS_i).

The inducible form of NOS is calcium independent and is found in platelets and macrophages; it is activated by cytokines, particularly TNF and interferon γ (Bryant and Elliot 1994) and by endotoxins. Nitric oxide action in macrophages is also mediated through cGMP. This is the mechanism responsible for countering infection but can also lead to the acute lowering of blood pressure seen in septic shock. Administration of inhibitors of the NO pathway have been given to patients suffering septic shock with some success (Petros *et al.*, 1991; 1992)

Once NO is produced it has a very short half life, less than 5 seconds, and readily diffuses through tissues. In brain and nervous tissue it acts as a neurotransmitter (Garthwaite, 1992). NO is thought to mediate the afferent responses of non-adrenergic, non-cholinergic nerves found in the gastrointestinal tract and elsewhere. These responses are of the type regulated by the bNOS which is independent of the eNOS and the endothelium.

In the endothelium, as in the nervous system, when nitric oxide is produced it has an immediate effect, and is relatively non specific. It is bound to haemoglobin and is inactivated by superoxide ions. There are however, synthetic donors of NO which do not depend on the intact endothelium to promote vasorelaxation. The action of these has been known for many years but the mechanism for vasodilatation was unclear until very recently. Amyl nitrate, nitroprusside and glyceryl trinitrate are renowned for their vasodilatory properties of different types and they have been used to treat a variety of physiological disorders in a largely empirical fashion (Lefer and Lefer, 1994). The synthesis of NO from L-arginine is endothelium dependent whilst NO derived from synthetic donors such as glyceryl trinitrate is endothelium independent. This attribute makes them ideal under conditions of endothelial damage such as in arteriosclerotic plaques or by previous exposure to endotoxins.

Glyceryl trinitrate is an organic nitrate used as a sublingual and transdermal treatment for angina pectoris (Parker, 1993 ; Neal, 1993; Reeves, 1995). Its molecular structure is shown in Figure 5 5

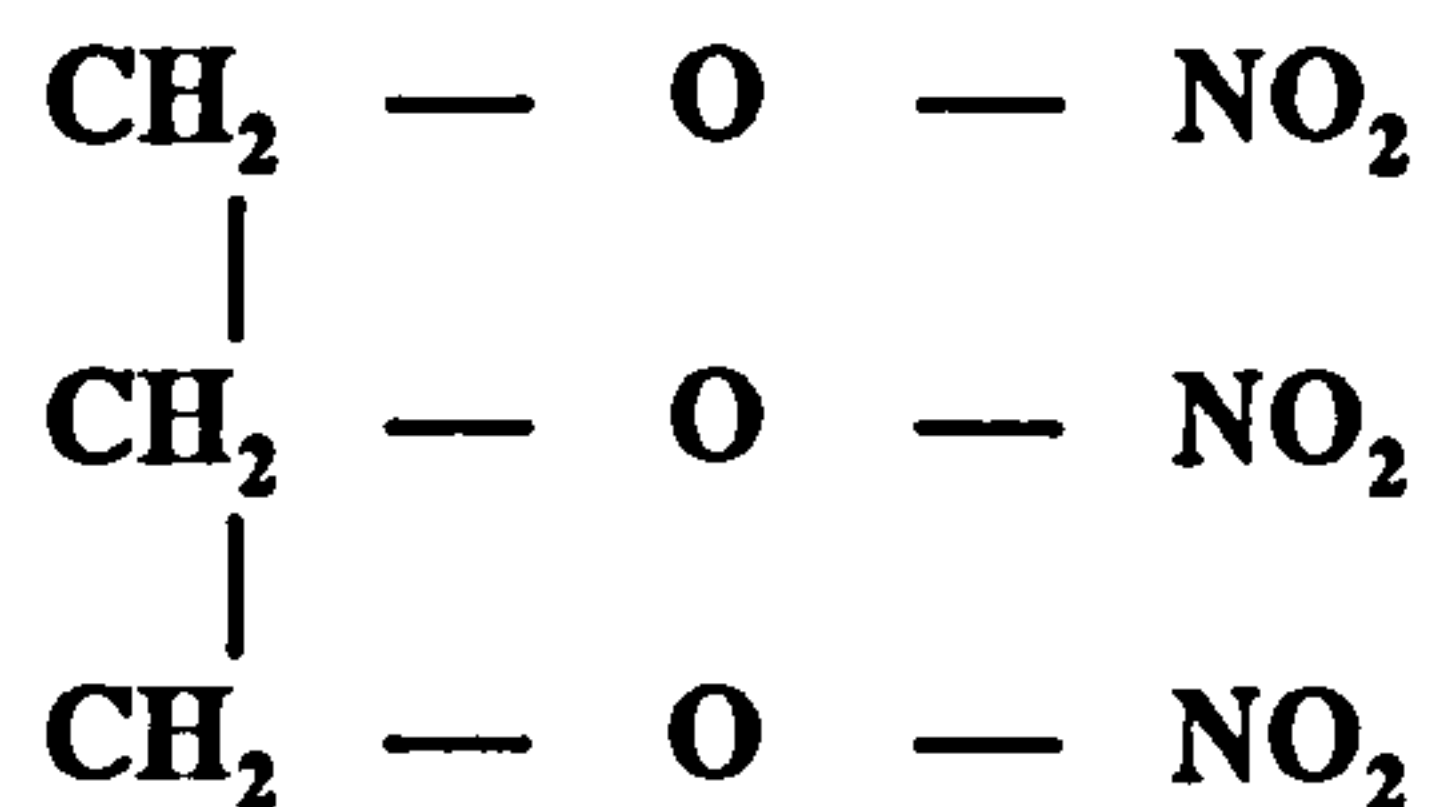


Figure 5.5 Molecular structure of glyceryl trinitrate

Glyceryl trinitrate releases a nitrite ion when in contact with tissue thiols, especially in cell walls (Rang and Dale, 1991; Neal, 1993) and further NO molecules can be released under enzymatic cleavage. When nitric oxide is produced it combines to form an active nitrosothiol intermediate which activates cGMP. This in turn causes relaxation of vascular smooth muscle. The action of GTN *in vivo* is rapid if taken sublingually but of shorter duration (about 30 minutes) than the slower release and more lasting duration of transdermal applications (Rang and Dale, 1994). GTN causes widespread vasodilatation of veins and arterioles with a reduction of central venous pressure and reduced cardiac output. In small doses the blood pressure remains normal as reduced cardiac output (reduced stroke volume) is compensated for by tachycardia. In larger doses, arterioles dilate and blood pressure will fall (Rang and Dale, 1994).

In the context of equine physiology, NO is likely to be an important factor in the regulation of pedal microcirculatory homeostasis. The *in vitro* responses of equine digital vessels to vasoconstrictive agents have been studied (Baxter *et al.*, 1989), and participation of NO in modulating reactions of the digital veins has been examined (Elliot *et al.*, 1994; Schneider *et al.*, 1994). Overall the responses of equine vessels resemble those of other species. Vessels from horses given endotoxins iv react differently to

endothelium dependent and endothelial independent donors of NO (Baxter, 1994a; Baxter, 1994b) in that they have attenuated responses to endothelium dependent agents. Since laminitis is a disease of a compromised microcirculation, endothelial derived hormones are potentially of vital significance. This study addresses, *in vivo*, the potential role of NO in regulating pedal blood flow under normal and laminitic circumstances.

5.1.2 Materials and Methods

The following investigations were undertaken:

1. Blood pressures and heart rates were measured non-invasively in normal ponies and during the treatment of acute laminitis with nitric oxide donors.
2. Pedal haemodynamics of a pony with acute laminitis were assessed non-invasively using Near Infrared Spectroscopy and compared with normal controls.
3. A 10% l-arginine solution and an infusion of 0.9% saline were infused intravenously into normal horses and pedal haemodynamics compared using NIRS.
4. The effects of intravenous administration of l-arginine on pedal haemodynamics during acute laminitis were examined with NIRS.
5. Glyceryl trinitrate was administered to ponies with acute laminitis of mild and moderate severity, and to one case of *post partum* laminitis. Responses were compared with those of untreated cases of mild laminitis.
6. The short term effects of l-arginine and glyceryl trinitrate on blood pressure and heart rate were recorded non-invasively
7. The metabolites of nitric oxide synthesis, nitrates, were measured in serum and urine in normal and diseased horses, before and after treatment.

Animals, management and measurements.

Animals. Table 5.1 summarises the animals used. Animals were kept as a group for at least 6 months prior to study and were subject to careful management regimes of feeding, anthelmintic prophylaxis and farriery. The animals were turned out to grass on unfertilised old pasture. Some ponies had a history of laminitis when at grass in previous years, but were not lame or in need of special farriery. Acute cases described (repeated attacks in 5 animals) were lame in four limbs and the lameness described by Obel (1948) which is outlined in the Introduction. In this study the categories were subdivided into half grades (eg 2.5) to describe the slight changes in lameness existing between the grades. In addition to the ponies suffering grass induced laminitis, there was one case of *post partum* laminitis which followed dystocia and endometritis. Details of this mare appear in Chapter 3, 6, 7 and 8, and the therapeutic regime is given in Appendix IV. Animals were restrained in a standard crush unless sedated. All procedures were carried out under the Animals (Scientific Procedures) Act 1986. Intravenous infusions were administered through indwelling catheters (Ohmeda, Hatfield, Herts.) placed in the left jugular vein, secured with superglue (Vetbond, 3M, UK) and tape (Treatplast, Animalcare Ltd., York).

Blood pressure and heart rate. Systolic, diastolic and mean blood pressures and heart rates were measured non-invasively using a tail cuff attached to a Dinamap Vital Signs Monitor Model 8711 (Critikon, Johnson and Johnson, Ascot, UK.). Animals had been accustomed to the routine over the previous 3 months during which time basal values for blood pressure were established. Mean blood pressures presented on tables represent multiple readings taken over a period of days or months.

Table 5.1. Description of animals used in the study and procedures

Name of animal	Breed	Procedures*	Normal / Clinical	Case No
Albert	Hunter	NIRS, sedated , BP	normal	-
Beckford	Hunter	l-arginine iv, BP, ECG	normal	-
Beau	Welsh cross	NIRS, l-arginine iv, GTN, BP	laminitis	1, 2, 3
Biscuit	Reg Irish Draught	GTN,BP	laminitis	13
Cobweb	Welsh cross	GTN, BP	normal/laminitis	7, 8, 11
Cracker II	Welsh cross	BP	normal	-
Despina	Welsh cross	NIRS, l-arginine iv, BP	normal	-
Domino	Appaloosa	BP	laminitis	9
Jasper	Welsh cross	BP	normal	-
Marron	Welsh cross	NIRS, sedated, saline iv, BP	normal	-
Maree Gray	Hunter	NIRS, sedated , BP	normal	-
Misty	Welsh cross	GTN, BP	laminitis	4, 5, 6, 12
Poppy	Reg Irish Draught	NIRS, unsedated	normal	-
Selluci	Welsh cross	BP	normal	-
Snowflake	Welsh cross	BP	normal	-
Tess	Welsh cross	BP	normal/laminitis	10

Key: BP - blood pressure measured non-invasively; NIRS - Near infrared spectroscopy; GTN - glyceryl trinitrate;

* see Table 5.2

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Non-invasive assessment of pedal haemodynamics using Near Infra Red Spectroscopy (NIRS)

The methods employed have been described in Chapter 4. and elsewhere (Hinckley *et al.*, 1995). A Critikon Cerebral Oxygen Utilisation Monitor Model 2000 (Critikon, Johnson and Johnson, Ascot, UK.) assessed vascular function within the hoof. An electro-optic cable connected the monitor to the sensor placed on the dorsal surface of the hoof. A neonatal sensor was chosen to match hoof size and to give an optimal strength of signal. Adhesive tape (Treatplast, Animalcare Ltd., York) or polythene adhesive tape (Gaffatape) ensured exclusion of ambient light and constant sensor to hoof coupling. Real time qualitative changes in deoxygenated haemoglobin (HHb), oxygenated haemoglobin (O₂Hb), cytochrome aa3 (cyt aa3) and total haemoglobin (tHb) were displayed on the monitor cathode ray screen and recorded on a portable computer (Thinkpad, IBM, UK). In some procedures , an improved version of the NIRS instrumentation , the Critikon Cerebral Redox Monitor Model 2001, was used to quantify changes (in $\mu\text{mol l}^{-1}$, μM). The two versions of the apparatus account for the differences in the figures shown in results. The methodology outlined by Essenpries *et al.*, (1993) is beyond the scope of this thesis, but the benefits in the ability to compare data between animals are clear. It should be recognised that quantification of the signals will result in some differences in the traces of the previous two instruments. These differences will show particularly on the cyt aa3 data, where changes will not be as great as those displayed on the trend device. This arises mainly from the fact that as the concentration of cyt aa3 in tissue is low, concentration changes will be small. A further experimental difference from the previous study was the use of adhesive pads (supplied by the manufacturer) which improve sensor function and prevent the detection of extraneous light. Sensor positioning was broadly the same as in the previous chapter, but a new instrument feature, signal status , allowed precise placement of the sensor to maximise signal strength. Data were again recorded on a PC and charts produced in a spreadsheet using Microsoft Excel.

Experimental Protocols

Preparation of l-arginine solution. A sterile 10% solution of l-arginine was prepared. 40g l-arginine hydrochloride were dissolved in 400 mls of 0.9% saline (Baxter, Thetford, Norfolk, U.K.) pH adjusted to 7.36, filtered through a 0.22µm membrane (Nalgene Filterware, Nalge Co., New York, U.S.A.) and stored sealed at 4°C. A similar solution of 12 % l-arginine was prepared in 0.9% saline, 0.1% glucose drip.

Effects of intravenous l-arginine. L-arginine solution was administered iv to three animals (two normal and one laminitic) and compared with a normal animal infused with the vehicle alone. The first horse - Beckford (550 Kg) - received a total dose of 231g of l-arginine (0.42 g/Kg) at a rate of approximately 50 mls/min (1 mg/kg/min). The normal pony - Despina (360 Kg) - was sedated, for assessment of pedal haemodynamics, by iv administration of 0.006 mg/kg detomidine hydrochloride and 0.012 mg/kg butorphanol. This animal received a total dose of 40g of l-arginine (0.12g/Kg) at a rate of approximately 27 mls/min (0.7mg/kg/min). The pony suffering acute laminitis - Beau (250 Kg) - was sedated, for assessment of pedal haemodynamics, by iv administration of 0.006 mg/kg detomidine hydrochloride and 0.012 mg/kg butorphanol. The l-arginine solution was administered through an intravenous catheter at a rate of approximately 40 mls/min (16 mg/kg/min) for 30 minutes. This pony (250 kg) received a total dose of 120 g of l-arginine (0.48 mg/Kg). Another normal pony - Marron (300 Kg) - was sedated as above and given the vehicle of 0.9% saline alone to act as control for the l-arginine.

Effect of transdermal glyceryl trinitrate (GTN). Ten cases of acute laminitis developed spontaneously whilst at grass. The severity of the disease varied between mild (two cases) characterised by Obel Grade 2 (Obel 1948) and moderate (ten cases) Obel Grade 3 or 4. On diagnosis, ponies were stabled, fed only hay, and other treatments or medication were not applied, except for one pony mare which received 1g oral Phenylbutazone daily because of minor accidental trauma. The caudal surface of the

pasterns was clipped and shaved over the area of the digital vessels extending medially and laterally, then rinsed and dried. The GTN patches were secured on the pasterns with stretch adhesive tape (Treatplast) to position them over digital vessels. Two, three or all four limbs were treated at any one time. In two cases the right hindlimb was bound with a dummy patch, without GTN, and served as a control. 2% Glycerol Trinitrate ointment was applied once daily to the pasterns as a "patch"; each aliquot of paste positioned over the two main digital vessels, covered with grease proof paper and secured in place with adhesive tape. Doses for individual animals are estimated under experimental protocols - these are obviously approximations since rates of cutaneous absorption are not yet known. Calculated doses of glyceryl trinitrate are shown in Table 5.2.

Table 5.2: Doses of glyceryl trinitrate given to acutely laminitic ponies

<u>Paste (ins)</u>	<u>Glycerol trinitrate</u> <u>Approx. weight(mg)</u>	<u>Total approximate amounts of Glycerol trinitrate mg/day</u>				
		<u>Applied in two aliquots to:</u>				
		<u>1 limb</u>	<u>2 limbs</u>	<u>3 limbs</u>	<u>4 limbs</u>	
3/4"	10	20	40	60	80	(dose a)
1/2"	7	14	28	42	56	(dose b)
1/4"	3	6	12	18	24	(dose c)

In all cases the treatment with GTN was carefully monitored both by blood pressure and by any response of improvement in lameness. If lameness markedly improved, blood pressure invariably fell and the dose of GTN was reduced. Each animal was treated individually to achieve maximal improvement without causing hypotension. Ten cases of laminitis were treated and two very mild cases were stabled but left untreated. Treated were compared with untreated animals.

Effect of intravenous arginine in combination with transdermal glyceryl trinitrate. The laminitic pony given 10% l-arginine iv was treated with GTN "patches" 12 hours after the l-arginine infusion. Patches were applied to three limbs only - right and left fore and

the left hindlimbs. The amounts given were as follows: on the first week a total of 60 mg GTN/day (0.3 mg/kg/day) was applied. This amount was reduced to 40 mg GTN/day (0.02 mg/kg/day) for five days, which was further reduced to 20 mg GTN/day (0.01mg/kg/day) for two days before the end of the treatment.

Transdermal administration of glyceryl trinitrate to cases of grass induced laminitis.

GTN was usually administered as an initial dose of 60 mg (0.3 mg/kg/day) for two days (dose a). If blood pressure decreased and lameness improved, it was reduced to 40 mg GTN/day (0.02 mg/kg/day) for two days (dose b) and then to 20 mg (0.01mg/kg/day) for two days until the end of the treatment (dose c). This regime varied slightly according to individual responses and severity of the disease.

Transdermal administration of glyceryl trinitrate to a case of post partum laminitis.

A five year old Registered Irish Draught Mare presented with slight acute laminitis following dystocia and retained placental membranes. Despite a course of parenteral antibiotics (25 mls/ day penicillin/ streptomycin im, Streptopen Pitman Moore, Cheshire, U.K) she developed mild laminitis 24 hours after foaling. The lameness was mild Obel 2 (Obel 1948) and there were no overt signs of uterine infection. The mare was treated with 12 mls flunixin meglumine iv, (Finadyne, Schering-Plough Animal Health, Suffolk, UK) and transdermal GTN paste at a dose of 1" paste x 2 on each pastern as described above (160mg). The mare received 10 mls flunixin meglumine 20 mls phenylbutazone solution iv (Tomanol) and 4 mls acepromazine solution (ACP) iv (C-vet , Bury St Edmunds, Suffolk, UK). The mare received uterine irrigation with 2L 0.9% saline and 2.5 mls (25 iu) oxytocin iv (Leo Animal Health Division, Princes Risborough, Bucks., UK) and 6mls Penicillin/ Streptomycin (Streptopen Pitman Moore, Cheshire, U.K) and 6 mls Framomycin (C-vet, Bury St Edmunds, Suffolk, UK). The analgesic routine of phenylbutazone, flunixin, oxytocin and ACP was repeated 4 times daily. The GTN patches were renewed once daily at half the dose on the second and third days. Uterine treatments were repeated once daily. The mare was kept on a deep bed of wood shavings and was showing marked clinical improvement and reduction of lameness by the

fourth day. On the fourth day, the dose of GTN was further reduced to 40 mg/day. Blood pressures were taken twice daily and the analgesic/ α -agonist therapy continued. GTN was reduced to 24 mg/day on the 7th day and repeated daily until the 21st day of the disease, when GTN was stopped. The other medication was continued except that the phenylbutazone was given orally at a dose of 4g/day rather than iv. Oral ACP was given instead of iv administration and blood pressure remained at a slightly higher than normal value.

Short term effects of iv l-arginine solution on heart rate and blood pressure of normal horses

One normal pony and two normal horses were given iv solutions of l-arginine as outlined above. Blood pressure and heart rate were monitored. The protocols are outlined above under the section entitled 'Effects of intravenous arginine'.

Short term effects of transdermal glyceryl trinitrate on heart rate and blood pressure during acute laminitis

Glyceryl trinitrate was administered transdermally to two animals suffering acute laminitis. These animals had received GTN the previous day and observations were made before, and after, the administration of the daily dose. Serial blood pressures and heart rates were recorded before administration of the dose and at 5 minute intervals for periods of up to 2 hours after.

Blood and urine samples

Blood samples were taken by jugular venepuncture into glass vacuum tubes (Vacutainer, Becton Dickinson) and allowed to clot. Samples were kept at 4° C until separation of serum and red blood cells was complete. Serum supernatant was pipetted into polypropylene tubes and stored at -20° C until analysis. Spontaneously voided urine was collected into a clean bucket and aliquots stored in polystyrene containers -20° C until analysis.

Serum and urinary nitrates

Nitrates were measured by ion chromatography using a 2000 I unit (Dionex, Farnborough, Hampshire) with conductometric detection and a AS4 Column (Dionex, Farnborough, Hampshire).

Statistical Analyses

Data are presented as Means \pm SE. A 2 sample Students t-test was used to assess statistical significance, assuming unequal variance and two tailed probability. Results were considered statistically significant when p was <0.05.

Drugs and other agents

The materials used and their sources were as follows:

L-Arginine hydrochloride (Sigma, Poole, Dorset, UK)

Glyceryl trinitrate ointment (2%) (Pericutol, Cusi (UK) Ltd., Haslemere, Surrey)

0.9% saline (0.1% glucose) drip (Baxter, Thetford, Norfolk, UK)

0.9% saline drip (Baxter, Thetford, Norfolk, UK)

Detomidine hydrochloride (Demosedan, Smith Kline Animal Health Ltd., Stevenage, Herts, UK)

Butorphanol (Torbugesic, C-Vet, Bury St. Edmunds, Herts, UK)

Phenylbutazone iv. (Tomanol, Intervet UK Ltd, Cambridge)

Phenylbutazone -oral (Equipalazone, Arnold, Essex, UK)

Acepromazine (ACP solution, C-Vet, Bury St Edmunds, UK).

5.1.3 Results

Blood pressures and heart rates of normal and acute laminitic ponies.

As seen in Chapter 3, heart rates varied between 43 and 45 beats/minute for normal ponies. Blood pressures and heart rates of animals during acute laminitis were significantly higher than normal, (Table 5.3). Changes in BP and heart rate following GTN administration are given later.

Table 5.3 Blood pressures and heart rate of normal and laminitic ponies before treatment

	Systolic	Blood pressure (mmHg)		Heart rate (bts/min)
		Diastolic	Mean	
Normal (n= 8) Number of tests = 63	107± 4	62 ± 2	78 ± 2	44 ± 1
Acute laminitic (n=10) Number of tests = 23	165 ± 5*	86 ± 2*	119 ± 4*	61 ± 3*

* (p≤ 0.001)

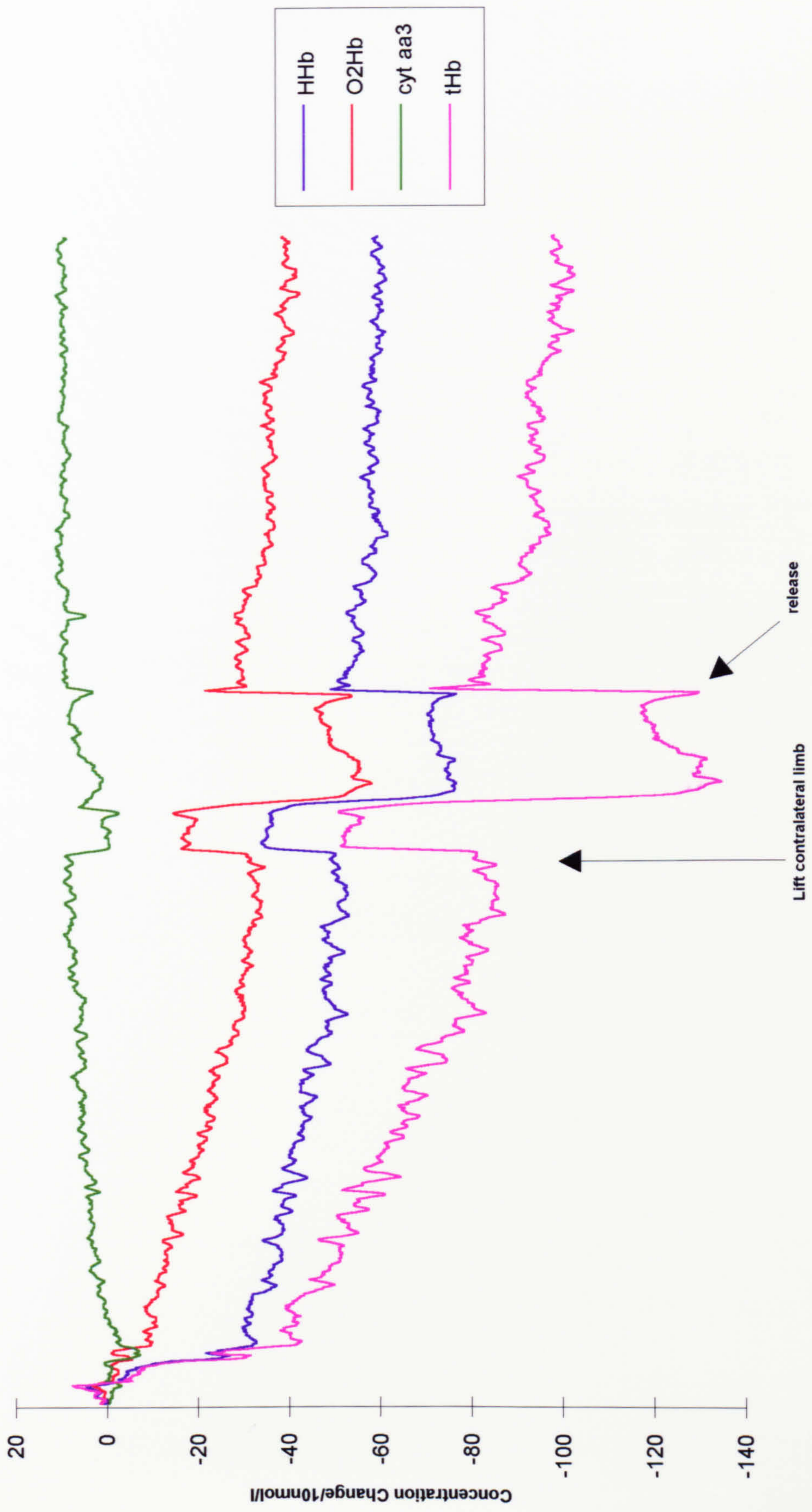
Non-invasive assessment of pedal haemodynamics using NIRS.

Basal values. Animals were monitored continually by NIRS during the period when they stood in the examination box. The traces of these animals displayed a gradual reduction in concentration of O₂ Hb, HHb, tHb and an increase in cyt aa3. to stabilise after approximately 30 minutes (see Chapter 4). The changes during sedation are shown in Figure 5.6. Sedated animals showed normal responses to manoeuvres designed to alter blood flow to the hoof such as lifting of the contralateral limb and manual occlusion of the digital vessels .

Figure 5.6 NIRS assessment of pedal haemodynamics of a sedated normal horse; traces were recorded over a period of 30 mins.

Key: HHb - deoxyhaemoglobin; cyt aa3 - cytochrome oxidase;
O₂ Hb - oxyhaemoglobin; tHb - total haemoglobin.

NIRS of pedal haemodynamics of a sedated normal horse.



Responses of a normal pony to intravenous 0.9% saline The usual initial changes were seen in a sedated normal pony. Traces showed the typical pattern during the iv infusion of 0.9% saline as a normal sedated pony (see Chapter 4). Saline infusion had no obvious effect on pedal haemodynamics (Figure 5.7) and manoeuvres to alter blood flow produced appropriate responses (Figure 5.8).

Responses of normal horses to intravenous l-arginine . There was increased concentration of O₂Hb during the infusion of l-arginine (Figure 5.9). This is concluded to reflect increased perfusion of the pedal vasculature following l-arginine administration, compared with the vehicle alone. Heart rate fell to 30 beats/min during the infusion and cardiac arrhythmia was observed. L-arginine 12 % infused into another normal horse produced transient hypotension, but this was followed by a slight but sustained increase in blood pressure. Heart rate fell to 30 beats/min and cardiac arrhythmia was also observed but other side effects in either instance were not apparent.

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Figure 5.7 NIRS assessment of pedal haemodynamics of a sedated normal horse during an infusion of 0.9% saline iv.; traces were recorded over a period of 30 mins.

Key: HHb - deoxyhaemoglobin; cyt aa3 - cytochrome oxidase;
O₂Hb - oxyhaemoglobin; tHb - total haemoglobin.

NIRS of pedal haemodynamics of a normal sedated pony during 0.9% saline infusion.



Figure 5.8 NIRS assessment of pedal haemodynamics of a sedated normal horse during an infusion of 0.9% saline iv and manoeuvres to alter blood flow.; traces were recorded over a period of 30 mins.

Key: HHb - deoxyhaemoglobin; cyt aa3 - cytochrome oxidase;
O₂Hb - oxyhaemoglobin; tHb - total haemoglobin.

NIRS of pedal haemodynamics of a sedated normal pony during 0.9% saline infusion and occlusion tests

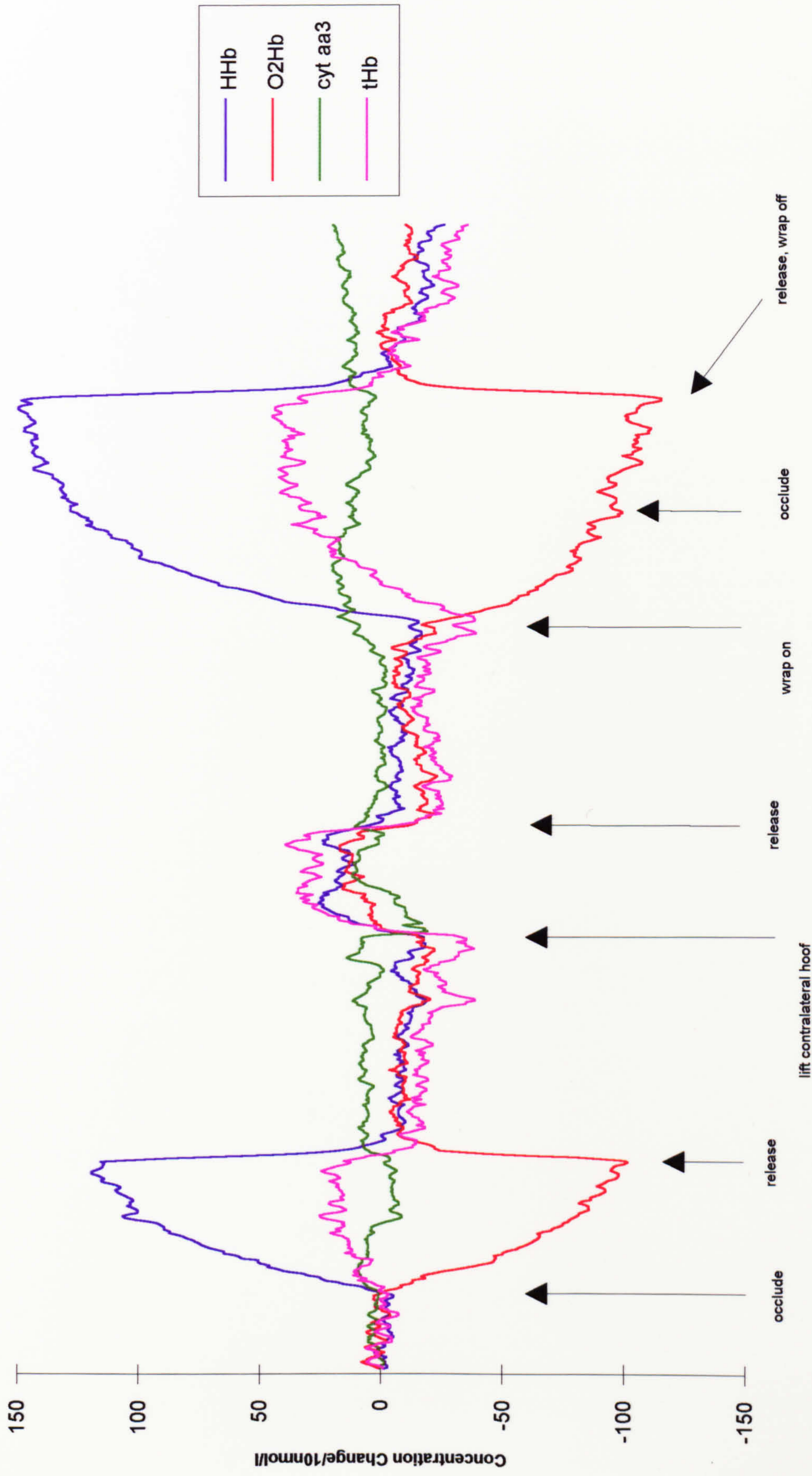
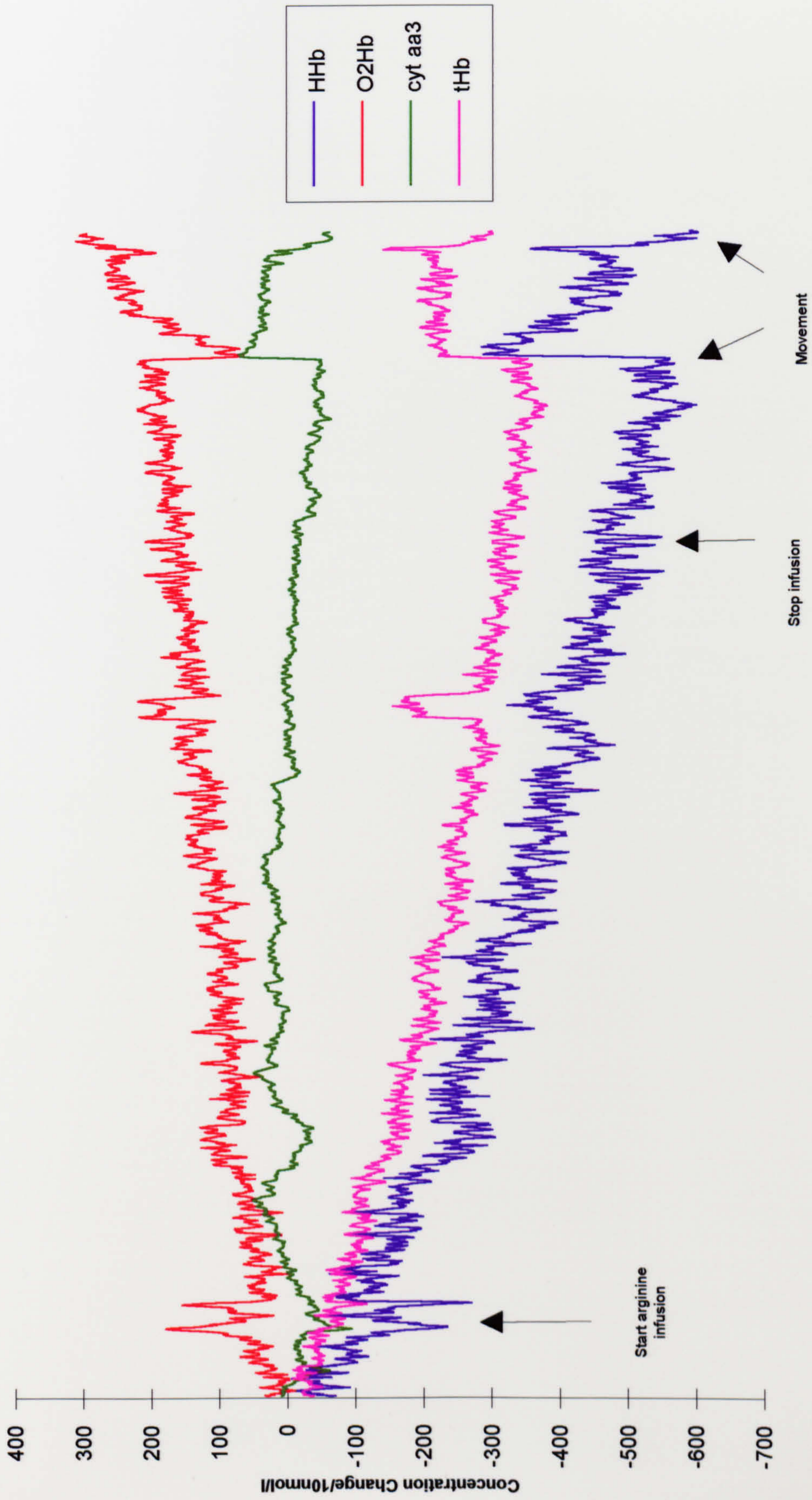


Figure 5.9 NIRS assessment of pedal haemodynamics of a sedated normal horse during an infusion of l-arginine; traces were recorded over a period of 20 mins, the infusion lasted 15 mins.

Key: HHb - deoxyhaemoglobin; cyt aa3 - cytochrome oxidase;
O₂Hb - oxyhaemoglobin; tHb - total haemoglobin.

NIRS of pedal haemodynamics of a normal sedated pony during an infusion of l-arginine



Vascular responses in acutely laminitic ponies. Baselines for oxyhaemoglobin, deoxyhaemoglobin, total haemoglobin, and cytochrome aa3 were obtained (Hinckley *et al.*, 1995) but these showed no responses to manual occlusion of the digital arteries, to lifting of the contralateral limb, or to general movement in the acute laminitic pony. The complete absence of responses to these manoeuvres suggests haemostasis. The qualitative changes in baseline values for normal ponies and an acutely laminitic pony are given in Table 5.3. The lack of responses during attempts to alter pedal blood flow during acute laminitis are shown in Figure 5.10.

Response to intravenous l-arginine solution during acute laminitis. O₂Hb, HHb, cyt aa3 and tHb changed within minutes of the arginine infusion (Figure 5.11). Laminal tissues were apparently reperfused after iv intravenous l-arginine in an acute laminitic pony. There was a transient hypotension during the infusion, mean blood pressure falling 12 ± 2 mm Hg. The animal showed initial signs of pain and sweating which was followed by shivering. The pony was rugged and given 5 mls phenylbutazone iv. and 0.05mg/kg iv. acepromazine 1 hour after ending the infusion. Movement responses were restored during the infusion.

Figure 5.10 NIRS assessment of pedal haemodynamics of case of acute laminitis (sedated) - no changes are seen in response to manoeuvres designed to alter blood flow. Traces were recorded over a period of approximately 2 mins. Each manoeuvre to alter blood flow lasted for 1 min. No movement artefacts are seen.

Key: HHb - deoxyhaemoglobin; cyt aa3 - cytochrome oxidase; O₂Hb - oxyhaemoglobin; tHb - total haemoglobin.

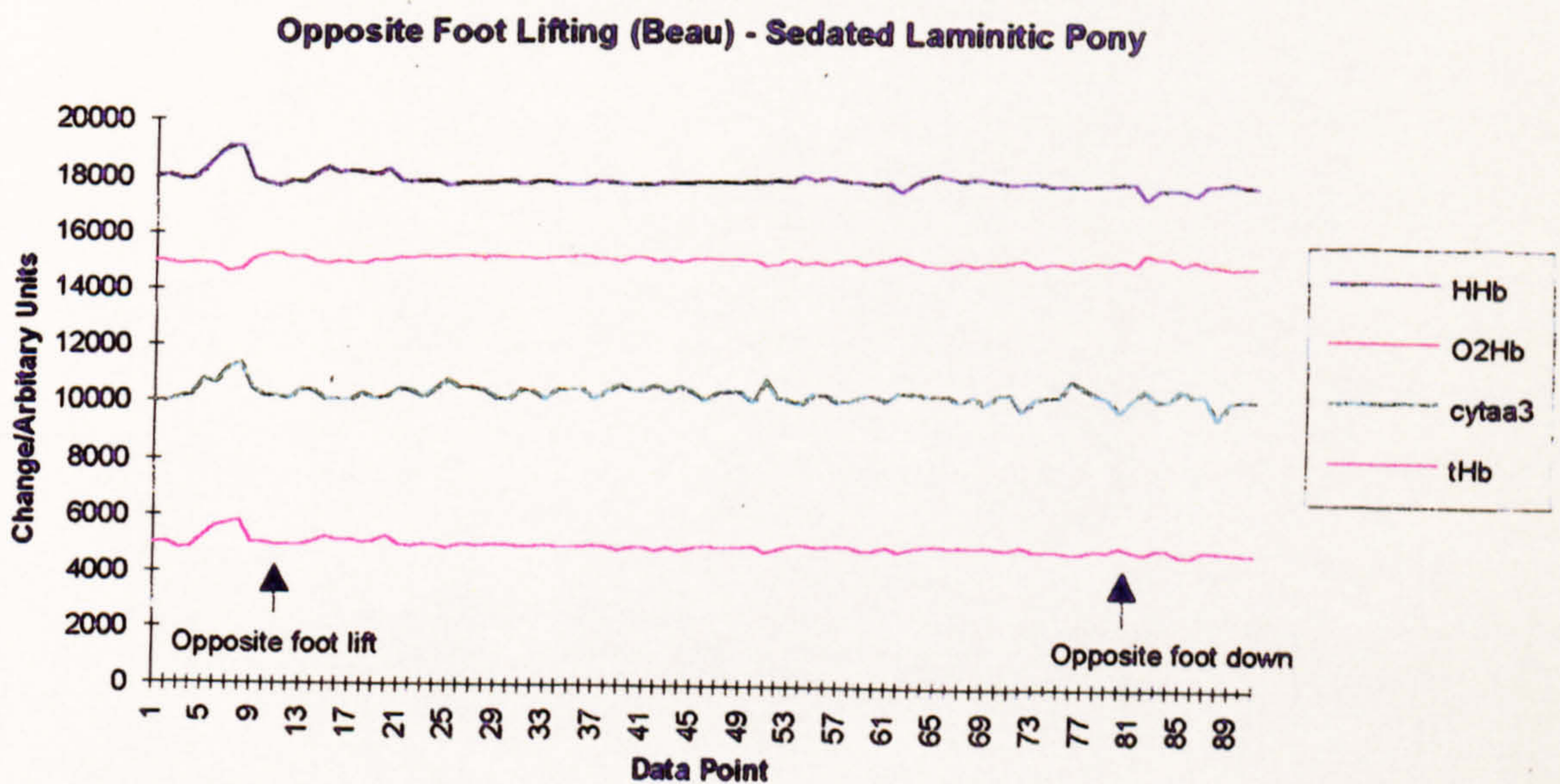
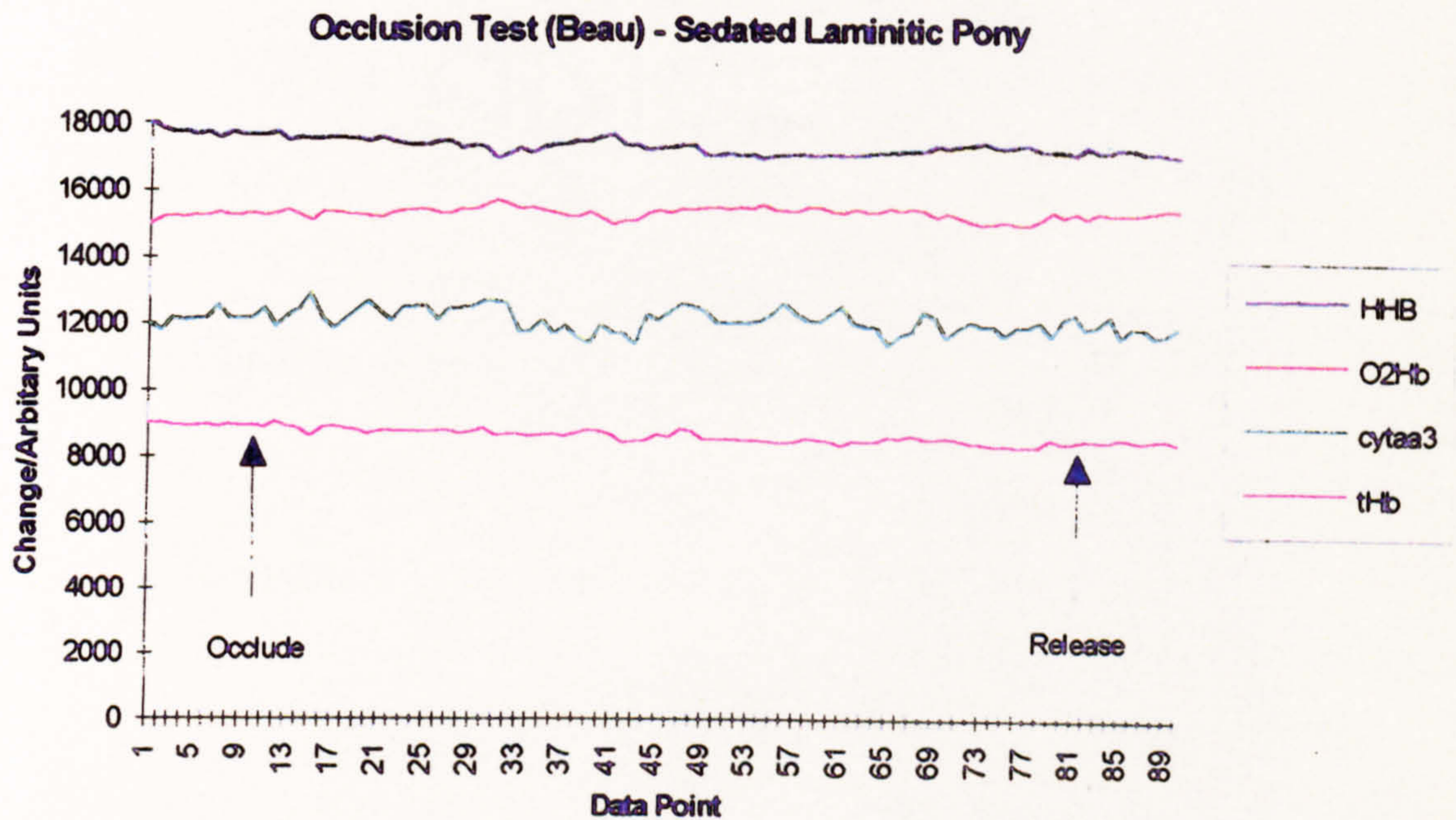


Figure 5.11 NIRS recorded responses of pedal haemodynamics of an acute laminitic pony (sedated) to an intravenous infusion of l-arginine; traces were recorded over a period of 20 mins . Key: HHb - deoxyhaemoglobin; cyt aa3 - cytochrome oxidase; O₂Hb - oxyhaemoglobin; tHb - total haemoglobin.

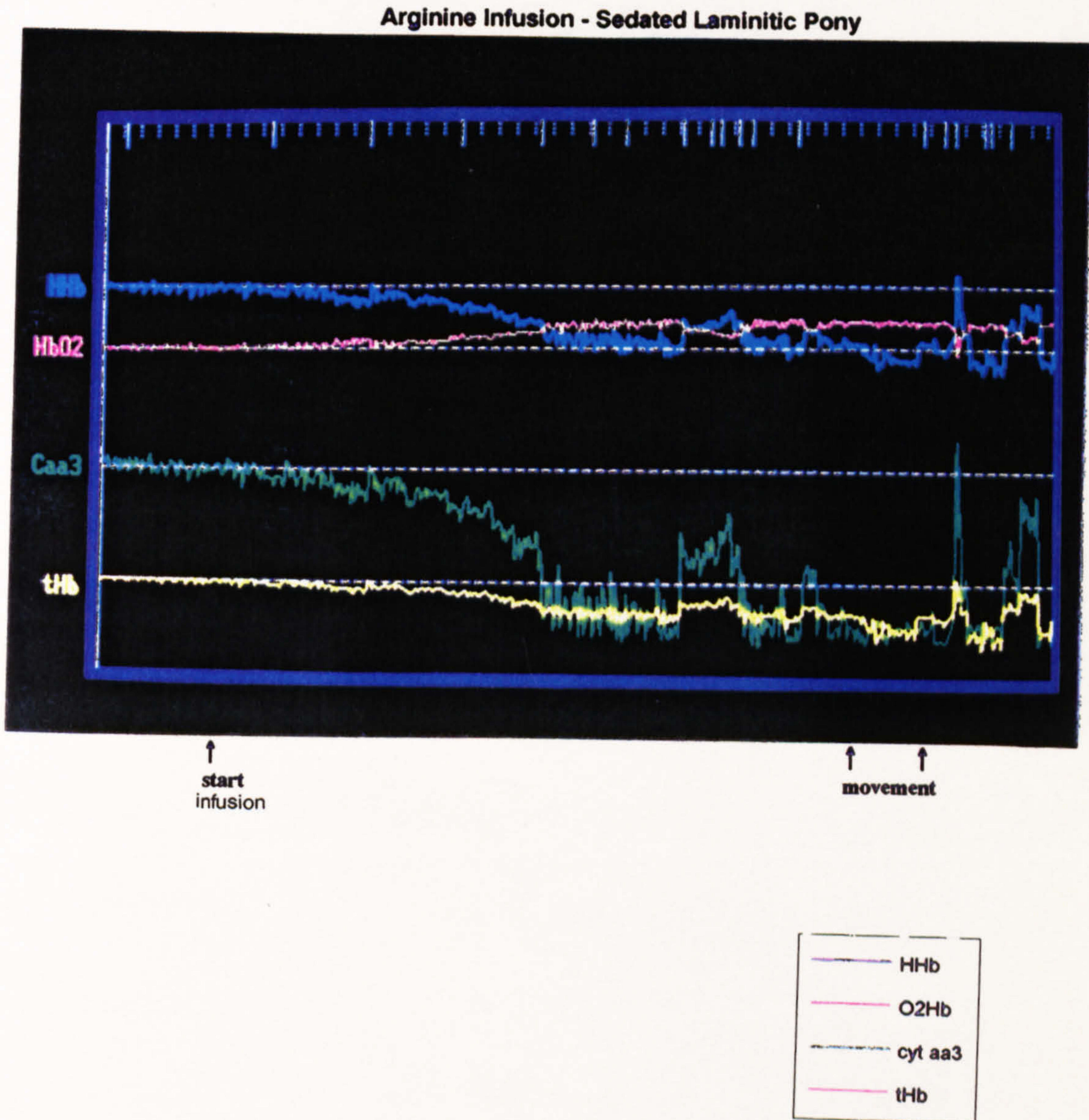


Table 5.4

Qualitative changes in haemoglobin and cytochrome oxidase in the hoof determined by Near Infra Red Spectroscopy. (i) unседated normal (ii) sedated normal (iii) sedated normal during iv infusion of 0.9% saline (iv) sedated normal during iv infusion of l-arginine (v) sedated normal during iv infusion of 0.9% saline and showing responses to manoeuvres designed to alter blood flow (vi) lack of responses of an acute laminitic pony to manual occlusion of digital vessels or lifting of contralateral limb (vii) sedated acute laminitic during iv infusion of l-arginine

		HHb	O ₂ Hb	cyt aa3	tHb
(i) ¹	<i>unседated normal</i>	(↓)	(↓)	(↑)	(↓)
(ii) ¹	<i>sedated normal</i>	(↓)	(↓)	(slight ↑)	(↓)
(iii) ¹	<i>sedated normal</i> (0.9% saline iv)	(↓)	(↓)	(↑)	(↓)
(iv) ¹	<i>sedated normal</i> (l-arginine iv)	↓	↑	=	↓
(v) ¹	<i>sedated normal</i> (0.9% saline iv) occlusion/ lift release	(↓) ↑ ↓	(↓) ↓ ↑	(slight ↑) = =	(↓) ↑ ↓
(vi) ²	<i>sedated acute laminitic pony</i> occlusion/ lift release	= =	= =	= =	= =
(vii) ²	<i>sedated acute laminitic pony</i> l-arginine infusion	↓	↑	↓	↓

Key:

↑	increase above baseline
↓	decrease below baseline
=	no change
(↑)(↓)	indicate slow changes in oxygenation over a period of time which are basal
O ₂ Hb	oxyhaemoglobin,
HHb	deoxyhaemoglobin,
cyt aa3	cytochrome oxidase,
tHb	total haemoglobin.

¹ Critikon Redox Monitor Model 2001² Critikon Redox Monitor Model 2000

Transdermal glyceryl trinitrate (GTN) in acute laminitis

Responses to iv l-arginine and transdermal GTN in acute laminitis Following iv l-arginine during acute laminitis, GTN paste was applied as "patches" to the pasterns of three limbs (Figure 5.12). This markedly improved lameness in treated limbs, but some lameness continued in the untreated limb over three weeks. The pony was able to trot on a concrete yard within two days of the acute phase despite the initial severity of the attack. A lowering of blood pressure suggests a possible systemic action of the transdermal treatment. No analgesics or other medication were necessary. The pony continued to improve and there were no apparent secondary changes, or sepsis, in the hooves.

Topical transdermal application of GTN in acute laminitic ponies. Topical application of GTN patches to the shaved pasterns of acute laminitic ponies reduced systemic blood pressure, alleviated bounding digital pulses and, after an initial worsening of lameness during the first day of treatment, improved lameness in treated limbs. Figure 5.13 summarizes data from individual cases. Clinically, the ponies seemed brighter and happier when the blood pressure was reduced and lameness improved markedly after the first day of treatment. Where animals had been treated on three limbs only, some slight lameness and digital pulses remained in the untreated limbs (Case 1 and Case 4) However, Case 8 that received transdermal treatment on only two front limbs did not have such a rapid reduction in blood pressure or improvement in lameness; this animal had only Obel Grade 2 at the outset of the disease and therefore was only slightly more severe a case than the untreated cases 9 and 10. Untreated mild cases did not show such a dramatic improvement as those that were treated (Figure 5.14). There seemed to be a direct relationship between the dose of GTN and mean blood pressure. In all cases, no special shoeing was needed at any stage and gross morphological changes, necrosis or abscessation were not seen in the hooves in the following nine months. All treated ponies improved steadily and were turned out to grass at the end of the treatment.

Figure 5.12 Glyceryl trinitrate "patches" in place on the pasterns of a pony



Figure 5.13 Case reports of acute equine laminitis treated with transdermal application of glyceryl trinitrate "patches". Doses (a, b and c) of glyceryl trinitrate are given in Table 5.2.

Key: ■ Systolic blood pressure □ Diastolic blood pressure.

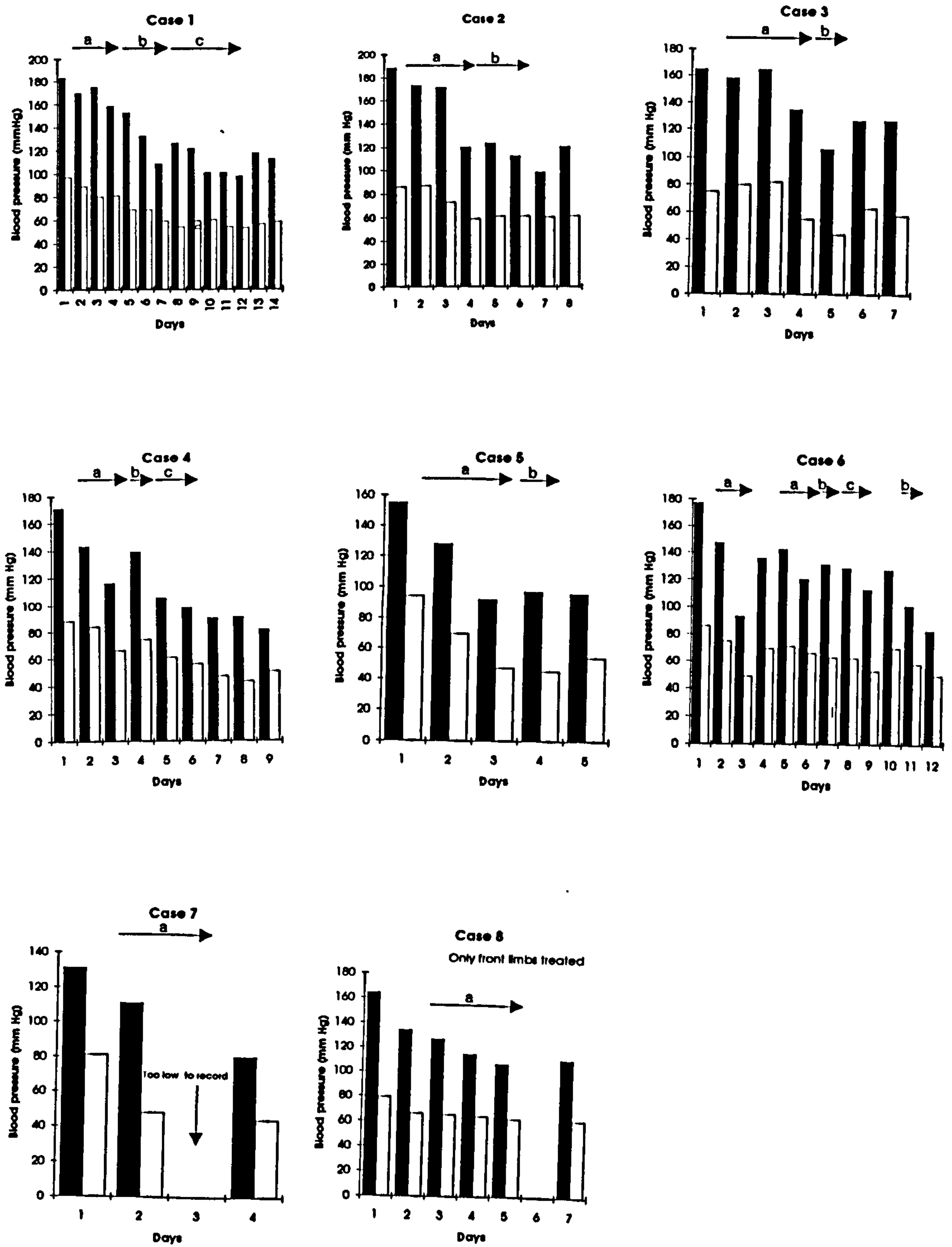


Figure 5.14 Case reports of mild acute equine laminitis left untreated.

Key: ■ Systolic blood pressure □ Diastolic blood pressure.

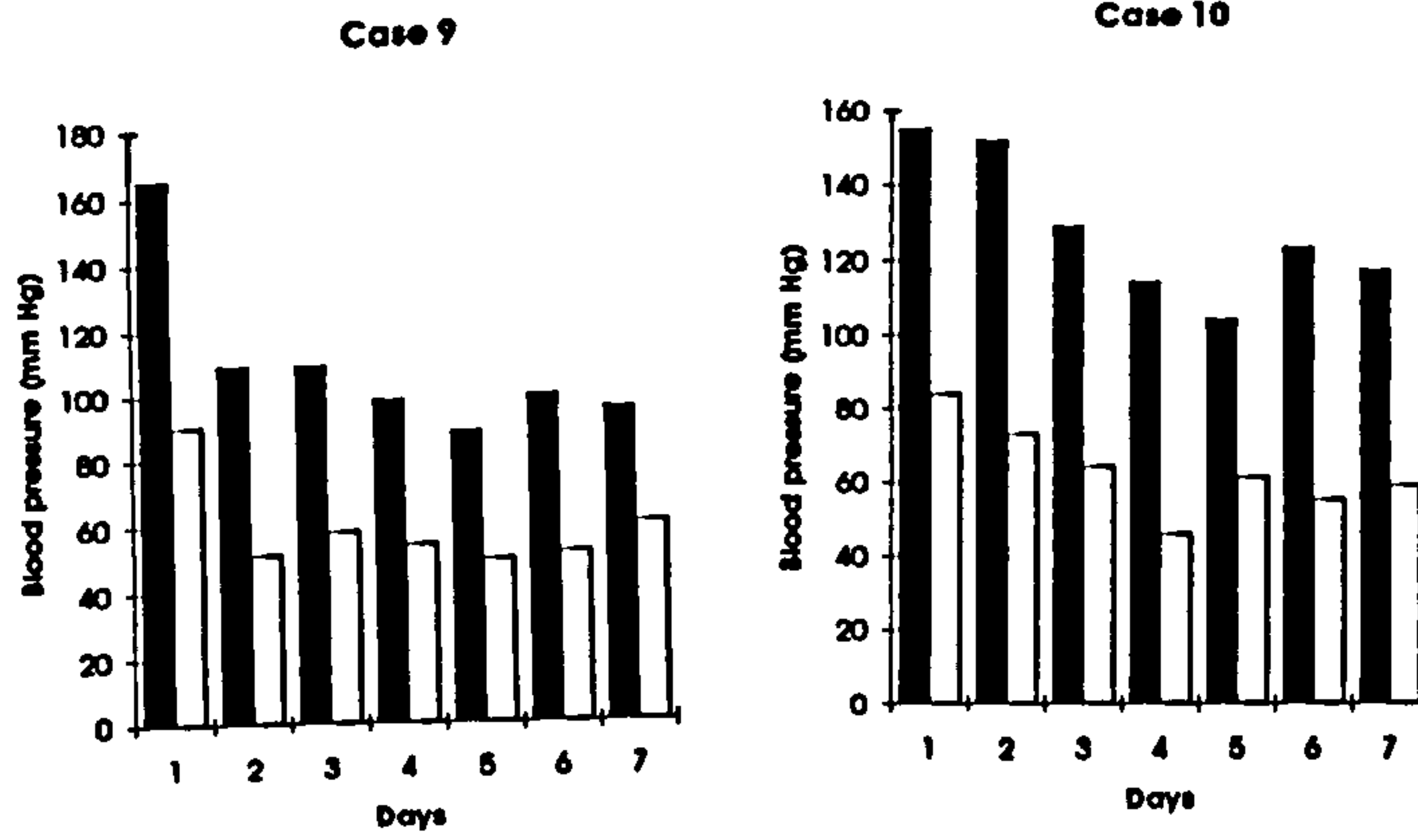


Figure 5.15

Case 1. Pony that received 10% l-arginine iv with glyceryl trinitrate "patches" in place two days after onset; this pony was Obel Grade 4 of lameness on the first day of acute laminitis, was Obel 2 on the second day of treatment and able to trot on concrete without analgesics.



Case report of GTN treatment of post partum laminitis.

A Registered Irish Draught mare presented with mild laminitis the day after foaling complications. The drugs administered are shown in Appendix IV. The mare showed clinical improvement by the evening. However, later that night the mare seemed anxious and was rather unsteady when standing, however she seemed to walk around the box without apparent lameness. By the next morning it had become apparent that the administration of GTN had not prevented the onset of severe acute laminitis. The mare was extremely distressed and was in severe pain and lame, Obel Grade 4. GTN treatment was used as an adjunct therapy to standard pharmacological regimes as outlined in the Materials and Methods section above. Changes in blood pressure are given below (Table 5.5), but of course they may not be directly attributable to GTN as many other drugs were given. Little change was seen between days 5 and 21, more detailed blood pressure records are given in Chapter 3 and in Appendix IV. If the ACP dose was reduced blood pressure increased and so the usual dose was restored

Table 5.5 Changes in blood pressure of a case of acute post partum laminitis given transdermal glyceryl trinitrate (GTN) as an adjunct therapy.

Day	Time	GTN (mg/day)	Systolic BP	Diastolic BP	Mean BP	Heart rate	Obel Gd	Comment
1	12.30	112	132	77	97	79	3 - 2.5	Mild acute laminitis.
	7.30	-	127	79	101	73	2.5	improvement
2	7.00	112	182	95	127	88	4	Severe acute laminitis.
4		56	140	78	107	67	3.5	improvement
21		ends	119	76	92	44	3	improvement

In the first few weeks, the mare's improvement was described as "wonderful" by the attending veterinary surgeon (R.Gunstone MRCVS) since cases of endotoxic aetiology are always severe and improvement is rarely, if ever, seen in early stages. The mare was turned out in a very small paddock with her foal on day 29 and was contented but not particularly mobile (Obel 3) for the next four weeks. During the period of improvement, the mare and foal escaped from their quarters and spent a whole night at liberty. When

recaptured, the mare and foal had done more walking than had ever been anticipated. After that time she showed signs of 'sinking' with a marked depression around the coronary band and increasing lameness. She was brought back into a deep littered box where her condition worsened and seven weeks after the initial onset of laminitis, the bony column had descended through the soles of both front feet which had prolapsed and the pedal bones were exposed. The mare was destroyed.

Short term effects of nitric oxide donors on blood pressure and heart rate in normal and laminitic equines.

(i) l-arginine

12% l-arginine in a glucose / saline iv drip produced transient hypotension in a normal unsedated horse; but this was followed by a slight but sustained increase in blood pressure. Heart rate fell from 47 to 30 beats/min and cardiac arrhythmia was also observed, making measurement of blood pressure with the Dinamap impossible during the infusion.

10% l-arginine in 0.9% saline iv drip was given to a sedated normal pony. This also lowered heart rate but not blood pressure. Heart rate fell from 51 to 28 beats/min during the infusion and cardiac arrhythmia was observed. The irregular heart beat stopped as soon as the infusion was stopped but the heart rate was still low. This returned to normal rate within 30 minutes after infusion. There were no obvious side effects in either instance. The effects of l-arginine on the blood pressure and heart rates of normal animals are shown in Table 5.6a.

L-arginine infused iv into an acutely laminitic pony lowered heart rate but did not produce irregularity. Blood pressure was only very slightly lowered. When the infusion was stopped, heart rate returned to pre-infusion levels. (Table 5.6b)

Table 5.6 (a) Changes in blood pressure and heart rate during iv infusion of l-arginine to two normal horses - one unsedated, Beckford and another sedated, Despina.

	Time	Systolic BP	Diastolic BP	Mean BP	Heart rate	Procedure	Comment
<i>Beckford</i>	10.30	101	61	74	45	basal	regular
	12.05	112	70	89	40	basal	regular
	12.06	112	56	75	39	infusion	
	12.11	ND	ND	ND	26	infusion	irregular
	12.22	ND	ND	ND	25	infusion	irregular
	12.33	ND	ND	ND	72	infusion	
	12.34	101	59	73	49	infusion	
	12.37	116	63	81	53	infusion	
	12.40	148	88	108	40	infusion	
	12.45	134	79	97	40	stop infusion	
	12.46	137	89	105	69		regular
	12.52	127	75	92	42		
	12.55	132	84	100	39		
	13.01	101	70	80	40		
	13.04	100	71	80	40		
	13.08	123	85	98	35		
<i>Despina</i>	11.30	84	49	58	51	basal	regular
	11.48	ND	ND	ND	28	sedated/ infusion	irregular
	11.50	ND	ND	ND	32	infusion	irregular
	11.52	ND	ND	ND	32	infusion	irregular
	11.58	ND	ND	ND	28	infusion	irregular
	11.59	ND	ND	ND		stop infusion	
	12.05	82	56	64	32		regular
	12.35	84	31	42	42		regular
	12.37	80	37	50	41		regular
	14.35	81	46	55	40		regular

Key:

BP - blood pressure in mmHg

ND - not determined because of irregular heart beat

Unsed.N - unsedated normal horse

Sed N. - sedated normal pony

Sed AL - sedated acute laminitic

Table 5.6 (b) Blood pressure and heart rate of a sedated acute laminitic pony during infusion of l-arginine.

	Time	Systolic BP	Diastolic BP	Mean BP	Heart rate	Procedure	Comment
<i>Beau</i>	12.25	172	93	171	75	AL	Resp 31/min
	12.37	209	118	175	74		
	15.00	186	90	122	75		
	15.45	177	92	133	76	sedated	
	15.50	179	86	123	71		
	16.00	186	103	137	70		
	16.08	181	94	134	70		
	16.15	181	99	145	61		
	16.22	177	101	136	64	infusion	
	16.25	174	95	126	64	infusion	
	16.30	161	85	119	68	infusion	
	16.40	172	91	131	68	infusion	
	16.45	168	80	121	68	infusion	
	16.49	176	101	146	72	infusion	
	16.51	169	109	136	74	Stop infusion	
	16.56	191	100	136	73		
	17.00	184	84	129	72		
	17.02	184	102	129	74		
	17.05	201	92	129	74		
	17.10	194	110	144	84		
	17.13	172	96	131	84		
	17.17	176	96	128	74		
	17.21	184	117	154	76		
	17.25	173	111	143	98		

The short term effects of transdermal of GTN on the blood pressure and heart rate of acute laminitis.

GTN did not appear to have any effect on blood pressure or heart rate when given transdermally. See Tables 5.7 (grass induced laminitis) and 5.8 (post partum laminitis).

Table 5.7 Serial blood pressures and heart rates after application of glyceryl trinitrate paste to a pony with acute grass laminitis

		Time	Systolic BP	Diastolic BP	Mean BP	Heart rate	Obel grade	Comment
<i>Beau</i>	unsed AL	16.00	215	117	161	64	3	untreated
		16.03	213	93	142	53		
		16.06	216	109	158	59		GTN 80mg
		16.09	218	110	162	58		
		16.12	220	122	163	59		
		16.15	222	123	165	57		salt appetite
		16.18	222	123	146	57		
		16.21	197	108	152	59		
		16.24	211	98	149	58		
		16.27	220	109	164	57		
		16.30	224	104	157	57		
		16.31	212	110	154	61		
		16.34	221	96	134	57		
		16.37	213	99	139	58		
		16.40	205	95	137	57		
		16.43	212	99	151	57		
		16.46	214	99	141	56		
		16.49	202	102	135	57		GTN40mg
		16.52	205	96	138	56		
		16.55	201	95	144	55		
		16.58	214	116	153	57		
		17.01	212	105	148	56		

There seems to be little observable difference between pretreatment values and those during treatment. The pony was given 2g oral PBZ that evening and made an uneventful recovery from laminitis over a period of 5 days.

Little difference in blood pressures and heart rate was seen when transdermal glyceryl trinitrate was applied to a case of *post partum/refractory* laminitis on the seventh day after onset of the acute stage (Table 5.8)

Table 5.8 Serial blood pressures and heart rates after application of glyceryl trinitrate paste to a mare with acute *post partum/refractory* laminitis

		Time	Systolic BP	Diastolic BP	Mean BP	Heart rate	Obel grade	Comment
<i>Biscuit</i>	Unsed AL	12.45	139	102	116	68	4	FX,PBZ,ACP
		12.46	154	106	128	67		
		12.50	169	108	127	66		
		13.30	146	94	115	65		80mg GTN
		13.33	153	95	116	65		
		13.36	127	88	102	66		
		13.38	146	96	114	66		
		13.40	137	94	109	65		
		13.42	132	85	101	67		
		13.44	133	88	105	63		
		14.00	133	91	109	63		
		14.01	146	89	111	64		
		14.02	149	97	117	68		
		14.03	161	95	115	70		
		14.04	144	97	115	66		
		14.05	137	83	116	66		
		14.06	136	81	109	68		
		14.07	136	91	111	70		
		14.08	149	88	114	67		
		14.09	135	86	104	70		
		14.10	126	81	98	70		
		14.11	108	74	91	67		
		14.12	114	78	93	66		
		14.13	142	83	117	70		
		14.14	123	80	98	70		
		14.15	126	79	96	75		
		14.16	111	72	102	74		

Key : FX - flunixin meglumine; PBZ - phenylbutazone; ACP - acepromazine; GTN - glyceryl trinitrate. See Appendix IV for full treatment details.

Serum and Urinary Nitrates

Serum and urinary nitrates of both normal and acutely laminitic increased immediately (within minutes) following iv infusion of l-arginine, and decreased 24 hours afterwards. The exception was Beau, who following an iv infusion of l-arginine, was given glyceryl trinitrate. The serum nitrates were higher 24 hours after the infusion and during glyceryl trinitrate treatment (Table 5.9).

Table 5.9 Changes in urinary and serum nitrate of ponies receiving nitric oxide donors compared with normal values.

Normal values

	Urine (ppm)	Serum (ppm)
<i>Tess</i>	13.30	0.31
<i>Cracker</i>	9.30	0.35
<i>Marron</i>	14.40	0.35
<i>Beau</i>	6.20	0.40

Infusion of 0.9% saline

	Urine (ppm)	Serum (ppm)
<i>Marron (before infusion)</i>	14.40	0.35
<i>(during infusion)</i>	12.50	0.40
<i>(after infusion*)</i>	-	-

Infusion of l-arginine (normal horses)

	Urine (ppm)	Serum (ppm)
<i>Beckford (before infusion)</i>	15.20	1.71
<i>(during infusion)</i>	28.50	3.51
<i>(after infusion*)</i>	35.30	2.71
<i>Despina (before infusion)</i>	12.40	1.31
<i>(during infusion)</i>	37.75	3.31
<i>(after infusion*)</i>	-	2.71

Infusion of l-arginine (acute laminitis)

	Urine (ppm)	Serum (ppm)
<i>Beau (before infusion)</i>	-	1.21
<i>(during infusion)</i>	10.00	5.35
<i>(after infusion*/ during GTN)</i>	-	8.01

*after infusion is taken to indicate 24 hours after the end of the infusion. - indicates that a urine sample was not obtained.

5.1.4 Discussion

The present study describes treatment of acute laminitis arising spontaneously at grass, a common aetiology of laminitis in native breeds of ponies. One case of *post partum* aetiology is also included. The pony herd studied was kept on unfertilized permanent pasture; animals were accustomed to being handled regularly by the investigators and were relaxed about the procedures. The success of the management regime is reflected in the narrow ranges of normal blood pressures and heart rates within the group (see Chapter 3).

The blood pressures of acute laminitics were related closely to the Obel Grade of lameness (Obel 1948) and are an indication of the varying degrees of severity (Table 5.3). The values can be used to gauge the efficacy of treatment (*vide infra*). Possible causes of hypertension are pain and/or stress induced sympathetic discharge (Stashak, 1987; Hood, 1979) with activation of the renin - angiotensin system (Miller, 1981; Clarke, 1982).

Near Infrared Spectroscopy (NIRS) overcomes difficulties of artefacts in measurement since it non-invasively determines changes in pedal haemodynamics and oxygen utilisation. Validation for this new methodology appears in the previous Chapter and elsewhere (see Hinckley *et al.*, 1995). Although sedation with detomidine and/or butorphanol has some effect on blood pressure and heart rate (Clarke and Paton, 1988), there were no apparent differences between unsedated and sedated normal controls; these aspects are considered in Hinckley *et al.*, (1995). NIRS revealed a number of important differences in the pedal haemodynamic responses to occlusion of the digital vessels of normal and laminitic ponies.

The pony suffering from acute laminitis showed no responses to manoeuvres designed to alter blood flow within the hoof although normal baseline values were present. Normal

horses show some movement artefacts (Hinckley *et al.*, 1995a) and these were absent in the laminitic pony (Table 5.4 and Figure 5.10). Failure to respond to a compromised arterial supply, together with no alteration of haemodynamics during lifting of the contralateral limb, indicates haemostasis within the dermal laminae and possibly the terminal and circumflex arteries.

Arginine is a known secretagogue for growth hormone, insulin and other hormones but the modes of action are uncertain (Merimee *et al.*, 1967; Rabinowitz *et al.*, 1968; Reichlin, 1974). Human blood pressure responses to infusion of arginine are equivocal. Baudouin *et al.*, (1993) found no differences in blood pressure, heart rate, skin temperature or urinary cGMP in normal volunteers and in patients with systemic hypertension during iv infusion of l-arginine. Nakaki *et al.*, (1992), however, reported hypotension and tachycardia in normotensive humans following an iv infusion of 0.5 g l-arginine/kg b.wt. over 30 minutes. Diseased human forearm vessels vasodilate following infusion of l-arginine (Creagher *et al.*, 1992). Böger *et al.*, (1994) showed a 43% increase in blood flow through normal human forearm vessels, together with decreased blood pressure during administration of l-arginine. In the present study the transient hypotension and a slight pressor response of a normal horse to an iv infusion suggests either that homeostatic cardiovascular mechanisms are compensating for a transiently decreased blood pressure and/or that slight hypervolaemia elicits neuroendocrine reflex regulation of blood pressure and/or that the slight stress of the procedure masked vasodilatory responses.

The intravenous infusion of 10% l-arginine initiated reperfusion of digital tissues of the acute laminitic pony. The pattern of reperfusion was similar to reperfusion seen after deliberately induced acute ischaemia in the hoof of a sedated normal horse - see Chapter 4.2 (Hinckley *et al.*, 1995) and to that seen in normal humans (Thorniley, 1988). L-arginine infusion produced the same trends in trace patterns that were seen during restoration of blood flow after the tourniquet used to deliberately induce ischaemia was removed. These changes were consistent with reperfusion patterns in human and equine

subjects (Hampson and Piantodosi, 1988; Hinckley *et al.*, 1995). This pattern is normally associated with hyperaemia after deliberate tourniquet induced forearm ischaemia (Hampson and Piantodosi, 1988). The marked decrease in cyt aa3 in the presence of an increased O₂Hb is inconsistent with the classical view of a simple oxygen dependence of the enzyme. Postulated explanations include: i) Rapid oxidation of the enzyme in response to oxygen inflow followed by return to baseline - this would be in accordance with the known kinetics of cyt aa3 oxidation (Brunori *et al.*, 1981); ii) Binding of NO to the copper ion associated with cyt aa3 resulting in inhibition of electron transfer and accumulation of cyt aa3 in its reduced state - the latter mechanism is consistent with the known reactions of cyt aa3 with NO (Brunori *et al.*, 1981), but at this stage, the physiological model and effect on NIRS measurements are speculative.

The pony suffering acute laminitis showed a very slight transient decrease in diastolic blood pressure when infused with l-arginine, but otherwise systolic or mean blood pressures and heart rate showed little change. There was no irregularity of heart beat detected as the heart rate was very high. Clinically, the pony experienced cold and shivering suggesting peripheral vasodilatation and cutaneous heat loss. Increased pain was evident during reperfusion. The pain on reperfusion may be compared to the phenomenon experienced by anyone who has felt leg pain on restoration of circulation after sitting awkwardly. Such pain accompanying reperfusion has been shown in horses after 2 hours of induced pedal ischaemia (Hood, 1995). Pain responses may account for elevated blood pressure twelve hours after the l-arginine infusion; there are obviously many potential neuroendocrine mediators of this response. The hypertension was not the result of decreased substrate availability for nitric oxide synthesis since arginine is only slowly cleared from plasma, remaining elevated for 24 hours (see section 5.2 below). These abnormally high values of circulating l-arginine no doubt provided maximal substrate for NO synthesis, which was probably only limited by availability of nitric oxide synthase (NOS) itself. If reperfusion was the result of NO synthesis then it may be that synthesis of nitric oxide was submaximal. The reasons for additional plasma l-arginine being able to upregulate the synthesis of NO are unknown and this is a puzzle for

pharmacologists (Moncada and Higgs, 1995; Elliot, 1996). Serum and urinary nitrates of both normal and acutely laminitic animals were raised above normal values. Urinary nitrates are a good indication of the synthesis of nitric oxide which binds to oxygen free radical to form nitrites and then nitrates. Serum and urinary nitrates have been shown to be a measure of the synthesis of NO from both endogenous and synthetic sources (Bode-Böger *et al.*, 1994; Wennmain *et al.*, 1993).. Levels of serum and urinary nitrates returned to normal values within 24 hours except in the case of the acutely laminitic pony, Beau. His values remained elevated, indeed increased 24 hours later. Increased NO synthesis may occur because NOS was upregulated when supplied with a huge quantity of substrate for NO production (see 5.2), and/or because transdermal GTN was given to the pony at a maximum dose.

Glyceryl trinitrate (GTN) is an alternative synthetic source of NO which is endothelium independent and is neither regulated by NOS nor by the integrity of the endothelium, which is damaged during the acute phase of laminitis (Roberts *et al.*, 1980, Templeton *et al.*, 1985) even prior to the appearance of clinical signs (Hood *et al.*, 1993). As long ago as 1955, the vascular effects of nitroglycerin were studied in vivo (Conway, 1955). GTN can therefore provide vasodilatation even when endothelial function is severely compromised. GTN has been used as a treatment for angina since the 19th century (see Vane *et al.*, 1990) and more recently as a safe prophylactic agent to prevent preterm labour (Lees *et al.*, 1994). Its vasodilatory action results from the molecule "donating" nitric oxide which relaxes smooth muscle (Katsuki *et al.*, 1977). The patches of glyceryl trinitrate may have both local (improving lameness and reducing the typical bounding pulse) and systemic effects (lowering blood pressure) (Figure 5.13). GTN lowered blood pressure long term and this was usually accompanied by a marked improvement in lameness but there was individual variation. If GTN was reduced or stopped prematurely then blood pressure increased and lameness worsened (Figure 5.13). It is not known whether lowering of blood pressure was a direct systemic effect of GTN or because pain was relieved. Little effect on blood pressure was seen in the short term studies on acute laminitis cases. Similarly, GTN has little effect on blood pressure short

term when given in small doses to humans, because the initial effect is on veins and there is compensation by increased cardiac output (Rang and Dale, 1994). Larger doses affect both veins and arterioles. It is quite possible that strategically applied GTN paste will act as a large dose locally and cause vasodilatation of both veins and capillaries in the equine digital circulation but that the total dose is not sufficient to lower blood pressure immediately. The precise location of the effect on the digital circulation depends on the location of the haemostasis, which presumably varies from case to case - see 5.4 *vide infra*. The long term effect may be because the local effect of vasodilatation is beneficial and reduces pain. The lowering of systemic blood pressure may be a secondary effect as, when pain is reduced, catecholamines, for example, are reduced. Secondary relapse of vasoconstriction is therefore prevented by gradual lowering of the GTN dose. If the GTN is withdrawn prematurely, then pain responses presumably account for the increase in blood pressure. The pharmacokinetics of GTN are unknown in horses as is the length of time it takes for the GTN to cross the integument. GTN seems to be rapidly absorbed (within minutes) in people and has a relatively short duration of action as evidenced by application of the paste to one's own skin and timing of the onset of a headache. Presumably, in the horse transdermal GTN will be absorbed more slowly (because of the thickness of the skin) and cleared slowly so it has a longer term local effect than systemic administration of GTN, or other nitrovasodilators. Clearly more studies are needed to investigate the pharmacokinetics of GTN in the horse.

Although the ponies showed rapid clinical improvement in lameness and reduced pain, distress and depression, such assessments are subjective. Unfortunately, the NIRS equipment was not available to study changes in haemodynamics during application of GTN. Further NIRS studies which investigate haemodynamic changes following GTN application to digital vessels are underway. Side effects of transdermal GTN were minimal; slight skin irritation sometimes occurred at the site of application and sometimes low blood pressure was associated with an increased heart rate. In both instances the treatment was discontinued without recurrence. GTN treatment showed no sedative effects and clinical assessment of improvement was much easier than with

acepromazine treatments. At the onset of GTN treatment, blood pressure sometimes transiently increased during the first 24 hours, and this was concurrent with some worsening of lameness, possibly resulting from reperfusion pain as seen during the infusion of l-arginine. The observation that pain occurs during reperfusion supports the theory that laminitis is primarily a vasoconstrictive condition (Hood *et al.*, 1993). Concurrent adjunct therapy with phenylbutazone may be needed for the first 24 hours of the disease to relieve the pain of reperfusion; otherwise analgesic therapy is unnecessary. Transdermal GTN has been reported to have direct anti-inflammatory and analgesic actions (Berrazueta *et al.*, 1994) when used as treatment for thrombophlebitis. Therefore, one advantage of GTN treatment is that the need for analgesics is markedly reduced and the side effects of analgesia, particularly renal damage will be largely overcome. The GTN patches are easy to apply and the problems of oral or invasive administration of therapeutic agents are obviated. Mild cases of laminitis recover with only changes in management but no doubt they would recover more quickly were nitric oxide vasodilator treatment to be instituted when symptoms first appear. The treated ponies were clinically normal after the treatment ended and there was no increase in blood pressure, or return of lameness after therapy. The ponies were turned out to grass. However, treated ponies were subject to further episodes of acute laminitis weeks or months after the initial attack. Subsequent bouts of acute laminitis received the same regime as before and none of the ponies had any permanent disability, sepsis in the hooves or apparent changes in the following 9 months.

Unfortunately, the same could not be said of the *post partum* case of acute laminitis, which was of endotoxic aetiology. The application of transdermal GTN did not prevent the onset of severe acute laminitis nor improve the lameness, or prevent secondary changes in the hooves. There are several possible reasons for this. In this case endotoxins, bacterial lipopolysaccharides (LPS), had a profound effect on the vasculature. LPS bind to vessels walls where they cause massive destruction and damage, and initiate a cascade of inflammatory responses. Endotoxaemia causes a range of associated disorders ranging from ischaemia, to organ failure, disseminated

intravascular coagulopathy (DIC), septic shock, and death. In this case the pathogenesis of endotoxaemia was not recognised until after the onset of the severe lameness. Irrigation of the uterus revealed copious quantities of frank pus. Such a volume of bacteria in the uterus when it is still capable of absorbing toxins into the systemic circulation must have produced a massive insult and challenge to the animal. It may not have been that the GTN was ineffective but that it could not possibly counter such an immense and sustained challenge. Similarly, GTN may have had a local vasodilatory effect on the hooves but for a limited period of time. The dose of GTN may have been cleared by the evening allowing vasoconstriction, mediated by a number of different agents. If the initial insult had been recognised and removed then the eventual outcome may have been very different. Despite the apparent ineffectiveness of GTN as a prophylactic agent, there was some improvement which is not normal in such cases. The eventual breakdown of the laminae allowing 'sinking' of the bony column through the hoof capsule was probably because the connective tissue structures within the hoof could not grow, and as the hoof exterior grew there was no connection within. Whatever connection was left could not continue to support the weight of the horse as it bore more weight further down the interior of the hoof capsule. Such a failure of growth perhaps reflects the damage done to the endothelium by LPS; endothelial cells may not only be damaged but the endothelium may be actually denuded in some parts of the vasculature. Arginine was not given to this mare initially because the laminitis attack seemed very mild and afterwards because of the risk of endotoxic shock. It is very hard to say in this clinical case whether or not GTN had any effects. Whether the mare would have been killed sooner without the adjunct therapy of GTN will never be known; it may even have worsened the case if there was bleeding within the microcirculation. Fluid exudate which discharged at the coronary band contained blood, a symptom which carries a very poor prognosis (Colles, 1991). If endotoxins had induced bleeding it is possible that GTN vasodilatation would exacerbate the condition. On the other hand, NO inhibits release of AII and endothelin and has an antiproliferative effect on the endothelium. Under these circumstances, NO would be beneficial to the health of the vascular system. It is unlikely that the devastation of endotoxaemia will be countered by any one agent

and a combined armoury of vasodilators, cyclooxygenase inhibitors, antiplatelet aggregating agents and perhaps an anti lipopolysaccharide antibody (Stegantox, Schering Plough, Welwyn Garden City, Herts, UK) would provide clinicians with a better chance of success. Similarly, adjunct therapy with agents that will reduce reperfusion injury caused by free radicals is sure to be of benefit - see 5.4.

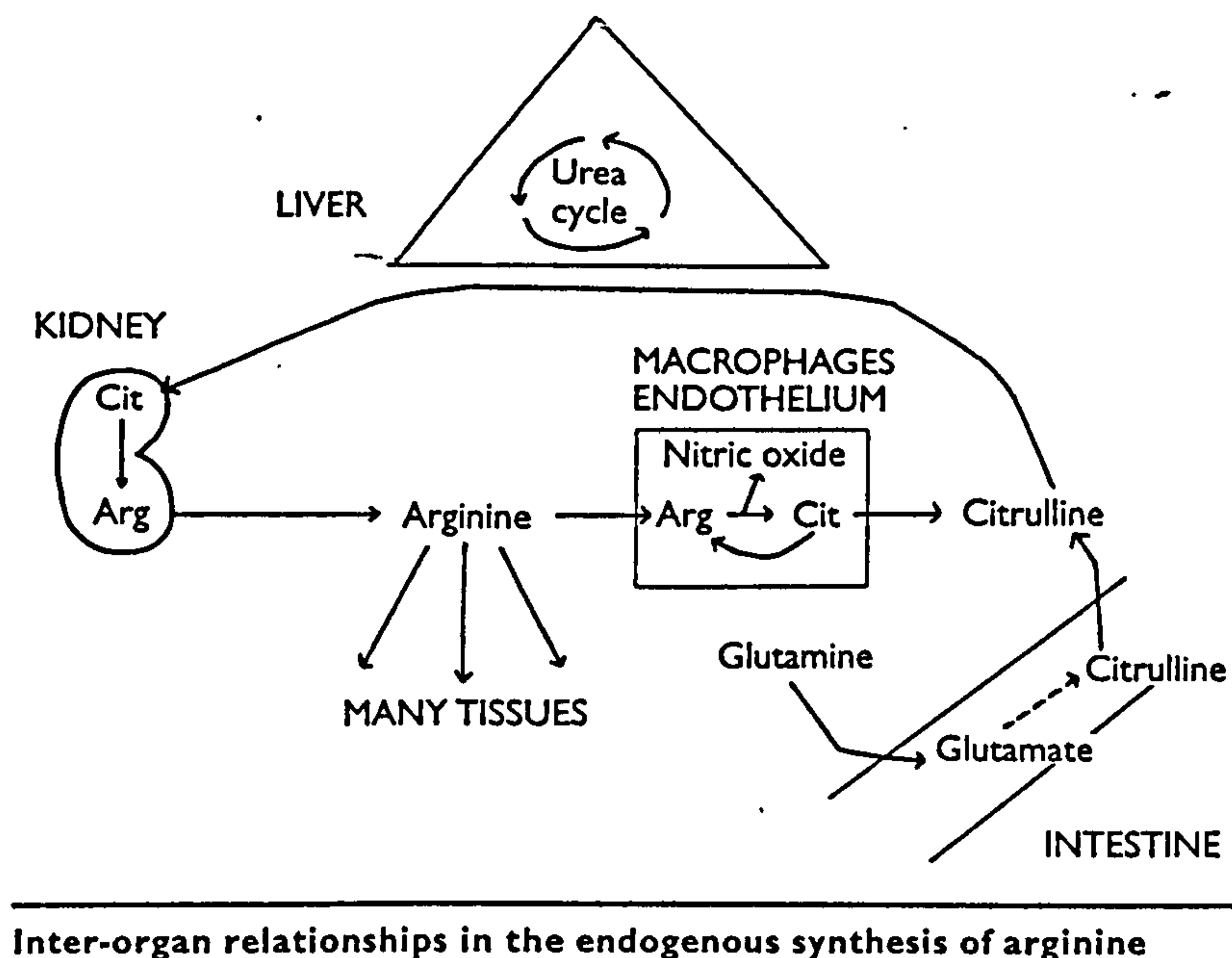
Although the pharmacological effects of transdermal GTN are at present equivocal, preliminary clinical results when used to treat grass laminitis are promising and warrant further investigation. Both scientific and clinical results of the effect of l-arginine on equine digital blood flow are encouraging. Nitric oxide donors are likely to form part of the clinicians' approach to treatment of the devastating disease of laminitis.

5.2 Plasma arginine and endogenous inhibitors of nitric oxide synthesis

5.2.1 Introduction

L-arginine is the substrate for the synthesis of nitric oxide *in vivo*. It is a strongly basic molecule with a molecular weight of 174 Daltons. There are two stereotypic isomers of l-arginine - the active l-arginine and the inactive d-arginine enantiomer. The sources of endogenous arginine *in vivo* are numerous. Arginine is ingested and some animals, such as the cat family, rely entirely on ingested sources (high levels are found in red meat) and cannot tolerate one meal without sufficient arginine since they have an enzyme deficiency of P-5-Carboxylase synthase and must ingest all arginine (Rogers *et al.*, 1985).

Although carnivores rely on the ingestion of red meat for their arginine requirements, omnivores and herbivores have different arrangements. They rely partly on dietary intake and partly on symbiotic association with gastro-intestinal tract micrororganisms which synthesise many amino acids. Herbivores can therefore survive winter months on poor quality roughage by digestion of cellulose either by rumination or hind gut fermentation. Other sources of arginine are physiological. Arginine is biosynthesised endogenously. The urea cycle involves the conversion of citrulline to ornithine and arginine but it is thought that this hepatic process does not provide the circulating substrate for basal nitric oxide synthesis (Brosnan *et al.*, 1992). Circulating arginine is thought not to be produced directly but by conversion from l-citrulline in the kidney (Brosnan *et al.*, 1992) (Figure 5.16). In addition, when endothelial cells convert l-arginine to l-citrulline and nitric oxide the reaction can be driven backwards in a reversible fashion and citrulline can be the substrate for l-arginine synthesis within the cell itself (Hecker *et al.*, 1990).



Inter-organ relationships in the endogenous synthesis of arginine

Figure 5.16. Renal biosynthesis of l-arginine from l-citrulline (Brosnan et al., 1993)

Exogenous l-arginine alters haemodynamics in normal and acutely laminitic ponies *vide supra*, therefore it might be possible that substrate levels are low prior to the onset of disease. Variation in plasma levels of the substrate, l-arginine, may influence blood pressure and vasodilatation in normal horses and be relevant in the pathophysiology of laminitis. Plasma levels of l-arginine were determined in groups of normal and chronically laminitic ponies when stabled and when they were turned out to grass. Variation in the quantity of ingested arginine is likely to vary alongside the horses' diet and integrity of the intestinal microflora.

If plasma arginine is at normal levels then it may be that the action of nitric oxide synthase (NOS) is inhibited during disease. Endogenous inhibitors of NOS include asymmetric $N^G N^G$ -di-methyl-l-arginine (ADMA) and N^G -monomethyl-l-arginine (L-NMMA) which are competitive analogues for the NOS enzyme. ADMA and L-NMMA are chemically similar to l-arginine (Figure 5.2 and 5.17)

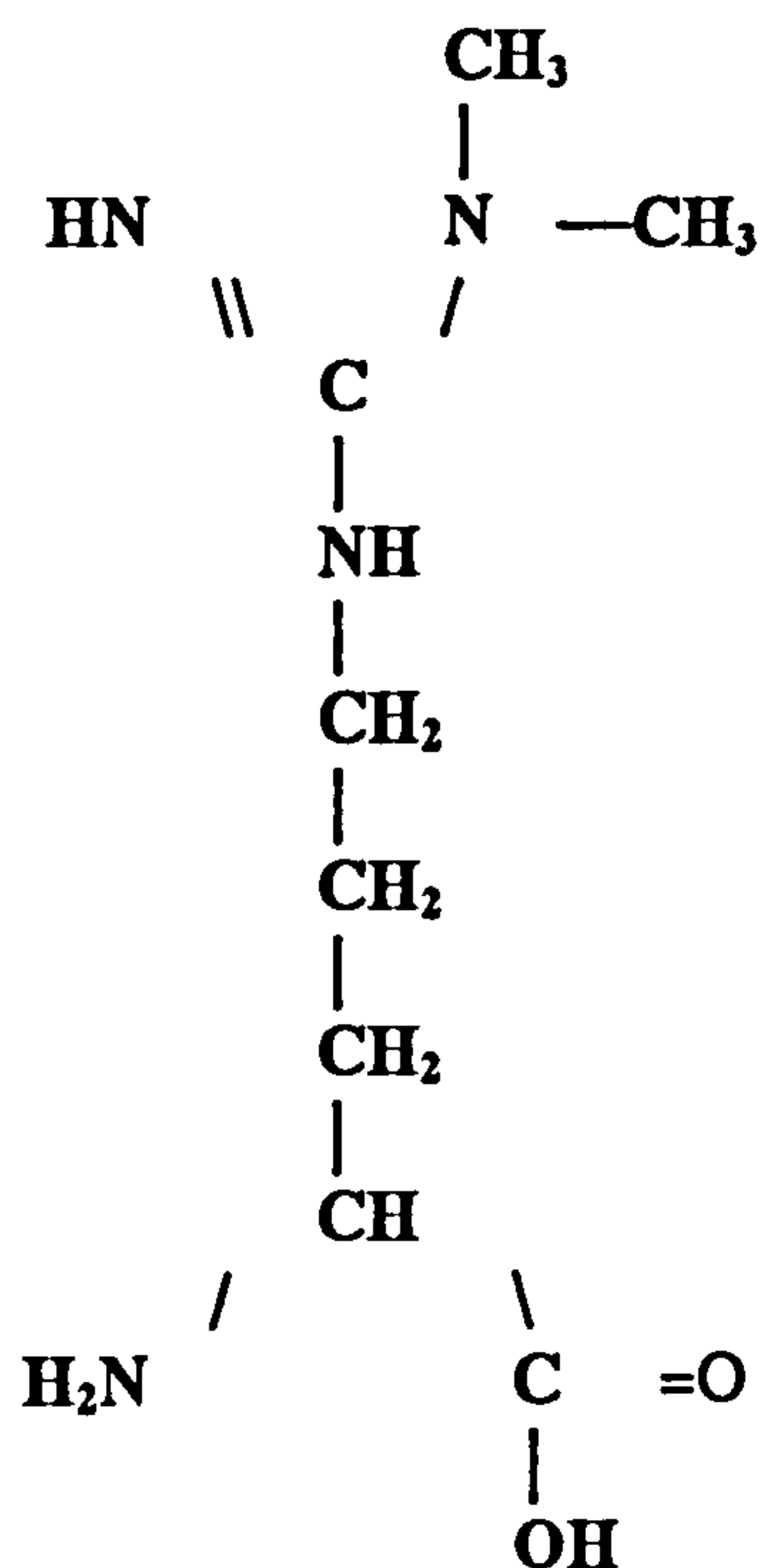


Figure 5 .17 Chemical structure of ADMA (Vallance *et al.*, 1992).

Endogenous l-arginine analogues, such as asymmetric dimethyl-l-arginine (ADMA) influence NO production (Fickling *et al.*, 1993; Vallance *et al.*, 1992) and therefore normal vasodilatation. The levels of plasma arginine and plasma ADMA were investigated in normal circumstances during summer and winter, and during disease.

5.2.2. Materials and methods

Animals

Eleven cross-bred ponies, eight mares and three geldings (aged four to eighteen years); three Registered Irish Draught mares (aged 6-23 years) and a hunter gelding were studied. Animals were kept as a group on designated premises for at least 6 months prior to the study and were subject to usual management regimes. The animals were either kept stabled on a diet of home grown hay or turned out to grass on unfertilised old pasture. An individual animal (Beau), classed as a chronic laminitic, was sampled from the period he was stabled, then at grass, and throughout the treatment of acute grass induced laminitis. Two animals were exercised under saddle when stabled. All procedures were carried out under the auspices of the Animals (Scientific Procedures) Act 1986.

Blood Samples

Blood samples were taken by jugular venepuncture into glass vacuum tubes containing 0.12 ml 0.34M ethylene diamine tetracetic acid (EDTA) (Vacutainer, Becton Dickinson). The samples were then mixed with inhibitor solution - comprising : 250 mM EDTA, 0.05 M 1,10 Phenanthroline (Sigma) in ethanol, and 1000 KIU/ml Aprotinin (Sigma) - in the ratio of 50 μ l/ml blood and kept on ice until centrifuged at 600g for 12 minutes. Plasma samples were stored in polypropylene tubes at -20° C.

Extraction

Extraction of l-arginine and dimethylarginines followed similar protocols outlined by Vallance *et al.*, (1992). L-arginine and ADMA were extracted from 3 ml plasma samples using 2 ml 'Bondelut SCX' columns (Analytichem, Cal. USA) then eluted in 50% ammonia/methanol solution and dried in a rotary vacuum drier (Univap) at 50° C.

Extracts were dissolved in 2 mls Nanopure water and loaded onto a 2 ml 'Bondelut CBA' column; L-arginine and dimethylarginines were dissolved in 10% ammonia/methanol solution and dried as above. The extracts were redissolved in 100 µl of running buffer and left overnight at 4° C. They were then stored at -20° C until used.

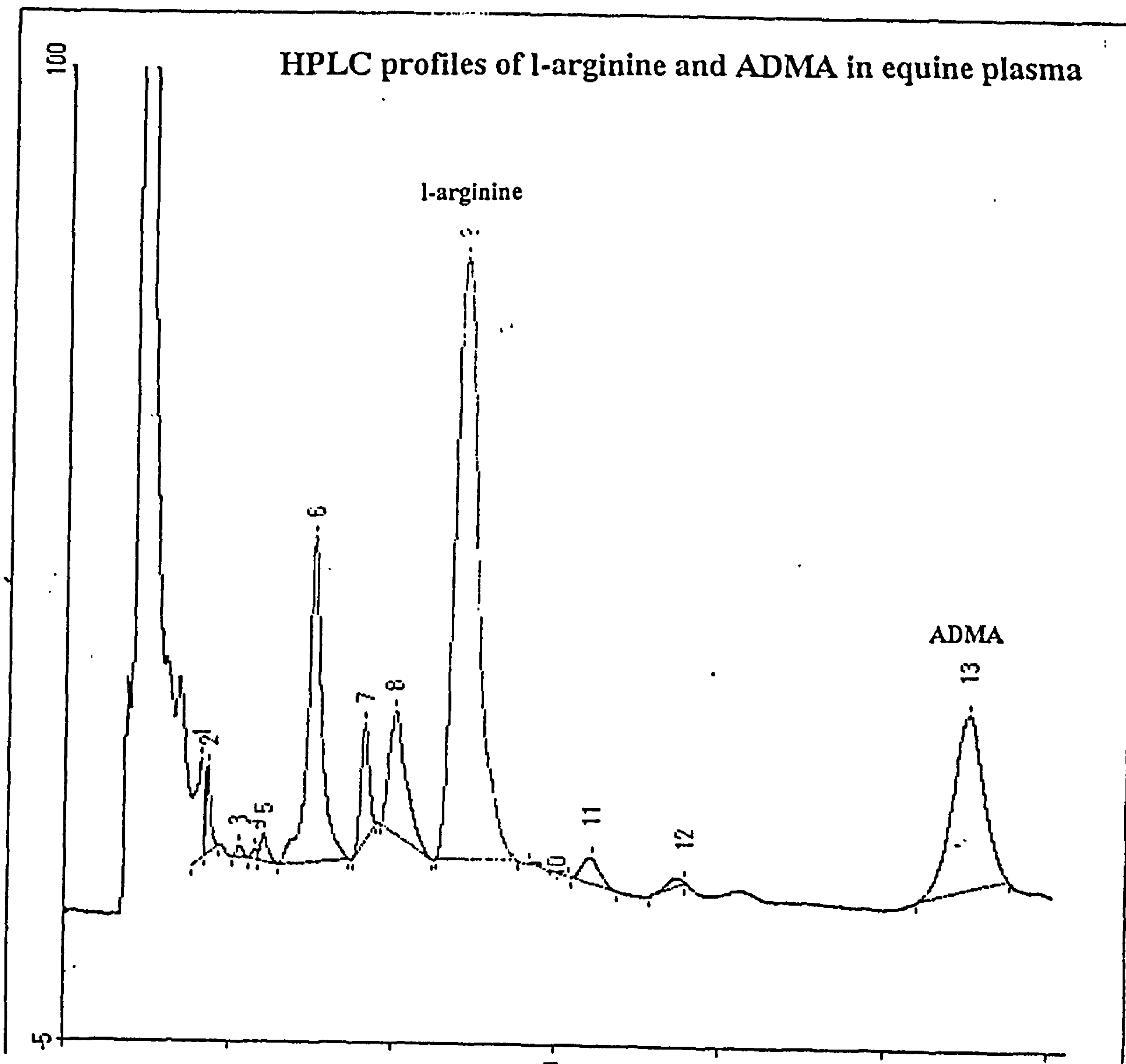
High Performance Liquid Chromatography (HPLC)

HPLC of L-arginine and dimethylarginine were separated by HPLC Phillips PU4100 pump (Phillips, Cambridge, UK) with a Rheodyne 7125 injector and Bio Rad 1305A UV detector. Phillips PU6000 software was used for data collection and analysis. An Apex 1 C18 25 cm x 4.6 mm analytical HPLC column (Jones Chromatography Ltd, Hengoed, Mid Glamorgan, UK) was used. UV absorption was measured at 205 nm x 0.04 abs. using a mobile phase of 100 % buffer consisting of 0.025M orthophosphoric acid (pH 5.0 with 5M KOH)-1.71 mls/l distilled water; 0.01M hexane sulphonic acid -1.88g/l distilled water and far UV grade acetonitrile 1% /vol. The flow rate was 2.0 mls /min. and the injection volume 30µl. Elution times for synthetic standards of l-arginine (Sigma, Poole, Dorset) and ADMA (Alexis, Affiniti, Nottingham, UK) were 6.5 minutes for l-arginine and 14.5 minutes for ADMA. Recovery was calculated by adding 250µm of l-arginine and 12.8µm of ADMA to 3 mls of dialysed plasma samples. After extraction, these samples were compared to known standards. Recovery for l-arginine was 5 % and 12 % for ADMA (n = 8).

5.2.3 Results

Clear peaks were obtained by HPLC for l-arginine and asymmetric dimethylarginine (ADMA) (Figure 5.18)

Figure 5.18 . HPLC profiles of l-arginine and asymmetric dimethyl arginine (ADMA) in equine plasma.



Plasma arginine levels of normal and chronically laminitic horses and ponies, when stabled and when at grass

Plasma concentrations of l-arginine were similar in control and laminitic groups. Plasma arginine increased compared with stabled values when both types were at grass (Table 5.10 and Figure 5.19). There was a slight but not significant difference in plasma arginine between normal and chronically diseased groups that occurred when animals were first turned out to grass in May. At this time levels were 124 $\mu\text{mol/L}$ in normal horses and 99 $\mu\text{mol/L}$ in chronic laminitics.

Plasma asymmetric dimethylarginine (ADMA) of normal and chronically laminitic horses and ponies, when stabled and when at grass, and after hard exercise.

Mean values for normal horses and ponies when stabled were $1.27 \pm 0.14 \mu\text{mol/L}$ and this fell to $1.02 \pm 0.13 \mu\text{mol/L}$ in May when at grass, and to $0.73 \pm 0.12 \mu\text{mol/L}$ during June. Full details appear in Table 5.11. Plasma ADMA values of the laminitic group were approximately 64% of control group values when stabled, decreasing to 56% of normal on turning to grass and regaining the previous value of 64% when the groups had been at grass for four weeks. Statistically there was no difference between the groups when the animals were stabled. However these differences became significant when the animals were first turned out to grass ($p < 0.05$) but were again not significant during summer months. Both groups showed parallel decreases in plasma levels when at grass compared to when stabled. Mean values are compared with normal values in Figure 5.20 and individual values shown in Table 5.10.

There was a significant ($p < 0.01$) difference in plasma ADMA of stabled exercised horses compared to stabled controls that were not exercised, mean values being 2.04 $\mu\text{mol/L}$ and 1.27 $\mu\text{mol/L}$ respectively.

Table 5.10 Individual values and group means for plasma l-arginine and plasma asymmetric dimethyl arginine (ADMA) of normal and chronically laminitic horses and ponies, when stabled and at grass. Exercised normal horse values are also shown.

	Stabled		At grass 2 weeks		At grass	
	L-arginine	ADMA	L-arginine	ADMA	L-arginine	ADMA
	$\mu\text{mol/L}$	$\mu\text{mol/L}$	$\mu\text{mol/L}$	$\mu\text{mol/L}$	$\mu\text{mol/L}$	$\mu\text{mol/L}$
Normal						
<i>Annabella</i>	-	-	-	-	96	0.71
<i>Cracker II</i>	-	-	236	1.05	177	0.82
<i>Despina</i>	52	0.91	-	1.45	119	0.96
<i>Jasper</i>	-	-	205	1.28	149	0.43
<i>Marron</i>	34	1.31	76	0.66	99	1.35
<i>Melody</i>	-	-	-	-	94	0.65
<i>Snowflake</i>	-	-	-	-	109	1.03
<i>Selluci</i>	65	1.61	76	1.01	110	0.55
<i>Tess</i>	74	1.24	237	0.66	197	< 0.1
Mean	56	1.27	163	1.02	128	0.73
SE	9	0.14	30	0.13	13	0.12
Range	(34-74)	(0.91 -1.61)	(76-237)	(0.66-1.45)	(94 -197)	(<0.1-1.35)
C.Laminitic						
<i>Beau</i>	-	-	48	0.19	43	0.35
<i>Cobweb</i>	67	1.46	77	0.79	67	0.35
<i>Domino</i>	83	0.15	138	0.60	115	0.54
<i>Misty</i>	-	-	82	0.70	-	-
<i>Rose Marie</i>	-	-	-	-	240	0.64
Mean	75	0.81	86	0.57	116	0.47
SE	8	0.66	19	0.13	44	0.07
Range	(67-83)	(0.15-1.46)	(48-138)	(0.19-0.79)	(43-115)	(0.35 -0.54)
Exercised Normal						
<i>Beckford</i>	141	2.05				
<i>Fury</i>	92	2.03				
Mean	117	2.04				
SE	25	0.01				

Figure 5.19 Seasonal differences of plasma arginine in normal and chronically laminitic ponies when stabled and when at grass.

N - normal horse or pony; L - chronic laminitic.

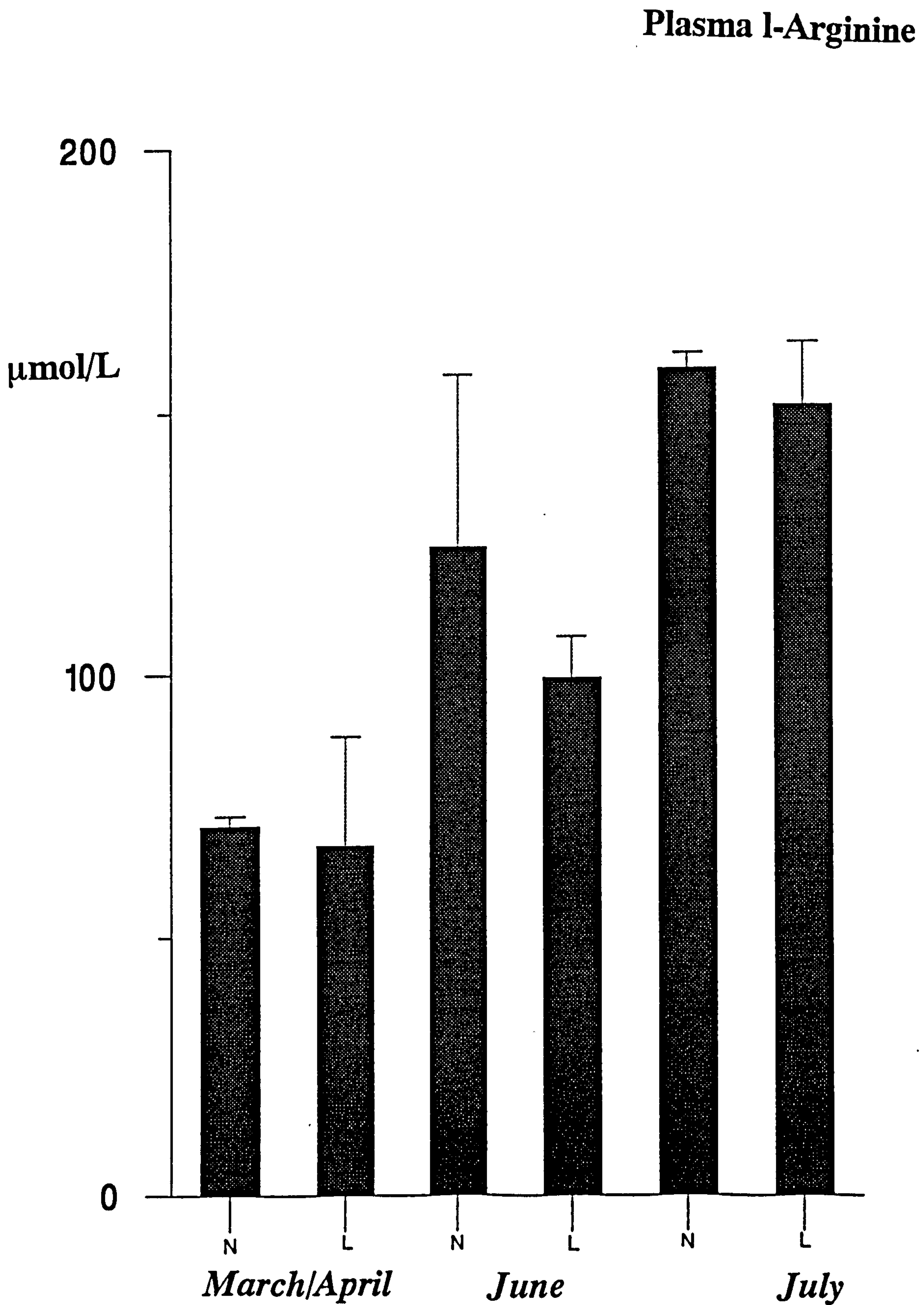
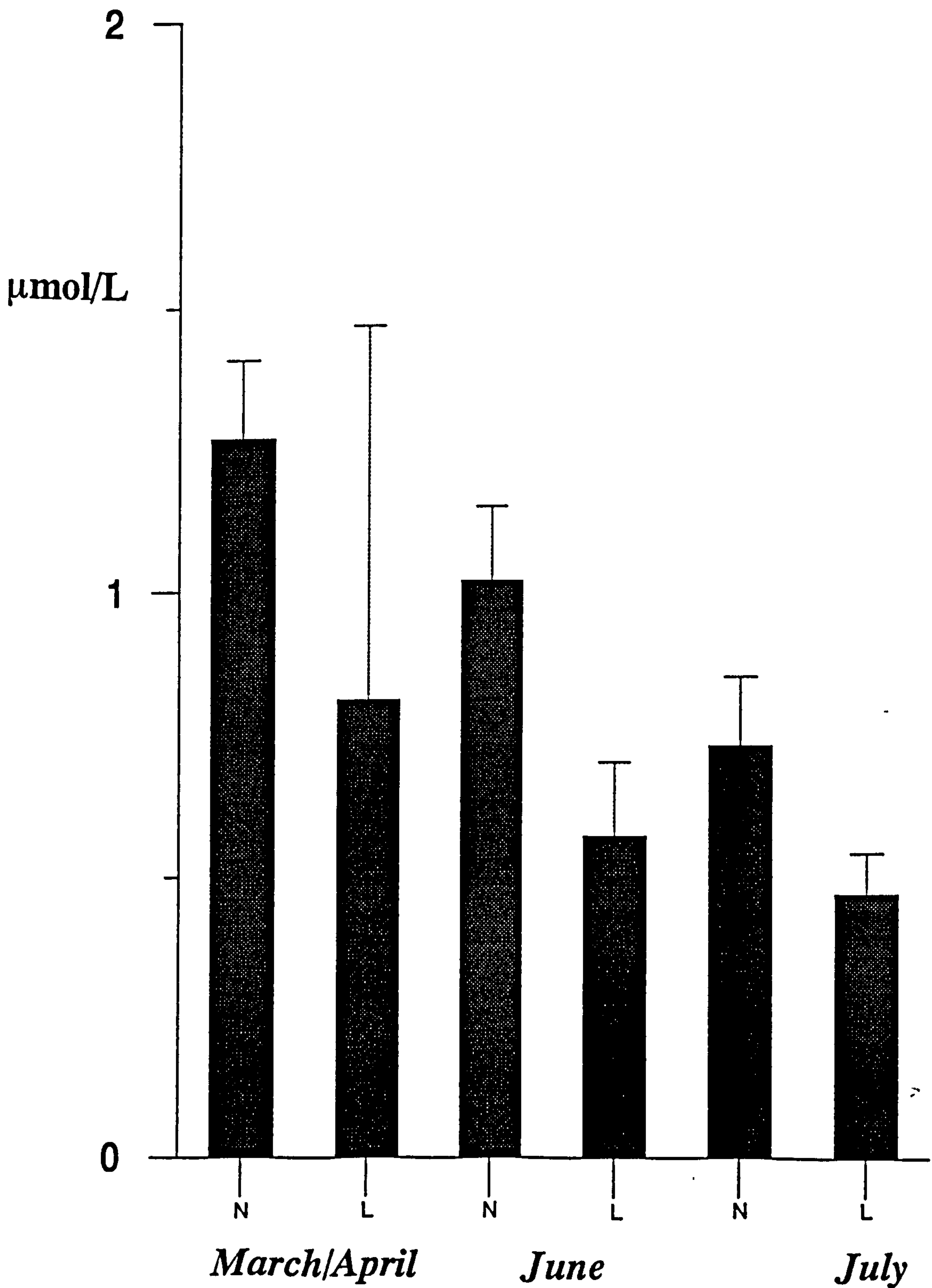


Figure 5.20 Seasonal differences of plasma in normal and chronically laminitic ponies when stabled and when at grass.

N - normal horse or pony; L - chronic laminitic

Plasma ADMA



Plasma l-arginine and ADMA during acute laminitis

The pony that developed acute laminitis had lower plasma arginine values $79 \pm 9 \mu\text{mol/L}$ than the mean of normal controls of $163 \pm 30.9 \mu\text{mol/L}$ for the month that he was at grass prior to the onset of the acute episode; this difference was not however statistically significant. When treated with l-arginine and transdermal GTN, plasma ADMA were lowered. The pony that developed acute laminitis showed a marked decrease in plasma ADMA when turned out to grass, mean levels being $< 0.1 \mu\text{mol/L}$, compared with previous mean levels of $0.71 \mu\text{mol/L}$ when stabled. ADMA values seemed to increase in response to vasodilatation caused either by arginine infusion or glycerol trinitrate patches to $1.26 \mu\text{mol/L}$ and $1.13 \mu\text{mol/L}$ respectively. When all treatment was completed levels again fell to $< 0.1 \mu\text{mol/L}$. (Table 5.11).

Table 5.11 Variation in plasma l-arginine and ADMA in a chronically laminitic pony (Beau) with seasonal change in diet and during a treatment of acute laminitis with exogenous l-arginine and transdermal GTN.

Date	L-arginine ($\mu\text{mol L}^{-1}$)	ADMA ($\mu\text{mol L}^{-1}$)	Comment (Obel Gd)
22.1.94	185	0.93	Stabled
16.3.94	171	0.48	Stabled
30.4.94	63	< 0.1	At grass
15.5.94	95	< 0.1	At grass
22.5.94	77	< 0.1	At grass
25.5.94	143	0.66	Symptoms AL (2)
30.5.94	190	0.3	Symptoms worsening
			Acute laminitis (4)
2.6.94	644	0.7	L-arginine infusion
2.6.94	3266	1.26	End of infusion
3.6.94	309	1.13	Stabled
4.6.94	137	< 0.1	Transdermal GTN
5.6.94	125	< 0.1	Transdermal GTN
7.6.94	126	0.51	Transdermal GTN
9.6.94	125	0.84	Transdermal GTN
12.6.94	130	0.82	Transdermal GTN
15.6.94	152	0.42	Transdermal GTN
26.6.94	132	< 0.1	Transdermal GTN

Low values of plasma arginine were seen when the animal was turned to grass for the month preceding the acute phase and ADMA were low in parallel. Plasma arginine

reflected the input of iv arginine and very high levels were recorded at the end of the infusion. These levels were cleared to normal values within 48 hours. When plasma l-arginine was administered, plasma ADMA rose to their highest levels and remained high alongside plasma arginine. When GTN was given, plasma ADMA fell to below detectable levels for 2-3 days before returning to normal values. Plasma levels of arginine were held at steady values while the animal was stabled but ADMA fell at the end of the study period.

5.2.4 Discussion

Physiological deficiency of l-arginine as a substrate for nitric oxide production may occur in several ways. First, there may be a deficiency of dietary arginine or disruption of physiological supplies of l-arginine and/or conversion of l-arginine to nitric oxide and l-citrulline may be inhibited. Nitric oxide production may therefore be limited by changes in diet or intestinal microflora, disruption of physiological supplies of l-arginine, or through inhibition of NOS by endogenous competitors such as ADMA (Vallance *et al.*, 1992, Fickling *et al.*, 1993).

Arginine is a major ingredient of graminaceous species of grass, especially during periods of rapid growth in Spring. Dietary arginine is therefore usually freely available. Mean levels of plasma arginine in the normal experimental group when stabled were $56 \pm 9 \mu\text{mol/L}$ which was comparable to results for equine plasma arginine in other publications. Pösö *et al.*, (1993) reported plasma arginine $64 \pm 3 \mu\text{mol/L}$ and Silver *et al.*, (1994) recorded plasma arginine of $227 \pm 44 \mu\text{mol/L}$ (albeit in whole blood which produces higher values) Plasma levels increased steadily after turning out to grass in May and reached their highest values in June. This suggests that there is both a plentiful supply of dietary arginine in the grass and that intestinal bacteria are not disturbed. These data suggest that generally speaking there are no differences in metabolism and/or utilisation of the substrate between normal and chronically diseased horses. Changes in metabolism or utilisation of the substrate can also occur when the biochemical components of grass change. When plants are "stressed" (by drought, frost, overgrazing or fungal parasites such as endophytes) during rapid growth periods (Hendry 1993) most of the arginine is lost and large quantities of water soluble carbohydrates are stored as long chains of fructose molecules - fructooligosaccharides, or fructans (McDonald *et al.*, 1991). Deposition of fructans happens quickly, usually overnight, and can constitute up to 60% dry matter of vegetative grasses. Fructans are known to disturb intestinal flora populations (Farnsworth, 1993) which may be relevant to the pathogenesis of equine laminitis. Indeed, endophytes are associated with laminitis (Rohrbach *et al.*, 1995) and

may induce laminitis both by the production of alkaloids and by the conversion of WSC to fructans which acutely disturb the digestive tract - in much the same way as an oral carbohydrate overload.

High levels of plasma arginine occur even when horses are stabled and their intake of arginine is low. This suggests that animals rely on intestinal microflora and symbiotic digestion of cellulose to provide physiologically normal levels of arginine or its substrate. Symbiotic digestion may be disturbed when horses are turned to grass and new populations of intestinal bacteria are established. Horses may rely heavily on ingested arginine when they are first at grass until new populations of hind gut microflora are seeded. Even horses already at grass are susceptible to changes in the composition of grass. Reduced herbal arginine is linked to increased levels of dietary fructans (Hendry, 1993). Ingestion of fructans, disturbed intestinal microflora and reduced ingested and symbiotic sources of l-arginine, or its substrate, thus may crucially combine to induce laminitis. The seasonality of grass induced laminitis and the fact that severe digestive disturbance cause laminitis (Stashak, 1987) supports this hypothesis. Although it has not been suggested that microflora provide normal circulating arginine levels in horses that are used to maintain vasodilatory tone, such a mechanism has been demonstrated in rats and other mammals (Brosnan *et al.*, 1992). It is therefore likely that digestive disturbance of microflora by carbohydrate overload or ingesting grass, which cannot be processed by the normal bacterial population, will disrupt the availability of l-arginine, reduce NO synthesis resulting in reduced vasodilatation.

The pony that suffered an acute attack of laminitis had reduced levels of plasma l-arginine when turned out to at grass during May compared to his stabled values before and after the attack. The reason for this individual response is unknown but one explanation is that the microflora of this particular pony was disturbed by the change in diet on transition from hay to grass fodder. Dietary fructans in the grass would certainly have this effect. The values would in this instance, reflect ingested arginine alone without the arginine normally synthesised by the microflora. When the gut had 're-

seeded' then the values returned to normal. As the plasma arginine was lowered for some weeks, it is possible that NOS was limited by substrate availability, and downregulated. Thus even when plasma arginine had returned to normal values, the enzyme would not be functioning at the correct rate; infusion of large quantities of l-arginine might be enough to upregulate the enzymatic activity and restore normality.

Urea cycle intermediates do not provide substrates for NO synthesis (Brosnan *et al.*, 1992) but arginine is synthesised from glutamate to glycine in the intestine. Glycine is in turn converted to citrulline, which is converted to arginine in the kidney (Brosnan *et al.*, 1992). In addition, microflora in the caecum and hindgut produce citrulline from various substrates which, after absorption, is quickly converted to arginine in the kidney (Brosnan *et al.*, 1992). Recently, oral l-arginine administration has been shown to increase nitric oxide synthesis, but not blood pressure, of rats *in vivo* (Bode-Böger *et al.*, 1994)

Endothelial cells convert N^G-monomethyl-l-arginine to l-citrulline and then to l-arginine (Hecker *et al.*, 1990). This mechanism may ensure that vasodilatation is not entirely dependent on plasma availability of l-arginine although but this pathway may be a comparatively short term measure. The fact that endothelial cells synthesise ADMA indicates a possible regulatory pathway for the control of NO synthesis at local level ie within the endothelium (Fickling *et al.*, 1993). This is the first occasion that ADMA have been measured in horses. Mean levels of 1.27 ± 0.14 $\mu\text{mol/L}$ for normal stabled horses compared with figures of 1.15 ± 0.13 $\mu\text{mol/L}$ for normal humans (Vallance *et al.*, 1992). Although plasma ADMA accumulate during renal failure (Vallance *et al.*, 1992) low levels are not usually considered relevant. It may be however that the significantly low levels of ADMA found in chronically laminitic ponies may be an indication that they are attempting to maximise vasodilatation. The putative causes for compromised vasodilatation are numerous and include glycation or fructosylation of vessel wall protein including hormonal receptors, since when at grass animals ingest considerable quantities of water soluble carbohydrates. This aspect is discussed further in Chapter 10. It has

been suggested that chronic laminitic animals have a different metabolism to non-laminitic animals (Jeffcott *et al.*, 1986; Freestone *et al.*, 1992), partly because of genetic/evolutionary considerations (ponies that have only recently been domesticated are better converters of feed to body fat) and partly because of previous exposure of the animals to summer winter cycles of feeding which upregulates intestinal transporters of sugars (Buddington *et al.*, 1987; Buddington and Diamond, 1992; Ferraris *et al.*, 1992). This aspect of laminitis deserves further study; for example *in vitro* culture of endothelial cells from normal and laminitic groups to establish differences in the synthesis of ADMA would verify or not the above hypothesis. The changes seen in the acute laminitis case are difficult to explain conclusively from *in vivo* evidence. There are many environmental variables and physiological changes taking place, some of which are beyond proper control. It could be that reduced ADMA in chronic laminitics is a response to compromised vasodilatation because of other factors including glycation (see Chapter 10). Alternatively, reduced ADMA could reflect increased enzyme activity of dimethylarginine dimethylaminohydrolase (DDAH) - which converts ADMA into citrulline which can be recycled to L-arginine and used for NO synthesis. Reduced levels of ADMA may reflect L-arginine insufficiency for whatever reason. Much more knowledge is required about the enzymology of NOS and related enzymes in this pathway. The increase in ADMA after exercise may reflect a regulatory response to increased blood pressure during exercise, including increased demand for NO or indicate impaired renal function. If ADMA are instrumental in hypertension at any stage of laminitis (especially the refractory stage) then inhibition of the synthesis of ADMA by the enzyme inhibitor named 4142W (MacAllister *et al.*, 1995) may be beneficial. ADMA inhibits arginine transport across cell membranes (Baydoun *et al.*, 1995) and increased intracellular levels of ADMA may prevent utilisation of plasma arginine for NO synthesis. Cultured endothelial cells may throw light on the regulation of vascular tone at local level which may be crucial to the susceptibility of an individual's predisposition to laminitis. Such regulatory processes may throw light on the cellular activities that are involved in the eventual prevention of acute laminitis

5.3 Synthetic inhibitors of nitric oxide synthesis

5.3.1 Introduction

The half life of nitric oxide *in vivo* is very short so it is both difficult to measure and assess its primary effect. The matter is further complicated by many other associated physiological factors. However, experimental inhibition of nitric oxide production can be assessed using the inhibitors of NOS. A synthetic analogue of l-arginine, L^o-nitro-L-arginine-methyl ester hydrochloride (L-NAME), competitively inhibits NO production and can be used to study compromised NO synthesis *in vivo* (Rees *et al.*, 1989, Calver *et al.*, 1992; Nafrialdi *et al.*, 1994; Gardiner *et al.*, 1994; Bode-Böger *et al.*, 1994; Moncada and Higgs, 1995). L-NAME solutions were therefore infused into a jugular vein of normal ponies to assess whether the symptoms of laminitis were induced and if so, whether they could be reversed with l-arginine.

5.3.2 Materials and Methods

Animals

Two pony mares, aged six and eight years each weighing 300 Kg, were used. An indwelling catheter (Ohmeda, Hatfield, Herts.) was placed in the left jugular vein and secured with superglue and tape (Treatplast).

L-NAME solution

Solutions of L-NAME were prepared under sterile conditions in a laminar air flow hood. The first solution comprised 225 mg of L-NAME hydrochloride (Sigma, Poole, Dorset, UK) in each 60 mls of 0.9% saline drip (Baxter, UK). The second solution comprised 600 mg of L-NAME (Sigma, Poole, Dorset, UK) in each 20 mls of 0.9% saline drip (Baxter, UK). The pHs of the 2 solutions were adjusted to 7.36 with sodium hydroxide. Solutions were filtered through a 0.22 μ m membrane (Nalgene Filterware, UK) and stored sealed at 4°C.

Infusion of L-NAME.

(i) The first pony received an initial dose of 10 mls solution containing 37.5 mg L-NAME over 8 minutes; 15 minutes later a second dose of 15 mls of solution containing 56.25 mg was given over 7 minutes. The total dose was 93.75mg (0.31mg/kg).

(ii) The second pony received an initial dose of 5 mls of solution containing 150mg L-NAME over 4 minutes; one hour later, a second dose of 10 mls of solution containing 300 mg L-NAME was given over 15 minutes. The total dose was 450 mg of L-NAME (1.5mg/kg).

5.3.3 Results

Infusion of L^o-nitro-L-arginine-methyl ester hydrochloride (L-NAME)

(i) Low dose

There was little observable effect at this dose and although there was a slight increase in blood pressure immediately after the injections, basal values varied. Basal values were: systolic 105 ± 6 mmHg; diastolic 68 ± 4 mmHg; mean 89 ± 6 mmHg and a heart rate of 47 ± 5 beats/minute. After infusion the values were: systolic 101 ± 3 mmHg; diastolic 59 ± 1 mmHg; mean blood pressure 75 ± 2 mmHg and heart rate 42 ± 1 beats/minute. A hypotensive episode was observed 30 minutes after the end of the second injection with a subsequent lowering of blood pressure to 80/57 (64) mmHg, heart rate fell to 46 beats/min and the horse appeared sleepy and was swaying.

(ii) Higher dose

Basal values were systolic 111 ± 6 mmHg; diastolic 63 ± 2 mmHg; mean blood pressure 80 ± 6 mmHg; heart rate 54 ± 2 beats/minute. After infusion values were systolic 141 ± 6 mmHg; diastolic 105 ± 3 mmHg; mean blood pressure 109 ± 3 mmHg; heart rate 41 ± 1 beats/minute. An increase in blood pressure to 142/93 (115) was seen 1 hour after the end of the first injection. When blood pressure was at its highest, the pony became very unsteady, seemed depressed and was swaying. Digital pulses in all limbs were apparent and retinal congestion and hyperaemia were noticed which had not been evident on clinical examination before the start of the procedure. The pony was taken for a walk but was most unsteady with an uncertain walk/gait and seemed stiff. One hour later the pony seemed to be anxious but quiet and blood pressure remained high - 142/86 (103) and 152/67 (95) but heart rate was steady at around 40 beats/min. The pony suddenly experienced an acute hypotensive episode with a sudden drop in blood pressure to 67/51 (56) and at this point the pony reared in the crush. Heart rate increased to 62 beats/min and there was a rapid increase in blood pressure afterwards to 137/52 (72) and 144/57 (122), however the heart rate returned to normal values of around 42 beats/min. At this point no digital pulses were evident. The pony was

completely normal at the end of the procedure and thereafter. Changes in blood pressure from basal values are all highly significant ($p < 0.01$). (Table 5.12).

Table 5.12 Effects of 1.5mg/kg i.v. infusion of L-NAME on blood pressure and heart rate in an unsedated normal pony

	Systolic	Blood pressure (mmHg)		Heart rate (beats/min)
		Diastolic	Mean	
BASAL	111±6	63±2	80±6	54±2
INFUSION				
15-95 mins.later	141±6*	105±3*	109±3*	41±1
100 mins. later (hypotensive episode)	67*	56*	56*	62
105-180 mins. later	141±4*	55±3	97±25	43±1

Mean values ± SE (*p < 0.005).

High iv doses of L-NAME increased blood pressure indicating that normal vasodilatation is compromised by l-arginine analogues. L-NAME increased blood pressure and, although bounding digital pulses were evident, laminitis was not apparently induced.

5.3.4 Discussion

Clearly, many factors may be involved in the pathophysiology of laminitis, but it is considered significant that L-NAME infusions did not overtly induce the acute condition; this probably reflects the multifactorial nature of the syndrome, only part of which involves the substrate for NO, L-arginine. Systemic inhibition of NO production by a competitive inhibitor of NO synthase, L^G-nitro-L-arginine-methyl ester hydrochloride (L-NAME) compromises NO production and causes vasoconstriction of specific vascular beds in rats (Gardiner *et al.*, 1993). L-NAME causes vasoconstriction in the rabbit ear which is reversed by L-arginine (Rossitch and Alexander, 1991) and has a similar effect on diseased vessels (Rossitch *et al.*, 1992). A similar analogue N^o-monomethyl -L- arginine (L-NMMA) causes vasoconstriction and ischaemia when injected into the brachial arteries of human volunteers (Calver *et al.*, 1992). Low doses of L-NAME had little effect on a normal pony but clinical signs were noted at a high dose. It was difficult to distinguish between the unsteadiness resulting from possible cerebral effects and that caused by muscular stiffness. Blood pressure showed a highly significant and prolonged increase following L-NAME administration. The increased blood pressure clearly demonstrates the role of nitric oxide in maintaining vasodilatation. Other hormones, such as bradykinin, atrial natriuretic factor and others, also play a crucial role in cardiovascular homeostasis. Such hormones may have been involved in the acute hypotensive episode - presumably a homeostatic reflex. Other relevant hormones are presently unidentified.

As laminitis was not induced, it is clear that reduced nitric oxide alone is not a single causative feature of the pathogenesis. Acute laminitis is therefore multifactorial, although attenuation of vasodilatation and impaired homeostatic mechanisms will undoubtedly contribute.

-

5.4 General Discussion

The realisation that the vascular endothelium is an endocrine organ began 15 years ago with the discovery that *in vitro* vessels stripped of the epithelial lining behave very differently to those with an intact endothelium (Furchgott, 1984; Furchgott and Zawadzki, 1990). Since then research in endothelial function has burgeoned (Marletta, 1989; Ånggård, 1990; Ignarro, 1989; Ignarro, 1990; Bredt and Snyder, 1992; Warren *et al.*, 1994; Moncada and Higgs, 1995; Dusting *et al.*, 1995). Nitric oxide is now recognised as both a neurotransmitter and is an essential component of the complex paracrine control of vascular tone (Moncada and Higgs, 1995). It interacts with many other paracrine agents such as endothelin, prostacyclin and other arachadonic derivatives and with systemic hormones such as angiotensin, vasopressin and atrial natriuretic factor (Vanhoutte *et al.*, 1993; Lüscher, 1994).

Vascular control mechanisms are clearly involved in the pathophysiology of equine laminitis (Hood *et al.*, 1993, Molyneux *et al.*, 1994) and it is now agreed that vascular alterations take place within the hoof during acute stage of the disease. This is certainly not a novel notion, but rather one that became unfashionable for a time - actually for a hundred years or two. The very earliest veterinary references to laminitis refer to alteration of blood flow within the foot :

"A horse is said to be foundred in his feet, when he has such a numbness, and pricking or tingling within his hoofs, that he hath neither sense nor feeling of his feet, but is in all respects like a man, that by hard or crooked sitting hath both his feet asleep (as we call it) during which passion we know we can neither well go nor stand, and even so fareth with a Horse in this case, for the course of the blood being stopped, those obstructions causeth this torment" (Gervas Markham, 1662).

Over three centuries later, similar observations were made with little more understanding of the mechanisms involved than in the previous reference :

" The vasospasm hypothesis is consistent with the clinical signs of laminitis. There is no lameness during the period of decreased blood flow in the developmental phase.

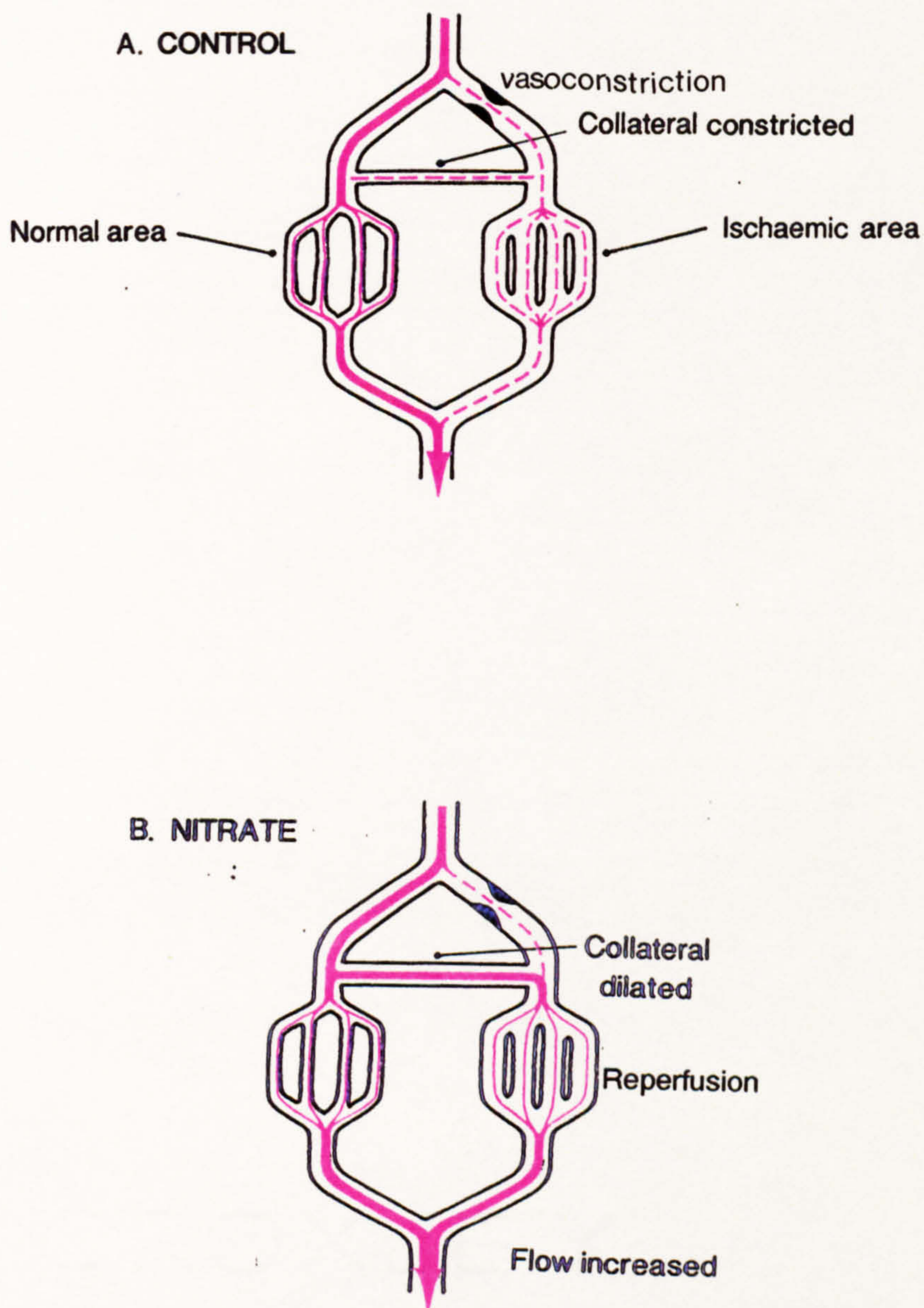
This is similar to the parasthesia experienced by 20% of individuals with iliac arterial occlusion, acute vasospastic disease of the digits in people, or when one's leg "goes to sleep" while sitting in a position that restricts perfusion to the limb. Tingling or pain occurs with restoration of tissue perfusion" (Hood et al., 1993)

The data in the present study support the observations outlined above giving, it is hoped, more insight into the vascular mechanisms involved. Obviously, there are moments within the developmental stages where feeling of the foot is lost as vasoconstriction occurs. This was observed at first hand. The mare (Biscuit) with endotoxic acute laminitis was seen to be unsteady and anxious in the developmental stages of acute laminitis. If the mare was lacking feeling in her hooves then it would be difficult for her to stand and/or walk without appearing unsteady. The case of grass induced laminitis that was treated with l-arginine iv, showed signs of pain on reperfusion. Hood *et al.*, (1993) stated that there was no pain during the original ischaemic period but there was pain on reperfusion, and this assertion is upheld by observations in the present investigation.

The aetiology of equine laminitis is clearly multifactorial and many hormones are certainly involved in the cascade of vascular events during the acute stage of the disease. The paracrine control of vascular tone is key in microcirculatory beds. Homeostatic mechanisms of normal horses are usually capable of overcoming potential vascular disturbances; in disease however, such homeostatic mechanisms may be impaired. During acute grass induced laminitis, vasodilatation is enhanced using NO donors to overcome haemostasis. The mechanisms involved are complex. NO may have a direct action on vascular smooth muscle and allow collateral restoration of circulation, similar to the relief of angina spasms of the coronary artery by GTN (see Rang and Dale, 1994). GTN redirects blood to ischaemic areas by restoration of blood through fairly large diameter vessels in contrast to other vasodilators like dipyridamole (Rang and Dale 1994) (Figure 5.21).

The restoration of blood flow by GTN through vasodilatation of vessels may be effective because other vessels, including AVAs, are already fully dilated and blood flows through these vessels are maximal. This is probable since the total blood flow to the foot is increased in acute phase of laminitis (Robinson, 1990) and the AVA will already be fully dilated. If AVAs are not fully dilated then perhaps extra vasodilatation by NO would activate shear stress activation of endothelin, sufficient to redirect blood flow back to ischaemic areas. This vascular interruption and ischaemia during acute equine laminitis is shown by Pollitt (1995) in the Introduction (Figure 1.22). The possible mode of action of nitric oxide is shown in Figure 5.21 and is likely to work in a similar fashion to that of coronary artery vasodilatation during angina. Nitric oxide will act at the areas of vasoconstriction within the equine pedal circulation and put vasodilatation in train along the vessel walls. Nitric oxide is known to vasodilate arteries, arterioles and veins (Moncada and Higgs, 1995). GTN has been used for nearly a century to treat angina without the mechanisms of action being elucidated (Vane *et al.*, 1990) and only recently has the mechanism of action of NO been realised (Parker, 1993; Dusting, 1995). The vasodilatory effect of NO may not only be a direct effect of NO itself but may also reflect inhibited activity of vasoconstrictor agents. GTN reduces both plasma endothelin and angiotensin II concentrations during acute laminitis (Hinckley and Henderson, 1995; unpublished observations, *vide infra*) and this has been described in other mammals (Lüscher, 1994).

Figure 5.21 Vasodilatory action of glyceryl trinitrate on collateral supply during ischaemia. (Rang and Dale 1994)



Many other paracrine mechanisms are certain to be involved, including interactions with prostaglandins interleukins and other cytokines. In addition, there will be beneficial effects of NO on the vessel wall. NO inhibits platelet aggregation and leukocyte adhesion and prevents proliferative damage to the damaged vessel lumen. Moreover a direct anti-inflammatory action of NO on damaged veins during thrombophlebitis has been reported (Berrazueta *et al.*, 1994). NO is not simply maintaining tonic vasodilatation but gives cardiovascular protection. The arginine - nitric oxide pathway, and use of synthetic NO donors, has been the subject of much research in terms of pharmacological treatment for arteriosclerosis, hypertension and diabetic complications (Dusting, 1995) and endotoxic shock (Curzon *et al.*, 1994).

This preliminary study suggests that grass induced laminitis can be treated in the acute stage with precursors of nitric oxide, such as l-arginine, or by synthetic donors of nitric oxide to induce reperfusion of digital tissues and markedly improve the clinical conditions (Chapter 5.1). Clearly more studies are required to assess nitric oxide donors as a possible therapy for grass induced acute laminitis and to evaluate pharmacokinetics and GTN tolerance, well documented in man (Goodman and Gilman, 1970, Rang and Dale, 1994). Tolerance to GTN in man usually occurs within a few days of chronic administration but is completely lost after 10 days without treatment. *In vitro*, the depletion of tissue thiols may explain the reduced relaxation of vessels to NO donors (Rang and Dale, 1994). Mechanisms of tolerance are likely to vary with species and diet. Ingested flavanoids and other organic compounds may influence the concentration of tissue thiols and influence tolerance. This is the subject of considerable study now that the mode of action of NO donors is known. (Mülsch *et al.*, 1995 a. b; Bassenge, 1995; Bassenge and Fink, 1995; Laight and Änggård, 1995). Dimethylsulphoxide (DMSO) reduces tolerance to nitrovasodilators by scavenging peroxy-nitrate radicals (Scatchkov *et al.*, 1995) and this may be an additional aspect to those already known of DMSO as minimising reperfusion injury by scavenging other free radicals (Baxter, 1992). The field of nitric oxide research is an exciting one which will certainly provide greater

physiological understanding in the future, despite the disadvantage of the lack of available methods to measure NO *in vivo*.

Urinary nitrate is a sensitive test for NO production which is even more accurate if GCMS is used (Böger *et al.*, 1993). Böger *et al.*, 1993 found that urinary nitrate excretion of healthy male volunteers was increased by 79% after an iv l-arginine infusion. Urinary nitrates increased by 29% in rats given l-arginine in their drinking water and is also correlated with values for cGMP (Bode-Böger *et al.*, 1994). In this study, increased serum/urinary nitrates are parallel to l-arginine administration and return to normal values within 24 hours. The case of acute laminitis given l-arginine had still higher levels serum nitrates and this may reflect increased NO formation because of the large dose of GTN that was given transdermally that day. It is also possible that NOS was upregulated by the availability of the substrate which was slowly cleared (*vide supra*). As nitrates were increased in the serum of the treated pony, NO probably has a systemic effect and this may account for the lowering of blood pressure in treated ponies.

Nitric oxide donors appear to be a useful therapy for laminitis of metabolic/nutritional aetiology but other types of laminitis of endotoxic/sepsis pathogenesis may not be so amenable. Endotoxic laminitis is particularly severe because of the cascade of inflammatory responses that are initiated by circulating lipopolysaccharides (see Chapter 10). Nitric oxide alone cannot be expected to cure a disease of this complexity and although endothelial damage may be reduced by nitric oxide donors, and ischaemia attenuated, other strategies are required to combat the devastation of sepsis. The *post partum* case of laminitis may not have been given sufficient GTN to have a long lasting effect to counter vasoconstriction. In retrospect it may have been wiser to have administered l-arginine initially but this not implemented because of the risk of vasodilatation of uterine vessels so soon after parturition and hence the risk of shock. Endotoxic shock is a major concern and administration of nitric oxide donors may be contraindicated in case the inducible form of nitric oxide is synthesised by macrophages to induce shock. In future, synthetic nitric oxide donors could be given together with

analogues of l-arginine, this strategy would inhibit the production of inducible nitric oxide from macrophages and vasodilatation would be enhanced alongside a reduced risk of shock. A specific competitive inhibitor of NOS, such as L-NAME, which has a sustained pressor effect on equine blood pressure (Hinckley *et al.*, 1994, *vide supra* Chapter 5.3) would be an ideal choice in such circumstances. It may also be useful to combine nitric oxide therapy with free radical scavengers such as DMSO (Baxter, 1992) and calcium channel blockers (Hood *et al.*, 1993) to minimise reperfusion injury. Nitric oxide seems to be a very useful therapy for grass laminitis and an adjunct therapy for endotoxic laminitis. Although further studies are required to evaluate the pharmacokinetics of GTN and the effects of GTN on the digital circulation. The investigation of the interactions between nitric oxide precursors and regulation of NOS activity by substrate availability or by inhibition of ADMA synthesis (MacAllister *et al.*, 1995) may be beneficial to those animals suffering chronic laminitis. One way or another, regulation of nitric oxide will help those horses and ponies afflicted by laminitis and will ease the considerable suffering that is now endured.

PAGE

NUMBERING

AS ORIGINAL

Chapter 6**Radioimmunoassay of plasma angiotensin in normal and laminitic horses**

	Page
6.0 Preamble	268
6.1 Introduction	268
6.2 Material and Methods	273
6.3 Results	
6.3.1. Angiotensin II-like immunoreactivity	281
6.3.2. Identification of suitable antisera for RIA	282
6.3.3. Angiotensin II in normal horses and ponies	285
6.3.4. Angiotensin II during chronic laminitis	288
6.3.5. Angiotensin II during acute laminitis.	293
6.5 Discussion	298

*“Why grass is green and why our blood is red
Are mysteries which non have reach’d unto”*

John Donne 1571 -1631

6.0 Preamble

The renin-angiotensin system (RAS), plays a crucial part in the physiological regulation of blood pressure, water and electrolyte balance in vertebrates (Corvol and Ménard, 1990). Both angiotensin and aldosterone have close relationships with other vasoactive hormonal systems with synergistic, permissive and antagonistic actions. Endothelin, arginine vasopressin (AVP), bradykinin, atrial natriuretic peptide (ANP) and nitric oxide have clear interplays (Vanhoutte *et al.*, 1992; Swales, 1994). The present chapter is concerned with the potential participation of the RAS in equine laminitis.

6.1 Introduction

The RAS is a vital physiological regulator of blood pressure and vascular tone. Not only is angiotensin a vasoconstrictor, it also participates in water and electrolyte homeostasis. Nearly 100 years ago, Tigerstedt and Bergman (1898) observed that saline extracts of rabbit kidney increased arterial pressure of recipient rabbits; they coined the term *renin* for this pressor substance and showed it to be heat labile and therefore probably a protein. Interest in this material waned for several decades, until Goldblatt and colleagues (1938), in studies of relationships in vascular disease, noted that certain manoeuvres that interfered with renal perfusion produced malignant hypertension. In particular, they showed that by narrowing the renal artery of the remaining kidney, after unilateral nephrectomy, increased blood pressure and this was reversible if normal renal blood flow was restored. These observations stimulated intense investigations around the world to identify the mechanisms

involved in these processes. A variety of approaches, from basic biochemistry, histology and pharmacology suggested that the kidney produced a substance that had profound effects upon cardiovascular function. Granular cells around the glomerulus of the kidney, had earlier been described (McManus, 1944) their granularity could be affected by salt and water status. Moreover, the active substance emanating from the kidney - renin - did not produce a rapid pressor response, rather a slow onset hypertension was characteristic. The active substance was shown to be a small molecule which was heat stable. Two laboratories, Braun-Menendez *et al.*, (1939) and Peart (1959), identified the material and termed it angiotonin and hypertensin respectively. The term angiotensin was agreed (see Robertson, 1994).

Skeggs (1957) demonstrated that the enzyme renin acted on the substrate angiotensinogen cleaving the tetradecapeptide molecule to produce angiotensin I. This in turn was converted to the potent vasoconstrictor hormone angiotensin II by angiotensin converting enzyme (ACE). Angiotensin II may in turn be cleaved to angiotensin III and inactive peptide fragments by angiotensinase. The most active component of the renin-angiotensin system is angiotensin II. Angiotensin II (A II) is an octapeptide, with a molecular weight of 1,045 D. The amino acid sequences of equine renin substrate and angiotensin have been reviewed by Brown *et al.*, 1983, Figure 6.2. A II is widely distributed in a variety of tissues (Dzau, 1988). The renin-angiotensin system is only part of the complex physiological homeostatic control of water and electrolyte balance and maintenance of vascular tone, both at paracrine and systemic levels (Figure 6.1). Renal release of renin is increased by a variety of stimuli:- decreased renal arterial pressure or blood flow; decreased sodium and/or potassium in the juxtaglomerulus; increased AVP; and local release of noradrenaline from renal nerves or cholinergic activation and is also influenced by renal sympathetic and cholinergic nerves (Davis and Freeman, 1976; Hladky and Rink, 1986). All these changes are sensed within the juxtaglomerular apparatus within the kidney. Renin release increases

with sodium restriction, hypovolaemia and reduced renal blood flow or ischaemia and initiates the cascade of events that result in the formation of angiotensin. At systemic levels, angiotensin may reduce sodium excretion within the kidney directly and indirectly through stimulating aldosterone secretion. The systemic actions of angiotensin act in concert with anti diuretic hormone but act in opposition to other systemic hormones, such as bradykinin (which is degraded by angiotensin converting enzyme) and atrial natriuretic peptide.

At a paracrine level, Angiotensin is just part of the extremely complex arrangements that maintain vascular tone Figure 6.1 (Vanhoutte *et al.*, 1992b).

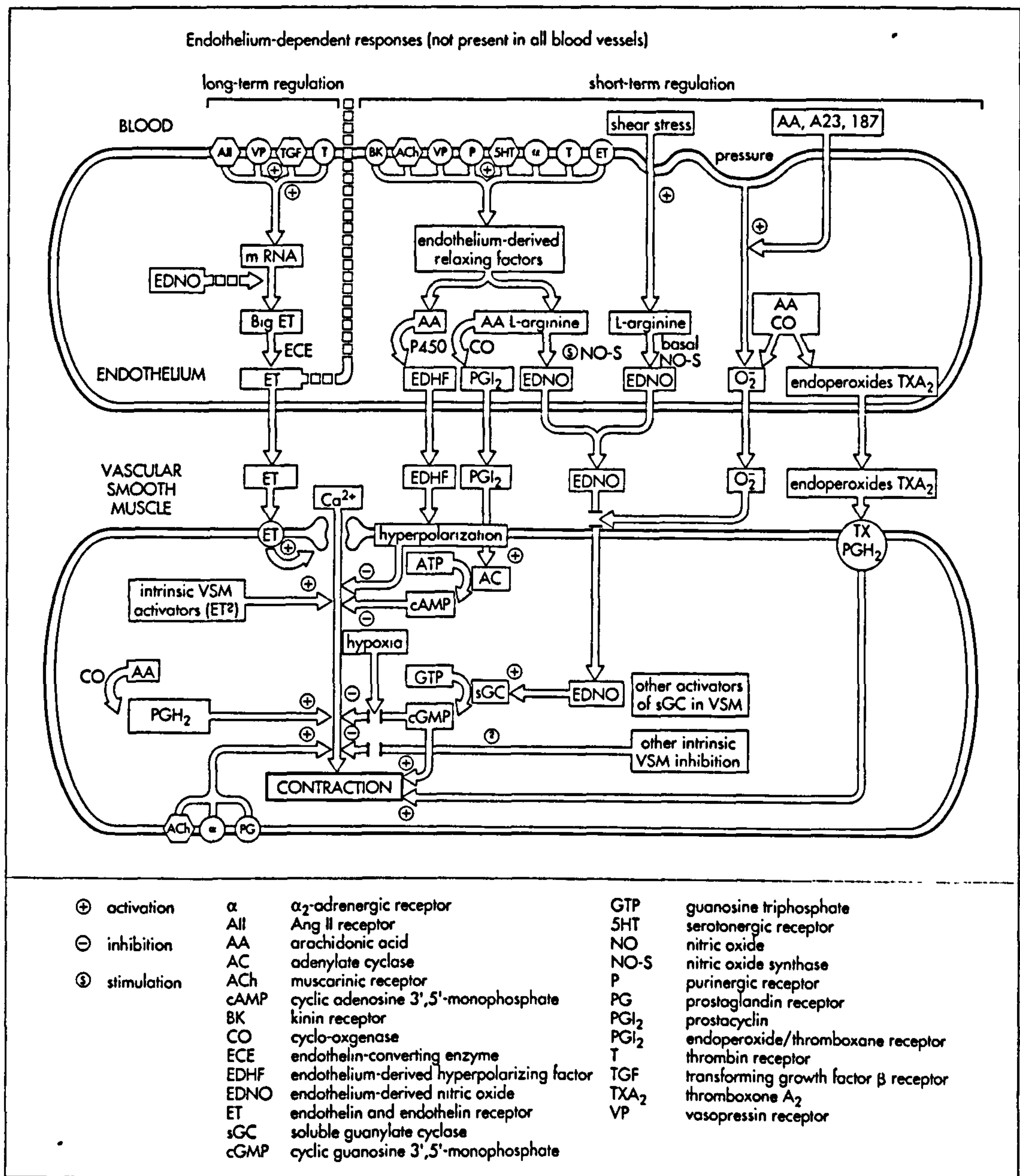


Figure 6.1 Endocrine regulation of vascular tone (Vanhoutte, *et al* 1993b)

Figure 6.2 Synthesis of angiotensins from angiotensinogen (after Robertson 1993)**Renin Substrate -Angiotensinogen (14 amino acids)**H -Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu- | *Leu-Val-Tyr-Ser* (equine)H -Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu- | *Val-Ile-His-Asn* (human)

↓ Renin

Angiotensin I (10 amino acids)

H -Asp-Arg-Val-Tyr-Ile-His-Pro-Phe | His-Leu- OH

↓ ACE

Angiotensin II (8 amino acids) and active fragments A¹⁻⁷, A³⁻⁸

H -Asp- | Arg-Val-Tyr-Ile-His-Pro-Phe- OH

↓

Angiotensin III (7 amino acids)

H -Arg-Val-Tyr-Ile-His-Pro-Phe- OH

↓angiotensinase

Inactive peptide fragments

Italics indicate differences between human and equine sequences of angiotensinogen
 | indicates site of cleaving the molecule

Equine angiotensin sequences are 100% homologous with human and rat sequences although angiotensinogen has different sequences at positions 11 -14 as do other species such as rat and mouse (Menard *et al.*, 1993).

Angiotensin I and II have never been measured in the horse and it is uncertain whether angiotensin is a central component in the aetiology of laminitis. Although angiotensin activity has been extrapolated from renin-activity (Hood, 1979; Miller, 1981; Clarke, 1982), difficulties in the development of a radioimmunoassay, of the unrealistic expense of alternatives such as GCMS, makes quantification of equine angiotensin difficult. Its role in laminitis has been also postulated by determination of aldosterone (Miller, 1981); the hormone itself has never been determined. It has been suggested that angiotensin is part of the pathophysiology of the disease (Hood, 1979, Stashak, 1987) but it is uncertain whether angiotensin is a secondary part of the pain/hypertensive response or central to the development of the disease. The use of angiotensin converting enzyme (ACE) inhibitors has been discussed (Stashak 1987; Purohit *et al.*, 1981). The aim of the present study was to establish a radioimmunoassay for the direct determination of equine angiotensin. The radioimmunoassay may then be used to establish basal values of angiotensin II for the horse and comparisons made between different levels of the hormone during developmental and acute stages of laminitis. Determination of angiotensin II during the developmental and acute phases may provide a clue as to whether it is immediately involved in the aetiology of the disease or whether it is merely a secondary factor.

6.2 Materials and Methods

Animals A total of 18 animals were studied. Twelve cross-bred ponies, 8 mares and 3 geldings (aged 4 to 18 years) and 2 hunter geldings (aged 15 and 17 years) were studied. Five Registered Irish Draught mares were also studied. Animals were subject to careful management (see Chapter 5). The animals were turned out to grass on unfertilised old pasture or stabled on a diet of hay *ad libitum*. Six ponies, and one Irish Draught mare, had a history of laminitis when at grass in previous years so were classified as chronic laminitic animals. The ponies were not severely lame - \leq Obel Grade 1 and not in need of special farriery but the chronic laminitic mare had Obel Grade 4 lameness. Five of the animals that had chronic laminitis, and one normal pony, suffered attacks of acute laminitis while at grass. An Irish Draught mare developed acute laminitis of endotoxic aetiology after foaling complications. In addition, clinical samples of cases of acute and chronic laminitis cases were provided by local veterinary surgeons. A group of adult male and female rats of Brattleborough, Long Evans and Milan Normotensive strains were reared under standard laboratory conditions and sacrificed to create a plasma pool.

Blood Samples Samples were collected between 10.30 am and 12.30 pm on the same day each week, the animals were standing quietly for at least 20 minutes before the sample was collected. Times of collection of blood samples from the *post partum* case are listed. The animals were accustomed to the procedure and not overtly stressed during the sampling. Blood samples were taken by jugular venepuncture into glass vacuum tubes containing 0.12 ml 0.34M ethylene diamine tetracetic acid (EDTA) (Vacutainer, Becton Dickinson). The samples were then mixed with inhibitor solution -: 250 mM EDTA, 0.05 M 1,10 Phenanthroline in ethanol, and 1000 KIU/ml Aprotinin - in the ratio of 50 μ l/1ml blood and

kept on ice until centrifuged at 600g for 12 minutes. The plasma samples were stored in polypropylene tubes at -20° C.

A plasma pool of rat samples was created. Samples of rat blood were collected by cardiac puncture under terminal anaesthesia (chloroform, inactin i.p. or halothane), or after stunning and cervical dislocation. These samples were collected using the methods described *vide supra* and assayed alongside the equine samples to assess accuracy.

Human control samples of AII in lyophilised sera were provided in the Nichols RIA kits and the actual values compared with those obtained by RIA.

Principles of Radioimmunoassay The principles of radioimmunoassay are described by Edwards and Rees (1994). In essence, the antibody recognises the hormone under investigation and binds to a selected site of the molecule. Radiolabelled hormone is added to the assay tubes and competes with the sample for the antibody. If very little hormone is present in the assay sample then most of the radiolabelled hormone will be bound to the antibody. Conversely, if there is a large amount of hormone present then a smaller proportion of the radiolabelled hormone will be bound to the antiserum.

Separation of the bound and free hormones is by centrifugation and therefore by molecule size. A secondary antibody, which attaches itself to the primary antibody, is used to increase the size of the bound molecule complex and enabling more accurate results to be obtained. A separation solution of polyethylene glycol and bovine γ globulin enables larger bound complexes to be separated from free molecules in a similar fashion during centrifugation. Calculation of the radiolabelled bound or free fraction is automatic on the gamma counter software although the principles of calculation are outlined below.

Total counts of the radioactive hormone added to each tube are measured. A standard curve of percentage binding at given dilutions of the standard is produced on a logarithmic scale, using known serially diluted standards of the hormone. Non-specific binding (NSB) to buffer solution molecules (ie when no antiserum is present) and binding when no standard is present (B_0) (ie binding of the antiserum to buffer solution molecules) is calculated. The lowest standard is calculated by subtracting the non specific binding from the zero standard. The percentage binding of the samples are compared with the percentage binding on the curve and the amounts of hormone in each sample calculated. Final figures are adjusted for concentration and recovery rate.

$$\text{Calculations are } \%B/B_0 = \frac{\text{Average counts of sample} - \text{NSB counts}}{\text{Average counts of } B_0 - \text{NSB counts}} \times 100$$

From the result of this calculation ie percentage bound, it is possible to find the appropriate percentage on the curve plotted and find the value of the sample compared with the standards given. In reality, the gamma counter software does this calculation and gives the result in pg/ml which is then adjusted for concentration and recovery.

Extraction of peptides

Dialysis Plasma (400mls), in sealed dialysis tubing of pore size 32/32mm was placed in a flask containing 4L of chilled distilled water. The flask was stirred at 4°C for 24 hours, the dialysate removed and the flask was refilled with distilled water. This process was repeated after 36 hours. Angiotensin II diffuses through the membrane and, after several changes of water, AII should be absent in the plasma compartment. Smaller volumes of plasma (10mls) and water surround (100mls) were sometimes used. The dialysate was dried under a vacuum in a Univap and reconstituted in 2.5 mls of assay buffer to give a twofold concentration of AII. The resulting solution was stored at -20° C. The dialysed plasma was used to assess cross reactivity of the various antisera to plasma proteins. The dialysate was

used to record values for equine AII that were not affected by the presence of plasma proteins. Each RIA protocol was assessed with both dialysed plasma and dialysate, if quantities allowed, and the results compared. Dialysed plasma was also used as a vehicle for "spiked" samples to calculate recovery rates during extractions.

Extraction protocols

Protocol I This method is outlined by Aptel (1993) and is the "in house" method for rat and human samples. Sep-Pak C18 cartridges were prepared with 5mls of Buffer B (70% Acetonitrile/ 0.1% Trifluoroacetic acid TFA) and washed with 10mls of Buffer A (0.1% TFA). Samples were thawed and acidified with 100µl/ml of 1%TFA. Three mls of acidified plasma were passed through the cartridge, retained and repeated so that the solution passed through the same column three times. The column was washed with 10mls of Buffer A and then eluted into polypropylene tubes with 2.5 mls of Buffer B. The samples were lyophilised at 40°C under a vacuum in a Univap.. Samples were reconstituted in 750µl of assay buffer, to give four fold concentration, and kept at -20° C until use. Recovery was calculated by 'spiking' a sample of dialysed plasma with radiolabelled AII. The cpm radioactivity after reconstitution, were compared with the equivalent volume of sample solution. Recovery for this protocol was 95%.

Protocol II (Dr J.J.Morton method) This method is standard in clinical evaluation of human AII and is described courtesy of Dr. J..J. Morton,(communication) Sep-Pak C18 plus cartridges (Waters, Millipore, Milford, MA, USA) were mounted on Vac-Elut extraction apparatus, connected to a vacuum pump. Cartridges were prepared by washing with 5mls methanol and then washing with 5mls of distilled water under a pressure of - 10 mmHg. The samples (volume 2 mls) passed through the cartridges once, then washed with 5mls 0.1% TFA solution. Samples were eluted in 2mls of 80 % methanol / 20 % 0.1% TFA and dried at 40° C under vacuum in a Univap. When dry, these samples were

reconstituted in 500 mls of 50mM Tris buffer, a four fold concentration. Reconstituted samples were stored at - 20° C until analysis. The recovery was calculated as above and found to be 94 % (Dr J.J. Morton, personal communication)

Protocol III (Nichols Institute) This method is described in the manufacturers literature which accompanies their radioimmunoassay kits (Nichols Institute Diagnostics B.V., The Netherlands). 1 ml of plasma is pipetted into a polypropylene tube and kept on ice. 5 mls of chilled ethanol are added to the sample and vortexed for 2 mins or vortexed briefly, placed on a mixer rack in an icebath for 30 mins at 2-8 °C. The tubes are then centrifuged for 15 mins at 2 - 8°C at 1500 - 2000g and the supernatant placed into clean polypropylene tubes. The supernatant is evaporated in a water bath at 3 °C under dry nitrogen gas. When dry the samples are reconstituted in 1ml of TRIS buffer and vortexed. The samples are at a 1:1 dilution and are kept at -20 ° C until required for RIA. Recovery was calculated using a similar protocol to the above (Nichols Institute directional insert) and varied between 34 % and 67%

Combinations of methods used in the different protocols were tried in an attempt to optimise results from equine samples i.e. different solutions used in extractions were exchanged from one protocol to another; buffers were exchanged and later the bound and free fractions were evaluated for each assay method.

Radioimmunoassay preparation and separation

Standard curves were prepared at different ranges according to the particular protocol. Separation methods were different in each protocol.

In Protocol I standard curves were prepared by serial dilutions with ranges in standards from 9.5 - 10 000 pg/ml. The radioimmunoassay was prepared by the addition of I¹²⁵-labelled angiotensin II (Amersham Ltd UK) and either laboratory stock antiserum or rabbit antibody 77 (Amersham Ltd, UK). Phosphate assay buffer (0.04M) is used. Total counts, non-specific binding and zero binding were measured in triplicate and samples in duplicate. Incubation was for 19 - 24 hours at 4°C. In this protocol separation of bound and free fractions was by addition of 0.1% bovine γ globulin and 1 ml polyethylene glycol solution, centrifugation of the tubes for 15 mins at 600g and aspiration of the supernatant. The pellet containing the bound fraction was counted by gamma spectrometry (Packard Auto-gamma)

Protocol II utilises a range of 3.5 - 400 pg/ml in the standard curve and 50mM TRIS buffer is used. Duplicate tubes were used for all standards and samples. The assay is incubated for 19 -24 hours at 4°C. The free fraction is separated from the bound with charcoal /dextran solution and centrifugation of the tubes for 15 mins at 600g. The free fraction contained in the charcoal pellet is counted by gamma spectrometry (Packard Auto-gamma).]

Protocol III (Nichols Institute) has a standard curve range of 0 - 400 pg/ml . Duplicate tubes were used for all standards and samples and TRIS buffer (Nichols Institute) is used. 400 μ l of sample were used in normal 1:1 concentration. 100 μ l of antiserum are added to all assay tubes except TC and NSB; then tubes are vortexed and incubated for 6 hours at 2 - 8°C. After the first incubation period 100 μ l of radiolabelled AII are added to all tubes. Tubes are vortexed again and incubated at approximately 4°C for 18 hours. After the second incubation, a secondary antibody is added - anti- rabbit -and the tubes are vortexed

and incubated at room temperature for 20 - 30 minutes. Then 1ml of water is added and the tubes mixed again before centrifugation for 15 mins at 600g. The bound fraction is the pellet and this is counted after aspiration of the supernatant by gamma spectrometry.

Cross reactivity of antisera with other vasoactive hormones

Cross reactivity of the laboratory antiserum with other vasoactive hormones has been established by N. Cougnon (1993) in this laboratory. Cross reactivity for Dr Morton's antisera has been established in his laboratory. Data on cross reactivity of the Nichols Institute Kit antiserum has been established by the company's own laboratory and are given below in Table 6.1

Table 6. 1

**Cross reactivity of Nichols Institute AII antiserum
with other vasoactive hormones**

		Cross reactivity (%)
Asp -Ileu -	Angiotensin II	100
Val-	Angiotensin II	100
Asn -Val-	Angiotensin II	30
Sar -Ileu-	Angiotensin II	0.02
	Angiotensin II (4 -8)	91
	Angiotensin II (3 -8)	70
	Angiotensin II (2 -10)	0.02
Ileu-	Angiotensin III	67
Asp -Ileu-	Angiotensin I	0.1%

Nichols Institute Antiserum, cross reactivity determined at 50% Bo. (Nichols Institute Diagnostics B.V. Directional Insert for radioimmunoassay kit, 1992).

Interassay variation Quality controls were used in all assays to assess interassay variation. The quality controls were made from a known standard to known dilutions of low, medium and high values designed to be equivalent in value to expected results. One batch of equine samples was assayed twice using protocol III and the individual values compared. The other protocols were assessed by quality controls of known standards which were run in each assay.

Intra-assay variation This was determined by multiple assays of the same sample extract within the assay.

Quality Controls. These were provided by Nichols Institute and were of given values. These were extracted and assayed with the equine samples.

Serial dilutions

Equine plasma extracts were, after lyophilising, concentrated in an appropriate quantity of assay buffer. For example, 2 mls of plasma would be reconstituted in 500 μ l of buffer to give a 4-fold concentration; or reconstituted in 250 μ l of buffer to give an 8-fold concentration. The solutions were sampled as concentrate and each sample serially diluted. These solutions were assayed using Nichols Institute antiserum and the results plotted on a chart.

Statistical Analyses

Mean values were calculated using spreadsheet software (Microsoft Excel) and are shown \pm standard error (SE). A paired two sample Students t-test was used to assess statistical significance, assuming unequal variance and two tailed probability. Results were considered statistically significant when p was ≤ 0.05 .

6.3 Results

6.3 1 AII-like immunoreactivity

The radioimmunoassay of equine AII, using protocol I and laboratory stock antiserum (R77), gave consistently higher values than those reported for other species and were therefore considered likely to be above physiological values. However, rat plasma samples determined in the same assay gave values of 24pg/ml and 54 pg/ml. The data on equine samples were unlikely to be solely for angiotensin II, and are judged to include cross reactive substances in horse plasma with the antiserum. Data are therefore “AII-like immunoreactivity”. Clinical samples showed differences in AII-like immunoreactivity before, during and after the acute phase of laminitis (Table 6.2). Plasma levels of AII like immunoreactivity were high during the acute phase of laminitis. However, serial dilutions from four fold concentration to one in four dilution were not linear. The stated values show changes during the course of the disease but must be viewed as purely qualitative.

Table 6.2 AII - like immunoreactivity values pg/ml of equine samples over the course of acute laminitis.

<i>Horse</i>	<i>Cheeky</i>	<i>Isadora</i>
Before onset	-	52.4
Acute laminitis	374.5	488.7
1 wk afterwards	271.5	244.8
1 month afterwards	108.7	-

6.3.2 Identification of suitable antisera for RIA determination of equine Angiotensin II.

Cross reactivity of the antiserum R77 with equine plasma proteins was evaluated by comparing Angiotensin II readings in neat plasma, dialysed plasma and dialysate. Tests using dialysed plasma and the dialysed AII revealed cross reactivity of the antiserum with equine plasma proteins (Table 6.3) The assay, repeated using commercially available antisera (Amersham), gave similar results (Table 6.3). Horse plasma samples gave values between 3382pg/ml and 4530 pg/ml at two fold concentrations, although rat plasma samples showed normal values.

The antiserum MRC 76/1B (J.J. Morton, Glasgow) also cross reacted with equine plasma proteins, but not rat plasma proteins, giving similar results to the R77 and Amersham antisera. Assays were repeated using both these antisera, after centrifugation of samples and pre-extraction filtration, but acceptable values could not be obtained without dialysis.

The antiserum MRC 30/ VP (J.J. Morton Glasgow), using protocol II, did not react with equine plasma proteins and gave normal equine values of 19 pg/ml. Dialysed plasma did not give reading on the curve range whilst dialysate gave a value of 11 pg/ml. Unfortunately, this antiserum was in very short supply and there was insufficient to assess all equine samples.

The antiserum used in the Nichols Institute RIA Kit for protocol III did not cross react with equine plasma proteins. Samples were assayed in one fold concentration/dilution and values adjusted for recovery. Equine values for two clinically normal horses were 16.8 pg/ml and 21.4 pg/ml respectively and human quality control values was 68 pg/ml (given range 32 - 68 pg/ml). Dialysed equine plasma were below levels of detection. This protocol was therefore

chosen as the most suitable method for determination of equine angiotensins. Therefore, all RIA were conducted using Nichols Institute RIA Kits in this study. Serial dilutions of concentrated samples are shown in Figure 6.3. The conclusions of the investigations into suitable protocols and suitability of the antisera are shown in Table 6.5

Table 6.4 Comparison of AII like immunoreactivity for extracted AII, dialysate (containing AII), dialysed and neat plasma to evaluate cross reactivity of various antisera with equine plasma proteins.

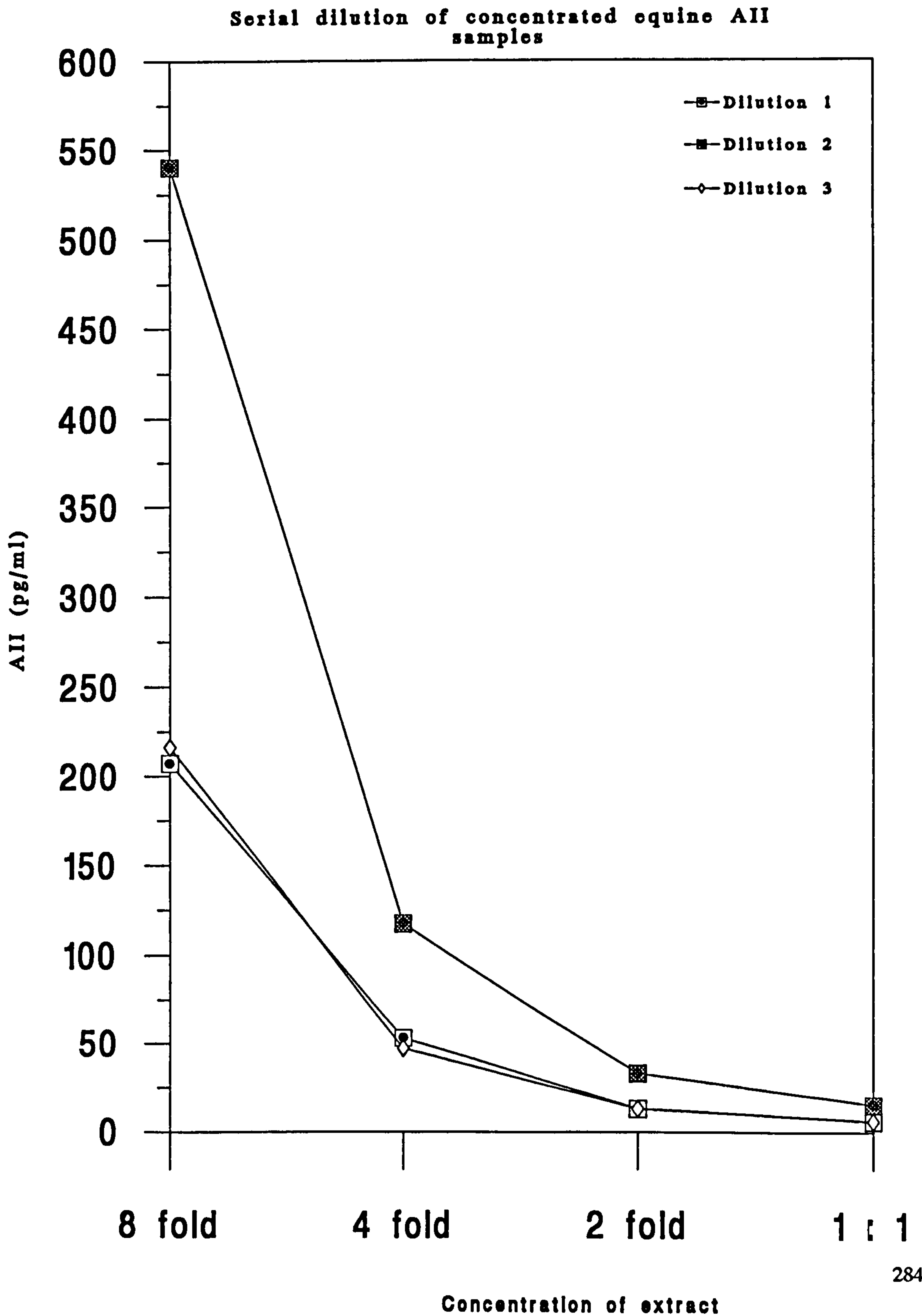
Antisera :	R77	Amersham	MRC 30/VP	Nichols Inst.
<i>Equine AII</i>	-	-	19 pg/ml	17 -21 pg/ml
<i>Dialysate (AII)</i>	11.33 pg/ml	22.66 pg/ml	11 pg/ml	-
<i>Dialysed plasma</i>	24.46 pg/ml	48.93 pg/ml	< 0.5 pg/ml	< 0.5 pg/ml
<i>Neat Plasma</i>	31.24 pg/ml	62.49 pg/ml	-	-
	Unsuitable	Unsuitable	Suitable	Suitable

Table 6.5 Summary of extraction methods and antisera suitable for RIA determination of equine angiotensin II

Suitable- No cross reactivity	Unsuitable- Cross reactivity with equine plasma proteins
Nichols Institute #	Amersham **
MRC, Glasgow 30/VP *	Peninsula **
	MRC, Glasgow 76/1B*
	Lab.stock R77**

Extraction/ separation methods: # Ethanol/ second antibody;
 * Methanol, Sep-Pak/ Charcoal dextran; ** Acetonitrile/ TFA/ PEG;

Figure 6.3 Serial dilutions of concentrated equine AII.



6.3.3 Angiotensin II in normal horses and ponies

Plasma concentrations of AII were determined for normal horses and ponies during summer and winter. During winter, all animals were fed hay but during summer months all animals were on a staple diet of grass.

Table 6.6 presents the AII data for normal individual horses and ponies over a 12 month period. Omissions from the Tables are either because the horses had not joined the group, because they were being treated for acute laminitis, or because those animals could not be caught on the day of sampling. Figure 6.4 shows individual distribution of plasma AII in the normal herd over a 12 month period.

Normal horses and ponies

The group mean of plasma AII in normal horses during winter months (Oct - Apr) was 39 ± 3 pg/ml (n = 12; number of tests = 37) compared with 14 ± 2 pg/ml (n = 12; number of tests = 50) of normal horses during summer months (May -Sept).

The concentrations are significantly lower in summer than the winter values of AII in normal equines ($p < 0.001$)

Equine Angiotensin II in plasma, pooling all the data is :

Normal equine AII = 24 ± 2 pg/ml

(range 1 - 84)

n = 12; number of tests = 88

Table 6.6 Plasma Angiotensin II values for normal horses and ponies (pg/ml)

Month	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Aug	Sep	Sep	Oct	Nov	Dec
Date	21.1.95	.	8.3.95	17.4.94	.	12.6.94	17.7.94	14.8.94	28.8.94	4.9.94	11.9.94	16.10.94	19.11.94	.
Albert	21	-	49	37	-	9	-	-	12	19	31	59	39	-
Annabella	-	-	-	-	-	1	4	8	11	20	20	-	-	-
Cracker II	26	-	32	37	-	8	5	8	8	7	15	27	38	-
Despina	41	-	37	38	-	3	3	11	9	7	20	37	33	-
Dolly	26	-	48	-	-	-	-	-	-	-	-	-	-	-
Jasper	11	-	-	26	-	3	10	11	11	11	-	50	11	-
Marron	35	-	27	33	-	28	6	9	8	12	8	85	35	-
Maree Gray	46	-	67	37	-	-	-	-	-	-	-	-	-	-
Selluci	-	-	-	-	-	8	15	11	11	15	16	40	35	-
Snowflake	-	-	-	-	-	9	4	12	66	15	33	74	30	-
Tango	42	-	51	-	-	-	-	-	-	-	-	-	-	-
Tess*	-	-	-	41	-	29	7	12	47	15	30	24	-	-
Gp Mean	31	-	44	36	-	11	7	10	20	13	22	50	32	-
SE	4	-	5	2	-	3	1	1	7	2	3	8	4	-

Tess* before the first attack of laminitis.

Figure 6.4 Seasonal variations of plasma angiotensin II (pg/ml) of normal horses and ponies..

6.3.4 Angiotensin II during chronic laminitis.

Values of plasma Angiotensin II for chronically laminitic animals are shown in Table 6.7

The group mean of plasma AII of chronically laminitic animals during winter months (Oct - Apr) was 35 ± 2 pg/ml (n = 6; number of tests = 27) compared with 14 ± 5 pg/ml (n = 6; number of tests = 28) during summer months (May -Sept).

Individual values and the distribution over a 12 month period is shown in Figure 6.5

As in normal horses and ponies, there is a statistical difference between summer and winter values of AII in chronically laminitic equines. $p < 0.001$. There is no difference between chronic laminitic and normal groups and the pattern of change in the two groups could be virtually superimposed one on another.

Table 6.7 Angiotensin II values for chronic laminitic animals (pg/ml)

Month	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Aug	Sep	Sep	Oct	Nov	Dec
Date	21.1.95	.	8.3.95	17.4.94	-	12.6.94	17.7.94	14.8.94	28.8.94	4.9.94	11.9.94	16.10.94	19.11.94	.
Beau	39		30	14		-	1	9	8	8	19	24	32	-
Cobweb	9		42	27		5	17	44	9	14	28	31	34	-
Domino	29		43	31		7	8	17	9	13	11	61	21	-
Misty	45		47	59		6	8	36	15	11	12	37	23	-
Rose Marie	-		53	50		4	6	9	43	7	1	33	30	-
Tess	20		40	-		-	-	-	-	-	-	-	42	-
Group Mean	28		43	36		6	8	23	17	11	14	37	30	
SE	6		3	8		1	3	7	7	2	4	6	3	

Figure 6.5 Seasonal variations of plasma angiotensin II (pg/ml) of chronically laminitic horses and ponies.

Seasonal plasma angiotensin II (pg/ml) of chronic laminitic ponies

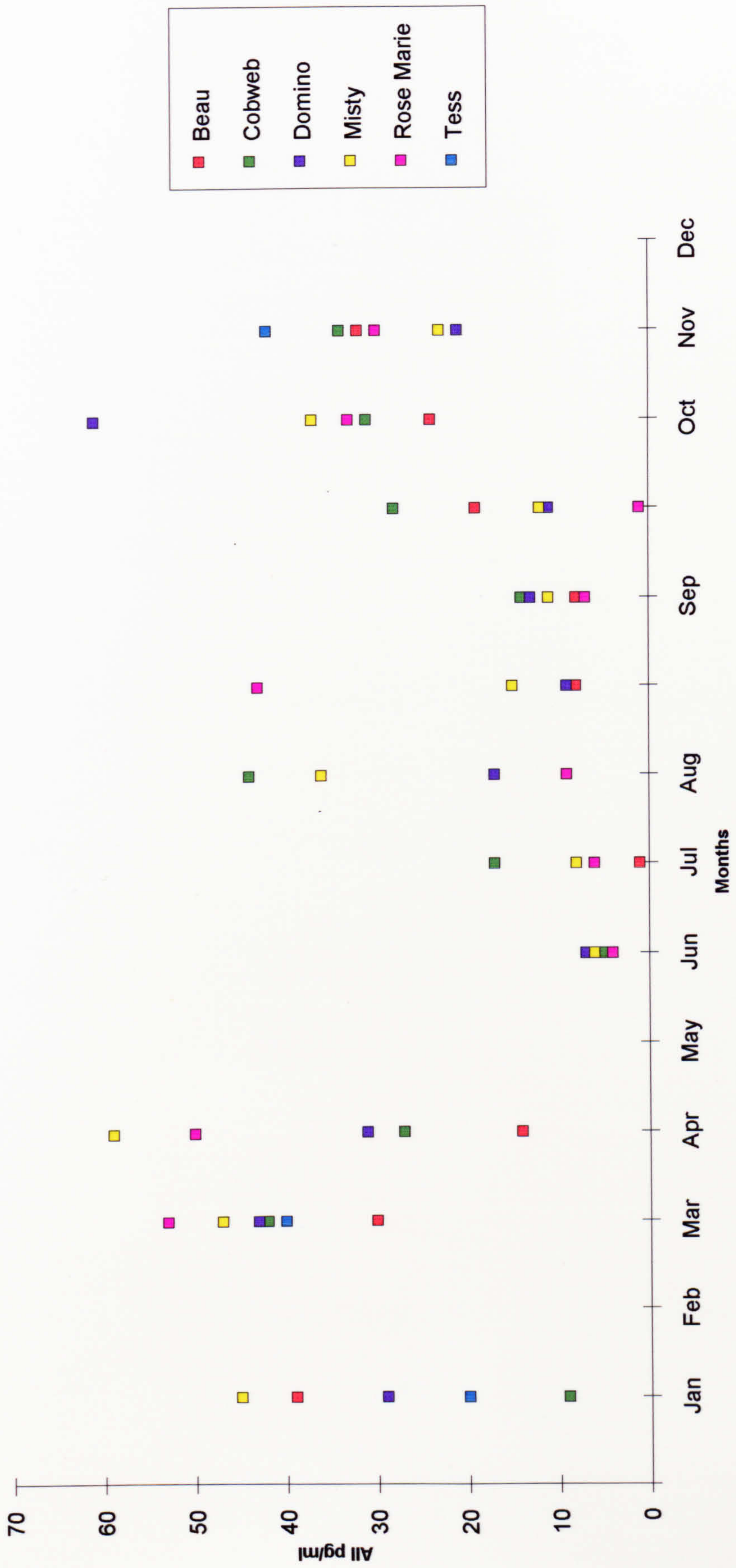


Table 6.9 compares plasma angiotensin II of normal and laminitic animals during summer and winter seasons and Table 6.9b compares the pooled data for the whole year in both groups.

Table 6.9a

Comparison of plasma angiotensin II concentrations (pg/ml) in normal and chronically laminitic equines.

	AII (pg/ml)	AII (pg/ml)
	Winter	Summer
Normal	39 ± 3	14 ± 2
n = 12; number of tests = 87		
Chronic laminitic	35 ± 2	14 ± 5
n = 6; number of tests = 55		

There is no statistical difference between normal equines and chronic laminitis cases during summer and winter months and the seasonal variation in mean values is shown in Figure 6.6

Table 6.9b

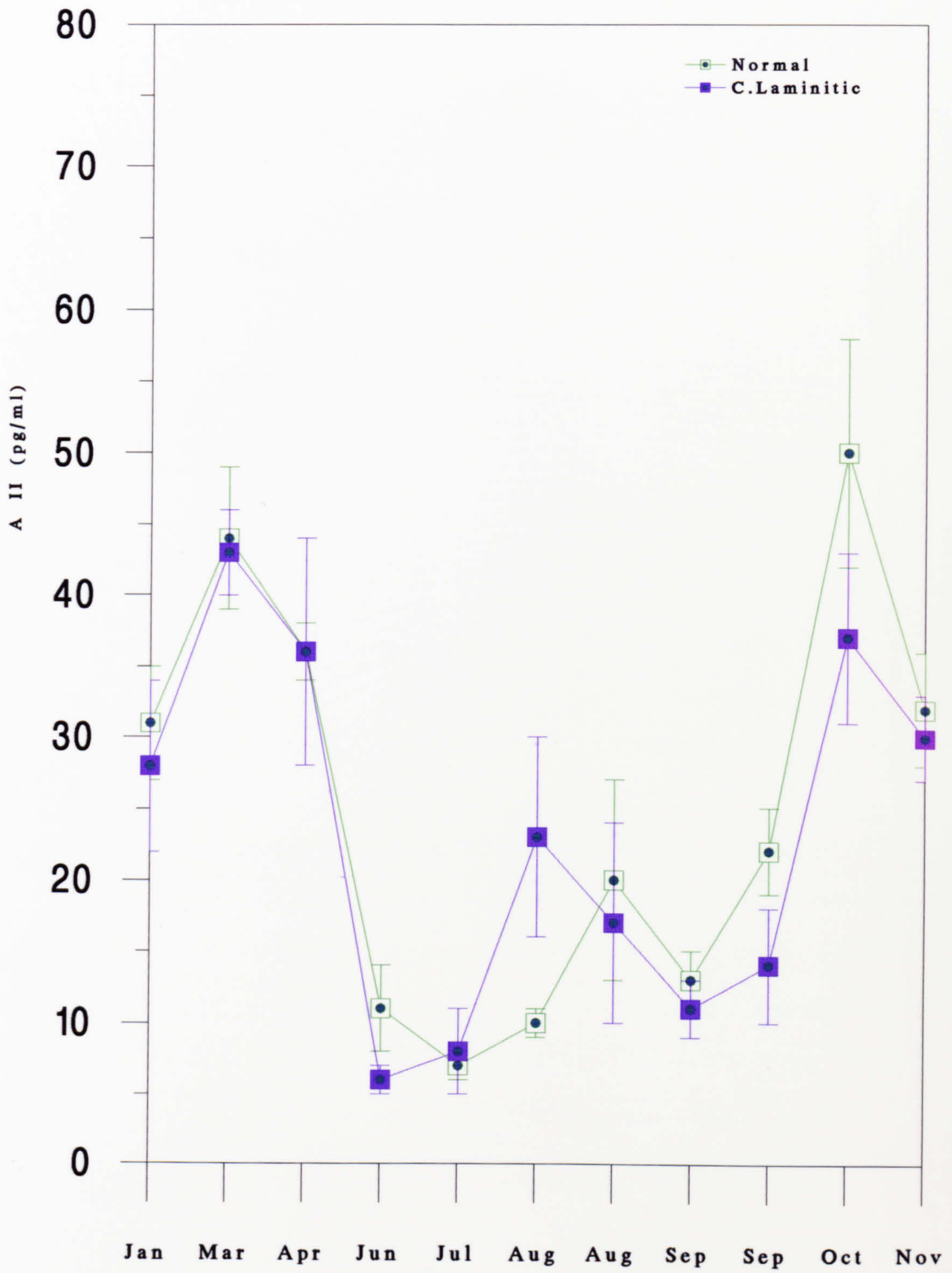
Comparison of plasma angiotensin II concentrations (pg/ml) in normal and laminitic horses and ponies.

Normal equine AII	24 ± 2 pg/ml
n = 12; number of tests = 88	(range 1 - 84)
Chronic laminitic AII	24 ± 2 pg/ml
n = 6; number of tests = 55	(range = 1 - 61)

There is no difference in mean values of normal equines and chronic laminitis cases.

Figure 6.6 Comparison of seasonal variations of mean plasma angiotensin II (pg/ml) of normal and chronically laminitic horses and ponies.

Seasonal differences in plasma angiotensin (A II) of normal and chronically laminitic ponies



6.3.5 Acute laminitis

Grass induced laminitis

Grass induced acute laminitis resulted in a slight elevation of plasma angiotensin concentrations but these were within normal ranges and the slight elevation only persisted until treatment was given (Tables 6.10, 6.11 and 6.12). During treatment AII returned to normal values immediately. Slight elevations in plasma AII were recorded when chronic laminitic ponies were kept at grass (36 pg/ml), but these values remain within normal ranges (Table 6.10).

Table 6.10 Comparison of plasma AII during acute laminitis with basal values for that individual and group mean values for that month

	Group Mean AII for that month (pg/ml)	Acute stage AII (pg/ml) before treatment	Individuals range of basal AII values for 12 months (pg/ml)
<i>Beau 1</i>	6 ± 1	21 - 28	1 - 39
<i>Beau 2</i>	8 ± 3	9	1 - 39
<i>Misty</i>	23 ± 7	32	7 - 59

Plasma concentrations of AII results for acute laminitis (Beau 1 and Misty), before treatment, were elevated compared with group values and the animals' own values for the time of year. The results for the acute stage were below winter values for the individuals concerned and the for the groups. AII was not increased during Beau's second attack. Changes before, during acute stage, during treatment, and after treatment are shown in Table 6.11

Table 6.11 Plasma AII during grass induced acute laminitis when treated with nitric oxide donors compared with normal values for the same horse in preceding and subsequent months (Beau).

<i>Beau</i>			
Date	AII(pg/ml)	Comments	Treatment
16.3.94	< 2	At grass	None
17.4.94	14	Stabled	None
30.4.94	3		None
6.5.94	13	To grass	None
15.5.94	< 2	Hyperlipidaemia	None
22.5.94	28	Lush grazing	None
25.5.94	21		None
2.6.94	3	Acute laminitis	Arginine iv , GTN
4.6.94	17	Stabled	GTN,PBZ
9.6.94	19	„	GTN
12.6.94	6	low BP97/53	End GTN
15.6.94	< 2	At grass	None
19.6.94	< 2	At grass	None
26.6.94	7	Acute laminitis	GTN
2.7.94	3		GTN
4.7.94	5		GTN
17.7.94	3		GTN
24.7.94	4		GTN
26.7.94	9		GTN
29.7.94	9	Salt appetite	↓GTN
31.7.94	< 2	Normal	↓GTN
7.8.94	4	At grass	None
14.8.94	4		None
14.3.95	13		None

Key : GTN - transdermal glyceryl trinitrate; PBZ - phenylbutazone; FXN - flunixin meglumine; ACP - acepromazine. ↑ - increase dose, ↑↑ - greatly increase dose, ↓ - decrease dose, ↓↓ - greatly decrease dose. Full details of therapeutic regimes are given in Chapter 5 and Appendix II.

*Grass induced acute laminitis (continued)***Table 6.12** Plasma ANP during grass induced acute laminitis when treated with nitric oxide donors compared with normal values for the same horse in preceding and subsequent months. (*Misty*)

<i>Misty</i>			
Date	ANP (pg/ml)	Comments	Treatment
15.5.94	9.6	Normal	None
30.5.94	12.5	At grass	None
12.6.94	11.0	Collision	Oral PBZ 1g/day
19.6.94	16.5	Stabled	Oral PBZ 1g/day
24.6.94	15.5	Stabled	Oral PBZ 1g/day
2.7.94	14.2	At grass	Oral PBZ 1g/day
9.7.94	31.7	Acute laminitis	Oral PBZ 1g/day
10.7.94	13.5	Stabled	GTN patches, Oral PBZ 1g/day
11.7.94	20.0	„	GTN patches, Oral PBZ 1g/day
12.7.94	15.0	„	↓ GTN patches, Oral PBZ 1g/day
13.7.94	17.0	„	GTN, Oral PBZ 1g/day
14.7.94	14.0	„	GTN, Oral PBZ 1g/day
15.7.94	14.0	„	↓ GTN, Oral PBZ 1g/day
17.7.94	8.0	„	GTN, Oral PBZ 1g/day
22.7.94	10.5	„	GTN, Oral PBZ 1g/day
24.7.94	22.5	„	GTN, Oral PBZ 1g/day
26.7.94	22.2	„	↓ GTN, Oral PBZ 1g/day
29.7.94	11.75	„	End GTN, Oral PBZ 1g/day
31.7.94	10.75		Oral PBZ 1g/day
7.8.94	18.75	At grass	Oral PBZ 1g/day
21.8.94	6.5	Stabled	Oral PBZ 1g/day
28.8.94	15	„	Oral PBZ 1g/day
4.9.94	11	„	Oral PBZ 1g/day
11.9.94	12	„	Oral PBZ 1g/day

Key : GTN - transdermal glyceryl trinitrate; PBZ - phenylbutazone; FXN - flunixin meglumine; ACP - acepromazine. ↑ - increase dose, ↑↑ - greatly increase dose, ↓ - decrease dose, ↓↓ - greatly decrease dose. Full details of therapeutic regimes are given in Chapter 5 and Appendix II.

The cases above correspond to Case references Beau 1 (Case Report 1) and Misty 1 (Case Report 2) in Chapter 5 and Appendix II. Full details of blood pressure are shown in Chapter 3 and Appendix II; and other endocrine analyses are shown in Chapters 7 and 8. Serum electrolytes are reported in Chapter 9.

*Post partum acute laminitis (including refractory stage)***Table 6.13** Plasma AII during normal months and during acute post partum laminitis.

<i>Biscuit</i>				
Date	Time	AII (pg/ml)	Comments	Treatment
21.1.95	11.30	8.7	Normal basal	None
20.4.95	11.30	8.6	Normal basal	None
23.5.95	11.30	14.1	Acute onset, mild, Obel 2	GTN 100mg/kg
24.5.95	7.00	13.4	Acute, severe, Obel 4	GTN 100mg/kg FXN
24.5.95	11.30	13.7	Some improvement. Salt appetite	GTN,PBZ, ↑FXN, ACP
24.5.95	19.00	12.0	Some improvement. Salt appetite	↓GTN, PBZ, FXN,ACP
24.5.95	21.00	13.2	Still Obel 4. Salt appetite	GTN,PBZ, FXN,ACP
25.5.95	19.00	18.2	Salt appetite	↓GTN,PBZ, FXN,ACP
26.5.95	19.00	18.0	Salt appetite	GTN,↓PBZ, FXN,ACP
27.5.95	19.00	16.4	Salt appetite	GTN,PBZ, FXN,ACP
28.5.95	19.00	14.5	Salt appetite	GTN,PBZ, FXN,ACP
29.5.95	19.00	16.8	Salt appetite	GTN,PBZ, FXN,ACP
30.5.95	19.00	12.9	Some improvement, Obel 3	↓GTN,PBZ, FXN,ACP
31.5.95	19.00	14.3	No salt appetite	PBZ, FXN,ACP
1.6.95	19.00	16.8	Some improvement, Obel 3	PBZ, FXN,ACP
2.6.95	19.00	9.9	„	PBZ, FXN,ACP
3.6.95	19.00	26.1	„	PBZ, FXN,ACP
4.6.95	19.00	13.8	„	PBZ, oral FXN,ACP
5.6.95	19.00	33.5	„	PBZ, oral FXN,ACP
12.6.95	19.00	19.8	„	PBZ, oral FXN,ACP
14.6.95	19.00	87.8	Obel 4 'Sinking' - bleeding coronary band	↑PBZ,↑FXN,ACP
19.6.95	19.00	7.3	Some improvement	↑PBZ,↑FXN,ACP
11.7.95	19.00	7.5	'Sinking'	↑↑PBZ,↑↑FXN,ACP
12.7.95	19.00	6.3	'Sinking' - mare destroyed	↑↑PBZ,↑↑FXN,ACP

Key : GTN - transdermal glyceryl trinitrate; PBZ - phenylbutazone; FXN - flunixin meglumine; ACP - acepromazine. ↑ - increase dose, ↑↑ - greatly increase dose, ↓ - decrease dose, ↓↓ - greatly decrease dose. Full details of therapeutic regimes are given in Appendix IV.

This mare had elevated levels of AII compared with her own normal levels for the time of year. Plasma AII remained slightly elevated compared with normal values for the time of year but within the normal range for winter stabled values (this mare was stabled during her acute stage and afterwards). Blood pressures are discussed in Chapter 3 and other endocrine analyses are shown in Chapters 7 and 8, together with electrolyte analyses in Chapter 9.

6.4 Discussion

Plasma Angiotensin II concentrations have not been previously determined in the horse. Hence a full validation is presented and is especially important. This study establishes a technique for assessing plasma Angiotensin II in horses. Extraction and radioimmunoassay of equine peptide hormones is particularly difficult as plasma viscosity varies; viscous samples are extremely difficult to pass through Sep-Pak extraction columns. Coupled with this problem, is the complication of finding suitable antisera. Although equine AII is 100% homologous with rat and human AII, difficulties arise when equine plasma proteins cross react with anti-AII antibodies. A combination of extraction methods that precipitate plasma proteins and the identification of antisera that are specific for Angiotensin II in equine samples allowed normal resting values of AII to be quantified. Any RIA for quantification of equine AII must be carefully validated. Linear serial dilutions of equine samples confirm the specific binding of the chosen antiserum to the AII molecule.

Extraction methods that use methanol or ethanol to precipitate plasma proteins, in combination with a very specific antiserum, were successful. The results using dialysed plasma when antisera had produced unacceptably high results, revealed that an ingredient in plasma caused false high values for AII. Equine plasma proteins are of different composition to other species as they contain higher concentrations of all immunoglobulins than other species (Jain, 1993). In addition, horses and ponies possess other immunoglobulins unique to equine species; for example IgGT and IgG(B) (Jain, 1993). Little wonder that the antisera need to be so specific for AII. The original clinical samples assayed showed marked AII immunoreactivity differences before, during and after the acute stage. These differences can be explained. Plasma proteins are known to rise during equine laminitis (Harkema *et al.*, 1978) especially C3c, C4, Hp and fibronectin (Edinger *et al.*, 1992). Increased plasma protein may be because of an immune response to endotoxins, because of endothelial

damage where proteins 'leak' into the plasma from vascular smooth muscle, or even intestinal leakage.

It is conjecture that Angiotensin II plays a key role in equine laminitis (Hood *et al.*, 1979; Miller, 1981; Clarke, 1982; Stashak, 1987) since there is no direct evidence. Plasma renin activity (PRA) and aldosterone concentrations increase during experimentally induced laminitis (Hood *et al.*, 1979; Miller, 1981) and during the acute phase of grass equine laminitis (Clarke, 1982). Although angiotensin and aldosterone plasma concentrations change during the course of the disease, it is unclear whether they are precipitating components in the aetiology or secondary factors. Angiotensin could precipitate vasoconstriction at an early stage and its involvement has certainly been discussed fully in this context (Stashak, 1987). On the other hand, the activation of angiotensin may be a secondary consequence of the acute phase of the disease because of a positive 'feed back' pain response (Hood, 1979). Unfortunately, all these studies are based on laminitis induced by oral administration of carbohydrate gruel (Laminitis Diet, Theracon Inc. Kentucky, USA) via naso gastric intubation. Induced laminitis invariably causes diarrhoea with associated water and electrolyte losses in the first instance and intense pain afterwards as the disease progresses. Garner, asked at the American Association of Equine Practitioners Congress 1974, about the similarities or differences between spontaneous and induced laminitis:

"Is the pathogenesis of founder in ponies on spring pasture the same as seen in your model?"

"I believe there may be some common denominator. However, one has to be very careful in extrapolation. I think it is a form of alimentary laminitis.."

(Garner *et al.*, 1974).

On top of the inevitable stresses of life at a veterinary hospital, such procedures and differences are likely to cloud results. Induction of laminitis using a method that causes digestive upset and electrolyte disturbance is not ideal when investigating the putative role of PRA, AII or aldosterone.

The present study has examined spontaneous laminitis arising within an equine herd kept under strictly controlled management. Postural variations in AII are precluded as horses spend nearly all their time standing. Diurnal rhythms evident in the horse (Clark, 1982) were obviated by collecting blood samples at the same time of day. Stress was minimal during blood sampling. Sodium intake was carefully observed - no additional salt was provided. No extra sodium was given unless the animal had an apparent pica or 'craving' evident by the horse avidly licking the walls. Such behaviour was observed during the days following acute laminitis of different aetiologies and will be discussed in the final Chapter. Clearly, these conditions are more typical of the disease of laminitis that occurs clinically and the AII values reflect the pathophysiology more accurately than the induced disease.

Normal values were established for the horse during summer at grass and during winter when the animals are stabled or in yards and fed dry fodder. Normal equine AII was established as 24 ± 2 pg/ml and this result compares with human values of 10 - 30 pg/ml (Williams, 1995) and 5.8 ± 2.3 pg/ml (Morton, 1993). The results in this study compare with the results 1 - 20 pg/ml for other species namely, rat, dog, pig, sheep and monkeys (Nussberger and Brunner, 1993). Variations in PRA and aldosterone have been observed after feeding (Clark *et al.*, 1982; Clark *et al.*, 1988) so animals in this study were not fed concentrates. Horses do not exhibit diurnal variation if feeding is *ad libitum*. There was a significant difference in the AII concentrations for individuals and for both diseased and normal groups ($p < 0.001$) between summer and winter samples.

The concentrations gradually increased as winter approached and decreased when the animals were turned out to grass in the Spring. The results are likely to be influenced by water, sodium and potassium content of the diet which have a seasonal variation (Table 6.14).

Table 6.14 Variation in sodium (Na⁺) and potassium (K⁺) content of dried feed and grass.(Data taken from Eye of the Master software; Clarke *et al.*, 1982).

Fodder	Electrolyte content	
	Sodium %	Potassium %
Grass	0.0	4
Wheat straw	0.39	2.33
Barley straw	0.13	2.16
Meadow hay	0.0	1.61
Seed hay	0.01	1.61
Clover hay	0.16	1.6
Ryegrass	0.0	1.34

The RAS influences primarily or secondarily sodium (Na⁺) and potassium (K⁺) ratios in blood and urine; plasma volume and hence blood pressure. The RAS is therefore susceptible to changes in blood pressure and renal flow, plasma osmolality - particularly Na⁺ and K⁺.

Obviously, the equine diet changes completely between the seasons. In winter the animals subsist on dried forage - hay and perhaps some straw- and the water provided in buckets which is not as potable as streams visited during summer months. Grass that grows in the Spring is sweet and lush consisting mostly of soluble sugars and water rather than dry matter. As the spring grass is so sweet, horses and ponies may gorge themselves and take in a water load during summer. The electrolyte content of grass is certain to challenge the RAS of herbivores. Herbivores therefore take in large volumes of water in spring and summer months and the AII plasma concentrations will decrease under these circumstances. All herbivores ingest grass which has a high K⁺ content - usually 3% - and a low Na⁺ content (Grunes and Mayland, 1977) Other studies have shown that grass contains K⁺

levels of up to 4% and virtually no Na⁺ (Clarke *et al.*, 1982; Dua and Care, 1995). When faced with a potassium load, PRA and hence AII will fall and K⁺ excretion (driven by aldosterone) will increase. Excess potassium must be excreted to maintain a plasma K⁺/Na⁺ ratio and sodium may also be excreted. Animals at grass will have a sodium deficient diet which would normally cause AII to increase, but high levels of ingested K⁺ will 'drive the system', opposing any increase in AII to attempt to conserve sodium, and in fact reducing PRA and AII. This assertion is supported by the findings that increased dietary K⁺ depresses renin release (McCaa *et al.*, 1975; Veyrat *et al.*, 1967; Williams *et al.*, 1970). This mechanism explains the low AII results that were evident in the summer months for both normal and chronically laminitic equine groups despite low dietary sodium. Since the reaction of aldosterone, under these circumstances, is independent of PRA and upregulates the density of AII receptors and their affinity for AII, it seems that plasma sodium is maintained by aldosterone activity rather than PRA. Aldosterone, a mineralocorticosteroid hormone secreted by the adrenal, is influenced by renin-angiotensin, ACTH and kalaemia. Plasma aldosterone concentration varies directly with K⁺ concentration in anephric humans (Vetter *et al.*, 1974). Equine aldosterone assays are already established and equine aldosterone has been measured (Hoffsis and Murdick, 1970; James *et al.*, 1970; Zolovick *et al.*, 1977; Gutherie *et al.*, 1980; Miller, 1981; Clarke, 1982) but the relevance of measured hormone levels during laminitis has not been addressed. The mechanism for the adrenal/aldosterone control of sodium under potassium loading is unknown (Corvol and Ménard, 1990) but final stage regulation of the conversion of corticosterone B to aldosterone by 18 hydroxylase and 18-hydroxydehydrogenase are suggested (Müller, 1971).

During the winter, the animals are on mostly dry feed and a relatively low water intake. Dry feed (hay) has a composition of 0 - 0.16% sodium and 1.61 - 1.34 % potassium; straw contains 0.39% sodium and 2.16 - 2.33 % potassium (Table 6.15) During winter months, animals ingest less potassium than in summer, and there is decreased intake of water, alongside a diet high in dry matter. Under these circumstances, the plasma AII levels

increase for both groups during winter months. Chronic laminitic horses and ponies seem to regulate sodium and potassium in the same way as the normal controls and there is no difference between the groups.

It has been suggested that aldosterone provides a unique regulation of the RAA axis in herbivores as an evolutionary mechanism to counter electrolyte stress (Clarke, 1982). In evolutionary terms, it is rare for herbivores to be subjected to electrolyte stress during summer months. One wonders though whether the plants that are ingested in the last 30 years handle potassium and sodium differently to the older varieties of grasses; or whether such commercial strains of grass alter their fluid composition of potassium and sodium when subjected to their own stress of drought or grazing. Certainly, old varieties of grass on old pasture are not 'lush' in Spring time and contain much less water. Do they contain lower potassium? Maybe this is another aspect of the difficulties that present day herbivores have to endure during domestication. It could be that if herbivores do regulate sodium by aldosterone, then this too has an occult role to play in the pathophysiology of laminitis.

During spontaneous grass induced acute laminitis ponies may: have suffered loss of electrolytes during digestive disturbance; have dietary sodium or potassium deficiency; have become stressed and/or are in pain. Under these circumstances, reduced plasma osmolality will increase PRA and hence increase plasma Angiotensin II. Pain will directly increase PRA and AII by sympathetic involvement through renal nerves. Noradrenaline directly activates renin release mediated through β - receptors, however β -blockade is not usual in acute laminitis cases - it was deemed less effective than a strategy of reducing pain with phenylbutazone (Hood, 1979). Treatment usually aims at analgesia through α - blockade although this will have little direct effect on renal adrenergic or cholinergic control of renin. Providing pain is controlled, then AII levels will not increase suggesting that AII involvement is secondary rather than primary. In the acute cases of grass laminitis studied the increase in AII was within the normal range for a twelve month period, which also

the increase in AII was within the normal range for a twelve month period, which also suggests that AII is not involved in the pathogenesis. Interestingly, although the increase in the acute cases showed a percentage increase on normal AII levels for that month, the AII showed a sharp decline when ponies were treated with nitric oxide donors. The initial increase in AII is nearly always the largest, and as the levels of AII decline in relation to the dose of GTN; pain is also attenuated, blood pressure falls and the GTN dose is reduced. At this stage AII levels rise again if the animal is still in pain (from reperfusion), but subside as the animals recovers. When the GTN treatment ends there is a slight increase in AII. NO donors are known to inhibit renin release (Vanhoutte *et al.*, 1993) and therefore GTN plays another, additional, role in the treatment of equine laminitis.

The *post partum* case of laminitis had similar values to grass induced laminitis but treatment at an early stage with GTN may have inhibited renin release; similarly, treatment with cyclooxygenase inhibitors (Flunixin and PBZ) may also inhibit renin through the inhibition of prostaglandins which have a synergistic relationship with the renin/ angiotensin system. The extreme complexity of this case makes it difficult to decide on any endocrine links. Under this treatment regime, AII levels remained within the normal range although the mare was hypertensive for weeks after the onset of the disease. This suggests that AII was not responsible for the maintenance of hypertension during the refractory period and that the hypertension was due to pain/increased heart rate. This assertion is supported by the highest levels of AII recorded when there was soft tissue breakdown within the hooves, causing uncontrollable pain. Even though these values were the highest recorded in the study, they were not much greater than one sample taken from a normal pony after he had been stressed and galloping prior to the blood sample being collected. The increased AII values seen during the painful 'sinking' period were markedly reduced when extra analgesia was administered.

The present data therefore contrast with the finding of studies of artificially induced laminitis. First, the induced form of the disease is not equivalent to the spontaneous disease for whatever reason, therefore conclusions drawn about the role of AII under such circumstances is not tenable. Secondly, under controlled conditions AII does not appear to have a primary role in the pathogenesis of equine laminitis and although it may contribute to elevated blood pressure, if pain is controlled and nitric oxide donors are used to treat laminitis then AII is not a major consideration.

Chapter 7 Atrial Natriuretic Peptide

	Page
7.1 Introduction	307
7.2 Material and Methods	316
7.3 Results	
7.3.1 Validation of ANP radioimmunoassay	323
7.3.2 Equine plasma ANP of normal and chronically laminitic horses and ponies	329
7.3.3 Plasma ANP during acute equine laminitis	336
7.4 Discussion	341

7.1 Introduction

Since William Harvey (1628) observed the action of the heart and the circulation, the control of blood pressure and vascular tone has fascinated physiologists. The heart regulates blood pressure by chronotropic and inotropic actions and as long ago as 1935 it was postulated that the heart must possess a mechanism for sensing the "fullness of the blood stream" (Peters, 1935).

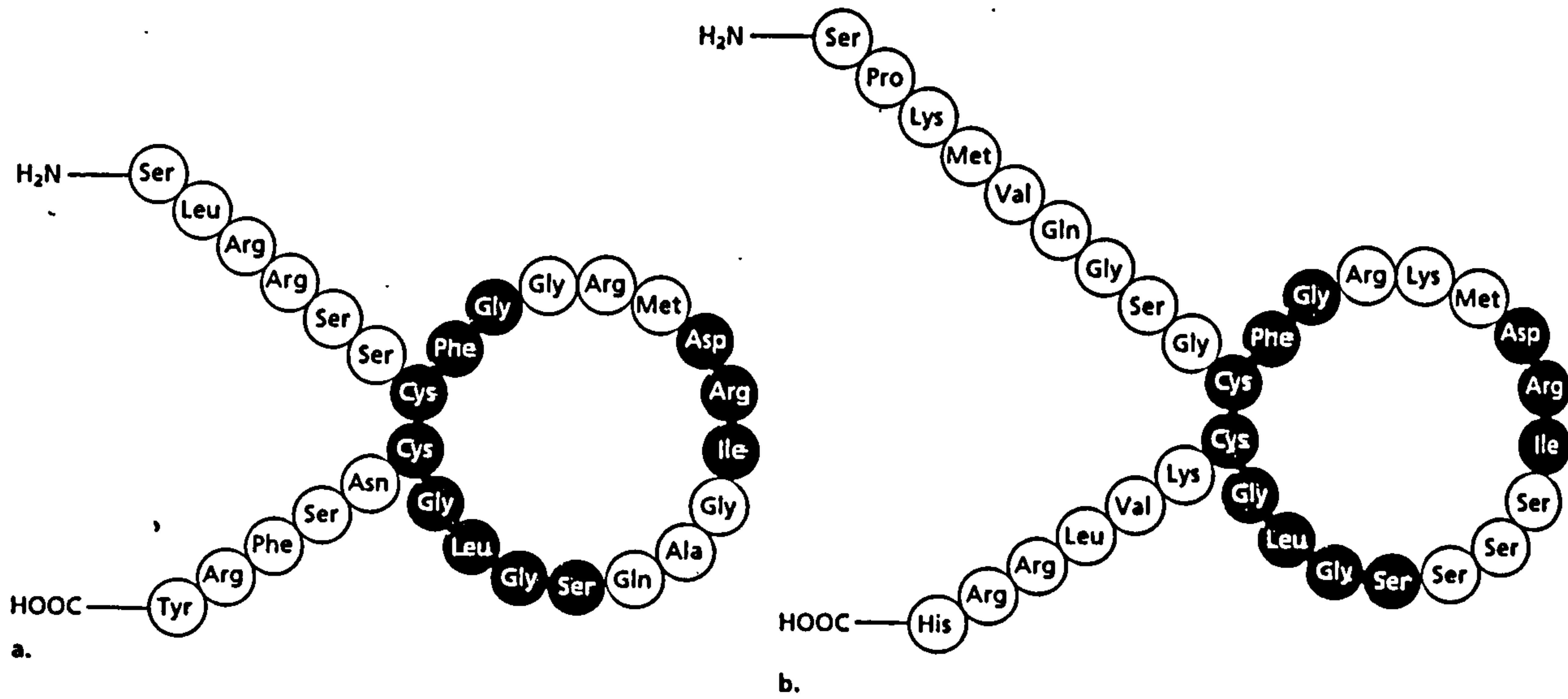
Blood pressure depends on blood volume, which is inextricably linked to plasma volume and osmolality, primarily regulated by renal control of electrolytes, especially sodium reabsorption and excretion. Although natriuresis and diuresis had been observed following atrial stretch (Cantin and Genest, 1984) and hormone-like granules observed in cardiocytes of man and mammals (Kirsch, 1956; Jamieson and Palade, 1964) the associations and mechanisms involved were not discovered until 1981 when de Bold, Sonneberg *et al.*, demonstrated natriuresis and diuresis in rats injected with homogenised rat atrial extracts. Since then much has been learnt about the hormone, named atrial natriuretic peptide (ANP) (Cantin and Genest, 1985; Mulrow and Schrier, 1987; Struthers, 1990; Adriaan *et al.*, 1993;) including the discovery that there are other closely related atrial peptides having a similar function - BNP and CNP (Sagnella and MacGregor, 1994).

Although the molecular biology of ANP is well described, the physiology of this family of peptides remains incompletely understood (Gardner *et al.*, 1990). Several different ANP molecules, ranging in size from 21 to 33 amino acids long were identified (Cantin and Genest, 1984; 1985; Needleman and Greenwald, 1986) and it was suggested that these molecules arise from a pre-pro hormone - pre-pro ANP - a molecule of 149 and 153 amino acids long; the cloning and transcription of ANP precursors and the active molecule itself is reviewed by Gardner *et al.*, (1990). The pre-pro ANP is processed to

the active form probably during the secretion of the hormone when it passes through the cell membrane (Imada *et al.*, 1988). The active form of ANP is the α ANP enantiomer. The active form is 28 amino acids long (Figure 7.1.) and has a disulphide bridge between the hemi-cysteine residues which is essential for its actions (Cantin and Genest, 1984; 1985). Several other vertebrate ANPs have been sequenced and have a high degree of homology (usually approximately 85%) and the molecule is highly conserved suggesting both a long evolutionary history and that the molecule has biological importance (Sagnella and Macgregor, 1994).

Equine sequences of ANP are listed on the Daresbury Database and the sequences shown in Figure 7.2). Equine amino acid sequences are 89.2% homologous with those of human ANP and 85.7% homologous with rat ANP (Henderson, 1996).

Figure 7.1 Molecular structure of atrial natriuretic peptide (ANP) showing a disulphide bridge between two cysteine molecules (Sagnella and Macgregor 1995).



Amino acid sequence of a. ANP and b. BNP peptides;

Figure 7 .2 Comparison of equine ANP sequences with hANP and rANP amino acid sequences

Human

Ser Leu Arg Arg Ser Ser Cys Phe Gly Arg Met Asp Arg Iso Gly Ala Glu Ser Gly Leu Gly Cys Asp Ser Phe Arg Tyr

Rat

Ser Leu Arg Arg Ser Ser Cys Phe Gly Arg *Iso* Asp Arg Iso Gly Ala Glu Ser Gly Leu Gly Cys Asp Ser Phe Arg Tyr

Equine

Ser Leu Arg Arg Ser Ser Cys *Phe* Ser Gly Arg Met Asp Arg Iso Gly Ala Glu Ser Gly Leu Gly Cys Asp Ser Phe Arg Tyr

In the equine sequence, Serine has been translocated from position 7 to position 9 and cysteine and phenylalanine translocated from positions 8 and 9 to positions 7 and 8. Rat and human ANP are only one amino acid different where isoleucine is substituted for methionine at position 12

Table 7.1 Three letter codes for amino acids from Henderson (1996).

Amino acid	Three-letter symbol	One-letter symbol
Alanine	Ala	A
Arginine	Arg	R
Aspartic acid	Asp	D
Asparagine	Asn	N
Asp and/or Asn	Asx	B
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Gln and/or Glu	Glx	Z
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Pyroglutamic acid	pGlu	<E
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Receptors sites for ANP have been described in renal , vascular and adrenal target tissues and these receptors activate guanylate cyclase as a secondary messenger *in vitro* (Waldman and Murad, 1989). *In vivo* infusions of ANP increase both plasma and urinary levels of cyclic GMP. There are three different types of ANP receptor each with a different affinity for ANP compared with the other atrial hormones BNP and CNP including a clearance receptor (Figure 7. 3).

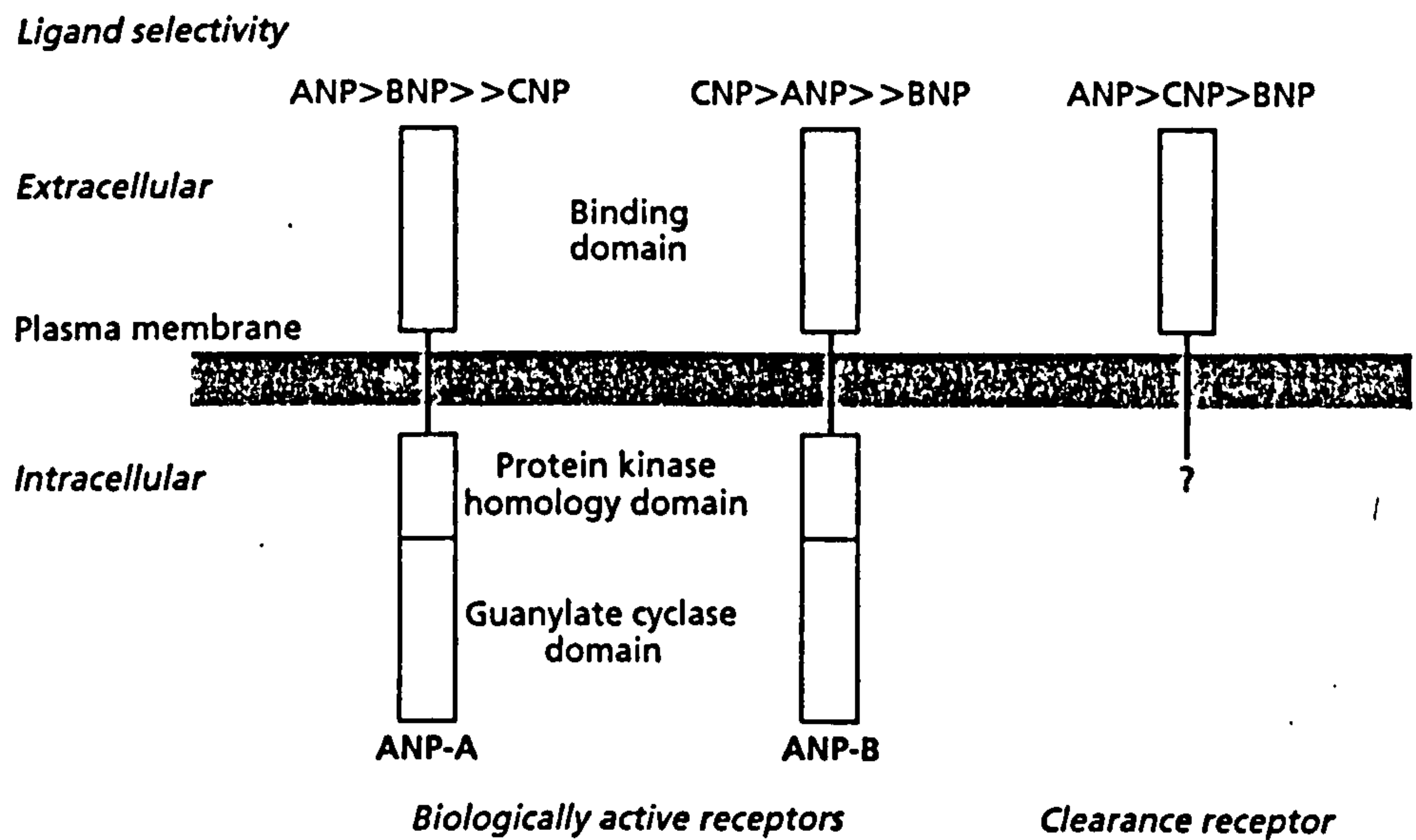


Diagram of three known ANP receptors and ligand selectivity for ANP, BNP and the C-type (CNP) natriuretic peptides. Two of these (ANP-A and ANP-B) display guanylate cyclase activity on peptide binding.

Figure 7.3 ANP, CNP and BNP receptors and their affinities for atrial peptides (Gardner and Macgregor 1994)

In physiological terms, the action of ANP is lyrically described by Rang and Dale (1994)

" Atrial natriuretic peptide appears to be, in therapeutic terms at least, the cardiovascular hero that stands opposed to the villainous intents of hormones such as angiotensin and vasopressin".

ANP is a vasodilator peptide that opposes the vasoconstrictor actions of AII and AVP. ANP opposes endothelin directly *in vitro* (Struthers, 1990) and *in vivo*, promotes natriuresis and diuresis thereby lowering blood pressure. ANP is synthesised by cardiac myocytes and the molecules can be visualised as granules within the cells. ANP is widespread in tissue and the atria are not the only sites of synthesis, which suggests that it may have an additional paracrine actions alongside the systemic one. Atrial stretch, usually caused by increased plasma volume due, for example, to sodium retention, causes release of ANP from granulocytes in the myocardium of the right atria (Wilkins, 1990). During chronic human hypertension, the ventricles may be recruited to synthesise extra ANP and left ventricular hypertrophy is often associated with increased plasma ANP. The ventricles of the heart are also a major source of BNP. Once released, ANP molecules bind to specific receptors causing a cascade of intracellular events (Green, 1990) that promote vasodilatation in cardiovascular smooth muscle and direct renal effects - binding to receptors in the collecting ducts to enhance excretion of water and sodium. The renal actions of ANP are debated (Green, 1990) and the results inconsistent. Arguments abound about actions on renal blood flow/GFR and the proximal and distal tubular function (where there are no ANP receptors!), but it is agreed that blood flow is redistributed to the renal medulla and this mechanism so inhibits reabsorption of water and solutes.

The half life of ANP is short, less than two minutes, and once released ANP will bind to either the active ANP_A or ANP_B receptors or to the clearance receptor, ANP_C (Sagnella and MacGregor 1994). Otherwise the peptide is subject to the endopeptidase enzymes which rapidly break down the molecule into inactive fragments. Most notable of the endopeptidases is neutral endopeptidase (NEP) and inhibition of the enzymatic activity has been the subject of intense pharmacological research since the enhancement of the half life of ANP could prolong the vasodilatory actions of ANP.

ANP in plasma is elevated during coronary heart disease (Winaver *et al.*, 1995), chronic renal failure and primary aldosteronism; healthy humans on a high salt diet or receiving mineralocorticoids develop a 2 - 3 fold increase in circulating ANP (Wilkins 1990). Other pathological conditions that are associated with increased plasma ANP include hypertension where hypertension is related to increased dietary sodium and/or increased renin activity, or plasma AII. Under these circumstances, ANP acts to promote natriuresis and diuresis.

The order of the increases is notable :coronary heart failure increases plasma ANP 20 fold but another cardiac natriuretic peptide BNP shows a 200 fold increase under these circumstances. Although brain natriuretic peptide (BNP) shows greater increases than ANP in pathological states, and may therefore be a better marker (Mukoyama *et al.*, 1990; Struthers, 1993). Circulating BNP is only 16% of circulating ANP (Struthers, 1990) and sensitive detection methods are required for its detection. Equine ANP and BNP has recently been identified in the equine atrium by immunohistochemistry and immuno-electron-microscopy (Mifune *et al.*, 1995).

Since acute laminitis cases are known to be hypertensive (Hood, 1993), see Chapter 3, it may be supposed that ANP may be secreted to restore normal blood pressure. This study tests the hypothesis that equine ANP may be elevated during acute laminitis to antagonise the effects of increased AII and corticosteroids, sodium retention and an increase in blood pressure/heart rate which are documented (Hood, 1993; Hinckley and Henderson, 1995). In other words, ANP may be involved in attempts to restore homeostasis during disease.

7.2 Materials and Methods

Animals

A total of 18 animals were the subject of this study. Twelve cross-bred ponies, 8 mares and 3 geldings (aged 4 to 18 years) and 2 hunter geldings (aged 15 and 17 years) were studied. Five Registered Irish Draught mares were also studied. Animals were kept as a group for at least 6 months prior to study and were subject to careful management regimes of feeding, anthelmintic prophylaxis and farriery. The animals were turned out to grass on unfertilised old pasture or stabled on a diet of hay *ad libitum*. Six ponies, and one Irish Draught mare, had a history of laminitis when at grass in previous years, but were not severely lame (\leq Obel Grade 1) or in need of special farriery. Six of the animals that had chronic laminitis suffered acute attacks of laminitis while at grass. All procedures were carried out under the Animals (Scientific Procedures) Act 1986. In addition, samples were provided by veterinary practices of cases of acute laminitis. These animals were of mixed sex, ages and breeds - mostly different pony breeds, and a warmblood mare, but of different aetiologies. The onset of acute was precipitated by grass ingestion, carbohydrate overload, pituitary adenoma and septicaemia and these are outlined in more detail in the results.

Blood Samples

Samples were collected between 10.30 am and 12.30 pm on the same day each week, the animals were standing quietly for at least 20 minutes before the sample was collected. The animals were used to the procedure and were not stressed during sampling. Blood samples were taken by jugular venepuncture into glass vacuum tubes containing 0.12 ml 0.34M ethylene diamine tetracetic acid (EDTA) (Vacutainer, Becton Dickinson). The samples were then mixed with inhibitor solution -: 250 mM EDTA, 0.05 M 1,10 Phenanthroline (Sigma) in ethanol, and 1000 KIU/ml Aprotinin (Sigma) - in the ratio of 50 μ l/1ml blood and kept on ice. Plasma was separated by centrifugation at 600 g for 12 minutes. The plasma samples were stored in polypropylene tubes at -20° C.

Extraction of ANP

This method is standard in clinical evaluation of human peptide hormones and is outlined by Dr. J.J. Morton, MRC Blood pressure Unit, Glasgow Royal Infirmary (personal communication). ANP was extracted from plasma by reverse phase chromatography using Sep-Pak C18 cartridges (Millipore UK Ltd.) mounted on Vac-Elut vacuum extraction apparatus connected to a vacuum pump. The cartridges were activated with 5mls of methanol and washed with 5mls of deionised water. Plasma samples (3mls) were drawn through the cartridge under vacuum pressure of -15 mmHg. The cartridge was washed with 5 mls of 0.1% TFA. Samples were eluted in 2 mls of 80 % methanol / 20 % 0.1% TFA and the eluate lyophilised at 40 ° C under vacuum in a Univap. When dry, samples were reconstituted in 750 µl TRIS assay buffer (50 mM tris [hydroxy-methyl] methylamine solution) adjusted to pH 7.4 with HCl and containing 5g/l bovine serum albumin and 2g/l neomycin sulphate. The reconstituted samples were kept in polypropylene tubes (LIP Ltd. West Yorks, UK) and stored at - 20° C until use. The recovery was calculated to be 78 % (Dr J J Morton, personal communication)

Preparation of antiserum

Rabbits were kept under normal laboratory conditions and injected subcutaneously with rat α -ANP (Sigma, UK) made up in Freund's adjuvant. They were given booster vaccinations at monthly intervals and blood was collected monthly. Blood samples were allowed to clot and the serum collected which was stored at - 20° C until use. The antiserum used in this study was from rabbit number 6, bleed number 7 1988 (R67).

Dilution of antiserum R67

Working dilutions of neat serum were made - 1:10; 1:100; 1:1000; 1:5000; 1:10 000; 1:20 000 and 1:40 000 . 100µl of each antibody solution were added to 100µl of labelled ANP (I^{125} r-ANP, specific activity ~2000 Ci/mMol (Amersham International plc, Bucks UK), diluted so that 100µl of radiolabelled ANP solution contained approximately 6000 cpm. 100µl of TRIS buffer were added to each tube to ensure the final concentration of antiserum was identical to that in assays. Assay tubes containing these solutions were made up in triplicate, together with total count and zero binding tubes, incubated for 19 -24 hours at 4° C. Bound was separated from free fractions by addition of 0.1% bovine γ globulin and 1 ml polyethylene glycol solution, centrifugation of the tubes for 15 mins at 600g and aspiration of the supernatant. The pellet containing the bound fraction was counted by gamma spectrometry (Packard Auto-gamma). The percentage of radiolabelled ANP bound at each dilution was calculated.

This procedure was repeated but 2500pg/ml of ANP standard solution was added to each tube as 100µl solution instead of the assay buffer and percentage binding at each dilution calculated. The best sensitivity is at approximately 30 -40% binding. When this was achieved, both dilution curves were repeated twice to verify results.

Standard curve validation

Standard curves were prepared by serial dilutions over the range 9.5 - 10 000 pg/ml at different dilutions of antiserum . Standard curves were prepared at working dilutions of antiserum as follows (Table 7.2)

Table 7.2

1:	5 000	(assay dilution	1: 15 000)
1:	10 000	(assay dilution	1: 30 000)
1:	20 000	(assay dilution	1: 60 000)
1:	22 000	(assay dilution	1: 66 000)
1:	30 000	(assay dilution	1: 90 000)

The standard curve was prepared by the addition of 100 µl I¹²⁵- labelled ANP solution, containing ~6000 cpm, to the 100 µl solutions of each standard. 100 µl of antiserum solution were added to each of the tubes except total counts and non-specific binding. Total counts, non- specific binding and zero binding were measured in triplicate. Incubation was for 19 - 24 hours at 4 ° C. Bound and free fractions were separated by addition of 0.1% bovine γ globulin and 1 ml of chilled polyethylene glycol solution (PEG). Sample tubes were centrifuged for 15 mins at 4°C and 600 g and the supernatant aspirated. The pellet containing the bound fraction was counted for 2 minutes by gamma spectrometry (Packard Auto-gamma)

When an acceptable assay standard curve was obtained at a particular dilution, the procedure was repeated at the same dilution of antiserum four times to ensure reproducibility.

Radioimmunoassay preparation and separation

Standard curves were prepared by serial dilutions over the range 9.5 - 10 000 pg/ml. The radioimmunoassay was prepared by the addition of I¹²⁵-labelled ANP (Amersham International Ltd, UK) and either laboratory stock antiserum R67 or anti-ANP antibody (Peninsula Ltd). Nichols Institute ANP antisera provided in their RIA kits were not suitable for the RIA of equine samples (Nichols Institute, personal communication). TRIS assay buffer was used. 100 µl I¹²⁵-labelled ANP solution, containing ~6000 cpm, to the 100 µl solutions of each standard. 100 µl of antiserum solution were added to each of the tubes except total counts and non-specific binding. Total counts, non-specific binding and zero binding were measured in triplicate and samples in duplicate. Incubation was for 19 - 24 hours at 4 ° C. Separation of bound and free fractions was by addition of 0.1% bovine γ globulin and 1 ml polyethylene glycol solution, centrifugation of the tubes for 15 mins at 600g and aspiration of the supernatant. The pellet containing the bound fraction was counted by gamma spectrometry (Packard Auto-gamma)

Cross reactivity of ANP antiserum with other vasoactive hormones

Standard curves were prepared as above but with identical serial dilutions (9.5 - 10 000 pg/ml) of standards of other vasoactive hormones - BNP, CNP, AVP, AII, and AI in place of r-ANP standard. Cross reactivity was calculated by first finding the displacement.

Displacement

$$= \% \text{ binding at 0 hormone concentration less } \% \text{ max. binding of peptide}$$

So, displacement

$$= B_0 / TC \quad - \% \text{ binding at max. concentration (10 000pg/ml)/TC}^*$$

* read from assay

For example, displacement of ANP

$$= B_0 / TC \quad - \% \text{ binding at max. concentration /TC}$$

$$= 38.5 \% - 6.8\%$$

$$= 31.7 \%$$

Cross reactivity was then calculated thus

Percentage of cross reactivity (CR)

$$= \frac{\% \text{ displacement of } x \text{ ng of cross reactant}}{\% \text{ displacement of } x \text{ ng of assay peptide}}$$

Intra-assay variation

Intra-assay variation was assessed by assaying several samples of the equine plasma pool within one assay

Interassay variation

Quality controls were used in all assays to assess interassay variation. The quality controls were made from a known standard to known dilutions of low, medium and high values designed to be equivalent in value to the results expected. One batch of horse samples was assayed twice and the values compared.

The inter - assay variation is estimated as :

$$\frac{\text{standard deviation}}{\text{Mean}} \times 100$$

Statistical Analyses

Data are presented as means \pm standard error . Students t-test was used to assess statistical significance of differences. Results were considered statistically significant when p was <0.05 .

7.3 Results

7.3.1. Validation of Radioimmunoassay

(i) Identification of suitable antiserum for the radioimmunoassay of equine ANP

Radioimmunoassay of equine samples using the Peninsula antiserum according to their own assay protocol, gave reasonable values for equine ANP and linear dilutions were obtained: four fold concentration, two fold concentration, and normal concentration gave values of 285 pg/ml, 143 pg/ml and 96 pg/ml respectively. However, dialysed plasma at four fold concentration gave a value of 142 pg/ml and a blank sample of assay buffer alone gave a value of 124pg/ml. It was concluded that there may be some cross reactivity with the antisera by equine plasma; other antisera were tried. Laboratory stock antiserum gave reasonable values for equine samples which diluted in linear fashion:- 47; 20 and 11 pg/ml at 4:1; 2:1 and 1:1 concentrations respectively (Figure 7 5) . The laboratory stock R67 antiserum was deemed suitable for further validation studies. These findings are summarised in Tables 7.3 and 7.4.

Table 7.3 Cross reactivity of Atrial Natriuretic Peptide Antisera with plasma proteins

Cross reactivity with equine plasma proteins

No cross reactivity

Peninsula**

Lab.stock 1988 R67**

Nichols Institute*

Extraction :

* Ethanol/second antibody;

** Methanol, SepPak/ PEG;

(ii) Optimum dilution of selected antiserum

Optimum binding of 30 - 40 % was seen at a working dilution of 1 : 20 000 and 1: 25 000 (assay dilution - in the assay tube - of 1: 60 000 and 1: 75 000). This was the range selected for the development of the standard curve (Figure 7.4)

(iii) Standard curves

Working dilutions of 1:10 000 and 1: 30 000 did not produce sigmoidal curves because of too much, or too little, binding. In other words, there was insufficient difference in percentage binding to show sensitivity. Working dilutions of 1:20 000 and 1:25 000 gave good sigmoidal curves with optimum sensitivity being obtained at a working dilution of 1:22 000 (assay dilution of 1:66 000). This curve is shown in Figure 7.4 .

Figure 7.4 Radioimmunoassay standard curve for ANP, 9.5 - 10 000 pg/ml using R67 antiserum at a working dilution of 1:22 000.

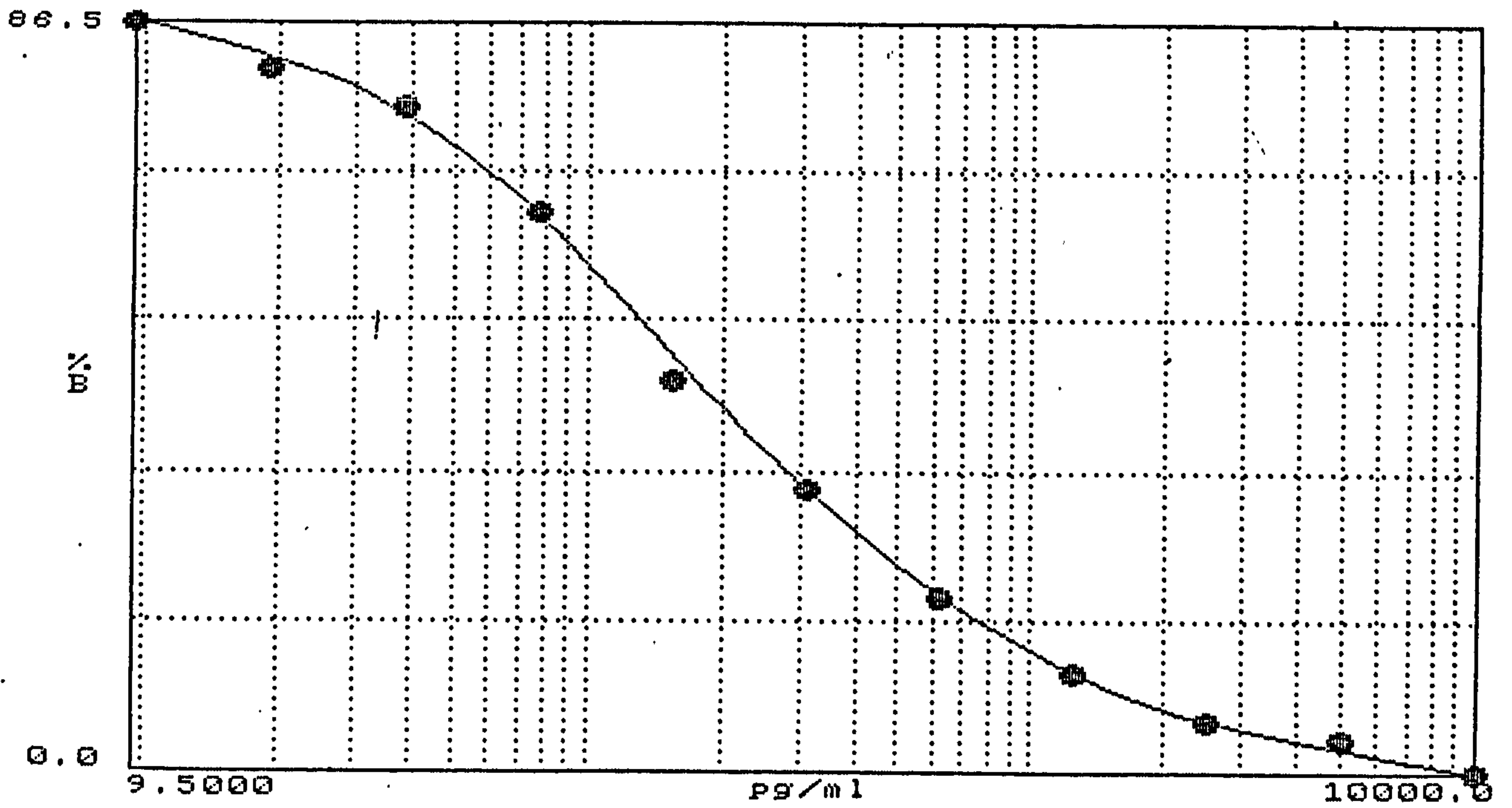
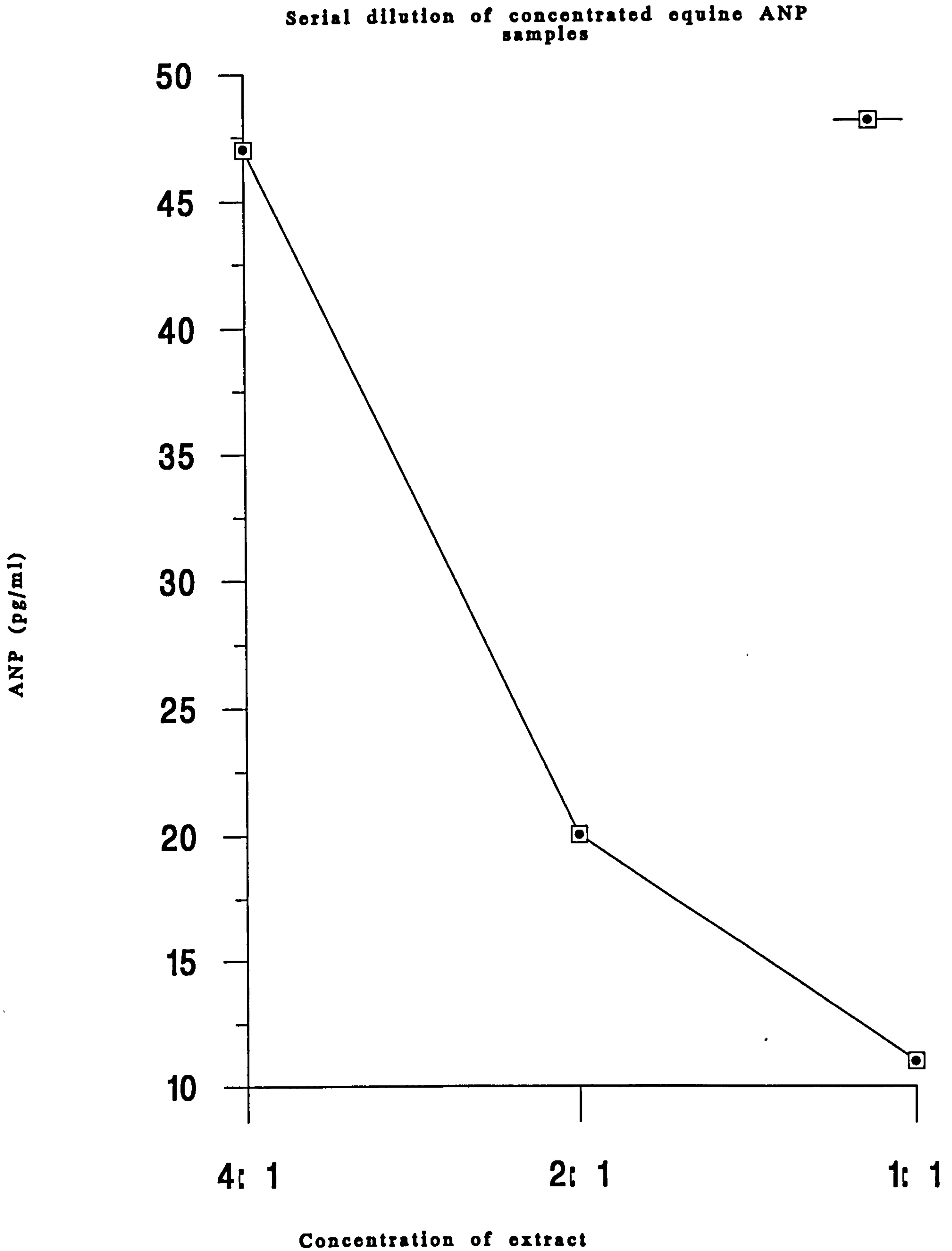


Figure 7.5 Serial dilution of concentrated extracts of equine Atrial Natriuretic Peptide, determined by radioimmunoassay using R67 antiserum.



(iv) Determination of Cross reactivity

Cross reactivity was calculated from the results of standard curves using ANP, BNP, CNP, AII and AVP as follows :-

First calculate displacement.

$$\text{Displacement} = B_0 / TC - \%B \text{ at max. standard}(10\ 000\text{pg/ml})$$

Therefore,

$$\text{ANP} \quad \text{Displacement} = 100 - 4 \quad = 96\%$$

$$\text{BNP} \quad \text{Displacement} = 100 - 88.3 \quad = 11\%$$

$$\text{CNP} \quad \text{Displacement} = 100 - 100 \quad = 0\%$$

$$\text{AVP} \quad \text{Displacement} = 39 - 37 \quad = 2\%$$

$$\text{AII} \quad \text{Displacement} = 37 - 39 \quad = 0\%$$

Percentage of cross reactivity (CR)

$$= \frac{\% \text{ displacement of } x \text{ ng of cross reactant}}{\% \text{ displacement of } x \text{ ng of assay peptide}}$$

Table 7.4 shows cross reactivity of R67 antiserum with other vasoactive hormones

Table 7.4 Cross reactivity of R67 antiserum

$$\text{Cross reactivity BNP (\%CR)} = \frac{11}{96} = 11\% \text{ at } 10\,000 \text{ pg/ml}$$

$$\text{Cross reactivity CNP (\%CR)} = \frac{0}{96} = 0\% \text{ at } 10\,000 \text{ pg/ml}$$

$$\text{Cross reactivity AII (\%CR)} = \frac{0}{96} = 0\% \text{ at } 10\,000 \text{ pg/ml}$$

$$\text{Cross reactivity AVP (\%CR)} = \frac{2}{96} = 2\% \text{ at } 10\,000 \text{ pg/ml}$$

If the cross reactivity of BNP is 11% at 10 000 pg/ml, and the cross reactivity of AVP is 2% at 10 000 pg/ml, then when measuring ANP at ranges of 10 - 200pg/ml the cross reactivity of BNP and AVP is very small and can be disregarded.

Table 7. 5 Reactivities of vasoactive hormones with selected ANP antiserum R67 at measured ranges of 10 -200 pg/ml

ANP antiserum (1988, R67)	ANP	100%
	BNP	0.2%
	CNP	<0.1%
	AII	<0.1%
	AVP	<0.1%

Recovery

Recovery varied. If extractions were performed by a technician the recovery was only 30% compared with more usual values of 87 % Low recovery reduced sensitivity in the assay but appropriate recoveries were calculated.

Intra-assay variation was calculated to be < 12% at four fold concentrations and < 25 % at two fold concentration. Variation is 1.2 pg/ml at low concentrations, 3.6 pg/ml at medium values and 7.2pg/ml at values above 60 pg/ml

Inter-assay variation varied with concentration. At four fold concentrations this was < 12 %, equivalent to approximately 1 pg/ml at usual concentrations

Sensitivity

The lowest detectable value was 6 pg/ml

7.3 2 Equine Plasma levels of Atrial Natriuretic Peptide

Plasma levels of ANP were determined in horses and ponies during summer months when at grass and also during winter months when the animals were stabled.

Results for individual animals, divided into either normal or chronic laminitic groups, are shown in the Tables 7.5 and 7.6 below. Group Mean values - for normal and chronic laminitic animals - are given each month and are shown \pm SE. Ranges are shown. The distribution of individual values is shown in Figures 7.5 and 7.6. Omissions in the data are usually because the animals suffered laminitis and were stabled. Under these circumstances they were not representative of their group and so were not included.

Table 7.5 Plasma ANP concentrations (pg/ml) for normal horses and ponies

Month	Jan	Mar	Mar	Apr	Jun	Jul	Aug	Aug	Sep	Sep	Sep	Oct	Nov
Date	21.1.9	8.3.95	10.3.94	30.4.94	12.6.94	17.7.94	14.8.94	21.8.94	28.8.94	4.9.94	11.9.9	16.10.94	19.11.9
	5										4		4
Albert	40	53	-	-		-	<6	-	26	13	7	103	95
Annabella	-	-	-	-		8	23	66	40	43	23	-	-
Cracker II	66	15	48	<6		<6	33	13	39	47	43	18	27
Despina	47	11	-	<6		<6	22	53	27	42	43	95	17
Dolly	42	54	-	-		-	-	-	-	-	-	-	-
Jasper	43	-	-	18		<6	<6	50	40	10	26	66	70
Marron	80	30	89	9		9	49	22	44	18	42	79	53
M. Gray	76	11	-	11		-	-	-	-	-	-	-	-
Melody	-	-	-	-		<6	<6	16	39	24	22	-	-
Selluci	60	-	-	<6		23	9	-	20	29	24	81	61
Snowflake	-	-	13	-		17	21	24	20	23	27	95	-
Tango	43	<6	-	-		-	-	-	-	-	16	-	<6
Tess*	-		8			32	24	18	31	22		25	-
Group mean	55	26	40	9		13	20	33	33	27	27	70	47
S. E	5	8	19	2		3	4	7	3	4	4	11	12
Range	40-80	6-54	8-89	6-18		6-32	6-49	13-66	20-44	10-47	7-43	18-103	6-95
No tests	9	7	4	6		9	10	8	10	10	7	8	7

Tess* before the first attack of laminitis.

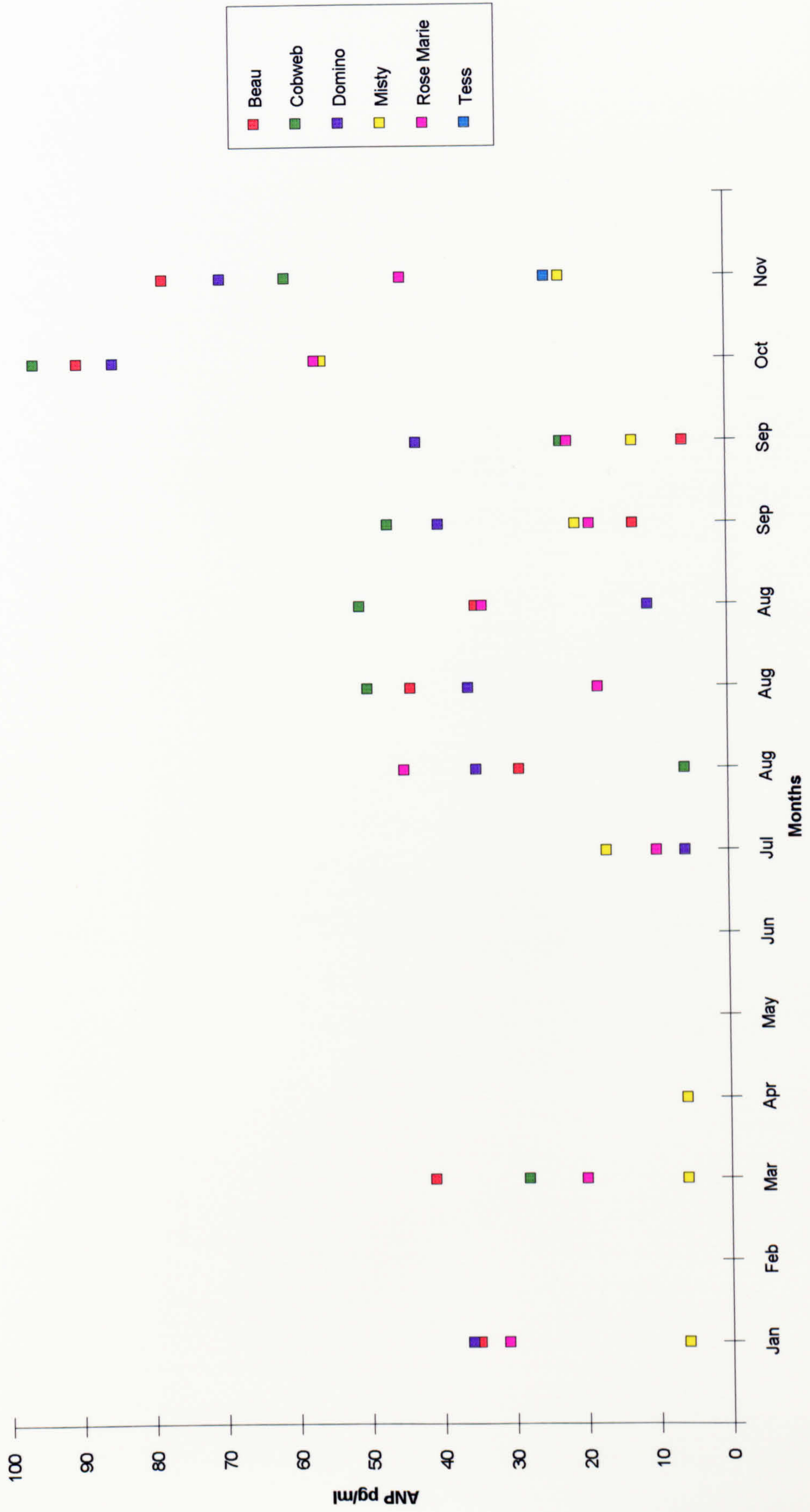
Table 7.6 Plasma ANP values for chronically laminitic animals (pg/ml)

Month	Jan	Feb	Mar	Apr	Jun	Jul	Aug	Aug	Aug	Sep	Sep	Oct	Nov
Date	21.1.95	-	8.3.95	30.4.94	-	17.7.94	14.8.94	21.8.94	28.8.94	4.9.94	11.9.94	16.10.94	19.11.94
Beau	35	-	41	<6	-	<6	29	44	35	13	<6	90	78
Cobweb	36	-	28	<6	-	10	<6	50	51	47	23	96	61
Domino	36	-	<6	<6	-	<6	35	36	11	40	43	85	70
Misty	<6	-	<6	<6	-	17	-	-	-	21	13	56	23
Rose Marie	31	-	20	-	-	10	45	18	34	19	22	57	45
Tess	-	-	-	-	-	-	-	-	-	-	-	-	25
Group Mean	29		20	<6		10	29	31	34	28	24	77	50
SE	6		7	0		2	8	8	6	7	8	8	9
Range	<6-36		<6-41	0		<6-17	<6-45	<6-50	11-51	13-47	6-43	56-96	23-78
No tests	5		5	4		5	4	5	5	5	4	5	6

Figure 7.6 Seasonal variation in plasma ANP in normal horses and ponies.

Figure 7.7 Seasonal variation in plasma ANP in chronically laminitic horses and ponies.

Seasonal plasma ANP (pg/ml) of chronic laminitic ponies



Comparison of plasma ANP of normal and chronically laminitic animals

(i) Normal horses and ponies

The group mean of plasma ANP concentrations in normal horses during winter months (Oct - Apr) was 44 ± 5 pg/ml (n = 13; number of tests = 41) compared with 30 ± 3 pg/ml (n = 13) for normal horses during summer months (May -Sept) (number of tests = 64)

There is a statistical difference between summer and winter values of ANP in normal equines. $p < 0.015$

(ii) Chronically laminitic animals

The group mean of plasma ANP concentrations of chronically laminitic horses during winter months (Oct - Apr) was 38 ± 6 pg/ml (n = 6; number of tests =25) compared with that of the same animals during summer months (May -Sept) of 26 ± 3 pg/ml (n = 6; number of tests = 28).

There is no statistical difference between summer and winter values of ANP in chronically laminitic equines. $p = 0.06$

Comparison of plasma ANP concentrations in normal and chronically laminitic horse and ponies.

Seasonal differences are compared between normal and chronically laminitic horses and ponies are compared in Table 7.7 and Figure 7.8

Table 7.7 Comparison of seasonal differences in plasma ANP concentrations (pg/ml) in normal and chronically laminitic equines

	Winter	Summer	All year
Normal , n = 13;	43 ± 5	30 ± 3	35 ± 3
range	(<6 - 103)	(<6 - 94)	(< 6 -103)
number of tests	41 tests	64 tests	105 tests
Chronic laminitic; n = 6	38 ± 6	26 ± 3	32 ± 2
number of tests			52
range	(<6 - 96)	(<6 -51)	(< 6 -96)

There is no statistical difference between normal and chronic laminitic groups.

Equine ANP in plasma

Normal equine ANP is 34 ± 2 pg/ml

(range < 6 - 103 pg/ml)

n = 19; number of tests = 158

7.3.3 Plasma ANP during acute equine laminitis

Grass induced acute laminitis in ponies

Changes in plasma ANP during acute grass induced laminitis are shown in Table 7.8 and these results are compared with normal values for that month. Changes in ANP during the course of treated acute laminitis are shown in Tables 7.9 and 7.10.

Table 7.8 Comparison of changes in plasma ANP concentrations during acute grass induced laminitis with mean values for normal horses in the same month.

	Acute stage (pg/ml) before treatment	Group Mean for normal horses for the same month (pg/ml)
<i>Beau 1</i>	86	10 ± 2 (6 - 17)
<i>Beau 2</i>	37	10 ± 2 (6 - 17)
<i>Misty</i>	15	10 ± 2 (6 - 17)
<i>Beau 3</i>	32	10 ± 2 (6 - 17)

Table 7.9 Plasma ANP during grass induced acute laminitis when treated with nitric oxide donors compared with normal values for the same horse in preceding and subsequent months.(Beau 1)

<i>Beau 1</i>			
Date	ANP (pg/ml)	Comments	Treatment
16.3.94	37	At grass	None
17.4.94	46	Stabled	None
30.4.94	37		None
15.5.94	40	To grass	None
22.5.94	50	Hyperlipidaemia	None
25.5.94	40	Lush grazing	None
30.5.94	70	Lush grazing	None
2.6.94	86	Acute laminitis	Arginine iv, GTN
3.6.94	43	Stabled	GTN
4.6.94	50	„	GTN
5.6.94	40	low BP 97/53	End GTN
6.6.94	63	At grass	None
7.6.94	20	At grass	None
9.6.94	20	At grass	None
12.6.94	20	At grass	None
15.6.94	40	At grass	None
19.6.94	37	At grass	None
26.6.94	37	Acute laminitis	GTN
2.7.94	23	Stabled	GTN
9.7.94	23	Stabled	GTN
17.7.94	43	Stabled	GTN
24.7.94	37	Stabled	GTN
26.7.94	43	Stabled	GTN
29.7.94	40	Salt appetite	↓GTN
31.7.94	24	Normal	↓GTN
7.8.94	63	At grass	None
14.8.94	24	At grass	None
21.8.94	50	At grass	None
28.8.94	46	At grass	None
2.9.94	25	At grass	None
4.9.94	50	At grass	None
11.9.94	13	At grass	None

Key : GTN - transdermal glyceryl trinitrate; PBZ - phenylbutazone; FXN - flunixin meglumine; ACP - acepromazine. ↑ - increase dose, ↑↑ - greatly increase dose, ↓ - decrease dose, ↓↓ - greatly decrease dose. Full details of therapeutic regimes are given in Chapter 5 Appendix III.

Table 7.10- Plasma ANP during grass induced acute laminitis when treated with nitric oxide donors compared with normal values for the same horse in preceding and subsequent months. (*Misty*)

<i>Misty</i>			
Date	ANP (pg/ml)	Comments	Treatment
30.5.94	31	Normal	None
5.6.94	16	At grass	None
12.6.94	23	Collision	Oral PBZ 1g/day
19.6.94	30	Stabled	Oral PBZ 1g/day
24.6.94	19	Stabled	Oral PBZ 1g/day
2.7.94	5	At grass	Oral PBZ 1g/day
9.7.94	15	Acute laminitis	Oral PBZ 1g/day
10.7.94	< 6	Stabled	GTN patches, Oral PBZ 1g/day
11.7.94	< 6	„	GTN patches, Oral PBZ 1g/day
12.7.94	12	„	↓ GTN patches, Oral PBZ 1g/day
13.7.94	5	„	GTN, Oral PBZ 1g/day
14.7.94	< 6	„	GTN, Oral PBZ 1g/day
15.7.94	24	„	↓ GTN, Oral PBZ 1g/day
17.7.94	21	„	GTN, Oral PBZ 1g/day
26.7.94	< 6	„	↓ GTN, Oral PBZ 1g/day
29.7.94	< 6	„	End GTN, Oral PBZ 1g/day
31.7.94	11		Oral PBZ 1g/day
7.8.94	15	At grass	Oral PBZ 1g/day
14.8.94	28		Oral PBZ 1g/day
21.8.94	32	Stabled	Oral PBZ 1g/day
28.8.94	15	„	Oral PBZ 1g/day
4.9.94	9	„	Oral PBZ 1g/day
11.9.94	13	„	Oral PBZ 1g/day

Key : GTN - transdermal glyceryl trinitrate; PBZ - phenylbutazone; FXN - flunixin meglumine; ACP - acepromazine. ↑ - increase dose, ↑↑ - greatly increase dose, ↓ - decrease dose, ↓↓ - greatly decrease dose. Full details of therapeutic regimes are given in Chapter 5 and Appendix II.

The cases above correspond to Case references Beau 1 (Case Report 1) and Misty 1 (Case Report 2) in Chapter 5 and Appendix II. Full details of blood pressure are shown in Chapter 3 and Appendix II; and other endocrine analyses are shown in Chapters 6 and 8. Serum electrolytes are reported in Chapter 9.

Post partum acute laminitis.

Changes in plasma ANP during the course of post partum acute laminitis of endotoxic origin are shown in Table 7. 11. Full details of blood pressure are shown in Chapter 3 and Appendix II; and other endocrine analyses are shown in Chapters 6 and 8. Serum electrolytes are reported in Chapter 9 and treatment details are given in Appendix III.

Table 7 11 Plasma ANP concentrations (pg/ml) during acute *post partum* laminitis compared with values for the same horse before the onset of the disease.

<i>Biscuit</i>				
Date	Time	(pg/ml)	Comments	Treatment
21.1.95	11.30	-ND	Normal basal	None
20.4.95	11.30	-ND	Normal basal	None
23.5.95	11.30	15	Acute onset, mild, Obel 2	GTN
24.5.95	7.00	27	Acute, severe, Obel 4	GTN , FXN
24.5.95	11.30	11	Some improvement. Salt appetite	GTN, PBZ, ↑FXN, ACP
24.5.95	19.00	-ND	Some improvement. Salt appetite	↓GTN, PBZ, FXN, ACP
24.5.95	21.00	15	Still Obel 4. Salt appetite	GTN,PBZ, FXN, ACP
25.5.95	19.00	20	Salt appetite	↓GTN,PBZ, FXN, ACP
26.5.95	19.00	22	Salt appetite	GTN,↓PBZ, FXN, ACP
27.5.95	19.00	26	Salt appetite	GTN, PBZ, FXN, ACP
28.5.95	19.00	10	Salt appetite	GTN, PBZ, FXN, ACP
29.5.95	19.00	23	Salt appetite	GTN, PBZ, FXN, ACP
30.5.95	19.00	11	Some improvement, Obel 3	↓GTN, PBZ, FXN, ACP
31.5.95	19.00	14	No salt appetite	Stop GTN, PBZ, FXN, ACP
1.6.95	19.00	16	Some improvement, Obel 3	PBZ, FXN, ACP
2.6.95	19.00	18	„	PBZ, FXN, ACP
3.6.95	19.00	26	„	PBZ, FXN, ACP
4.6.95	19.00	24	„	PBZ, oral FXN, ACP
5.6.95	19.00	13	„	PBZ, oral FXN, ACP
12.6.95	19.00	-ND	„	PBZ, oral FXN, ACP
14.6.95	19.00	-ND	Obel 4 'Sinking' - bleeding coronary band	↑PBZ, ↑FXN, ACP
19.6.95	19.00	-ND	Some improvement	↑PBZ, ↑FXN, ACP
11.7.95	19.00	-ND	'Sinking'	↑↑PBZ, ↑↑FXN, ACP
12.7.95	19.00	-ND	'Sinking' - mare destroyed	↑↑PBZ,↑↑FXN,ACP

Key : GTN - transdermal glyceryl trinitrate; PBZ - phenylbutazone; FXN - flunixin meglumine; ACP - acepromazine. ↑ - increase dose, ↑↑ - greatly increase dose, ↓ - decrease dose, ↓↓ - greatly decrease dose. Full details of therapeutic regimes are given in Chapter 5 and Appendix IV

Clinical cases of acute laminitis of different aetiologies.

Clinical cases were of the following aetiologies:

grass induced - Cheeky, Rambo, Glencora;
 septicaemia - Isadora;
 grain overload - Kerry;
 pituitary adenoma - Rainer
 chronic laminitis - Jubilee

The plasma ANP concentrations of clinical cases over the course of the disease are shown in Table 7.12. Plasma ANP were within normal ranges but decreased during acute stage.

Table 7.12 Plasma ANP concentrations (pg/ml) of clinical cases of acute laminitis

Name /Type	Date	(pg/ml)	Treatment*
<i>Before onset</i>			
Isadora	10.4.93	50	Stabled
<i>Day of AL attack</i>			
Isadora	10.5.93	10	Grass/Stabled,PBZ, ACP
Cheeky	23.3.93	5	At grass, ACP, PBZ
Rambo	18.5.93	8	At grass, PBZ
	Mean	8 ± 1	
<i>10 days after AL</i>			
Cheeky	24.4.93	24	Grass/stabled, PBZ, ACP
Cheeky	26.3.93	28	Grass/stabled, PBZ, ACP
Kerry	16.5.93	33	Grass/stabled, PBZ, ACP
Glencora	11.5.93	29	Stabled PBZ/ACP
	Mean	29 ± 2	
<i>Chronic laminitis</i>			
Rainer	5.6.93	16	Stabled
Jubilee	19.5.93	12	Stabled, PBZ
	Mean	14 ± 1	

Key : AL acute laminitis; PBZ - phenylbutazone; FXN - flunixin meglumine; ACP - acepromazine. ↑ - increase dose, ↑↑ - greatly increase dose, ↓ - decrease dose, ↓↓ - greatly decrease dose. * standard doses/ course of PBZ and ACP as directed by the clinician.

7.4 Discussion

The antiserum R67 gave satisfactory and reliable results at a working dilution of 1: 22 000 to produce a standard curve and to assay samples. R67 had low cross reactivity with similar peptide hormones such as BNP or CNP and produced results in the normal accepted physiological ranges for ANP with accurate results for quality controls of 100, 500 and 2000 pg/ml.

Mean equine plasma values of 34 ± 2 pg/ml (range 6 - 103 pg/ml) in this study are similar to human plasma ANP values of 10 - 60 pg/ml (Richards, 1987; Richards *et al.*, 1987) and are nearly identical to human values obtained by Cody *et al.*, (1986) of a mean of 37 pg/ml (range 6 - 93 pg/ml). Human samples show considerable variation depending on laboratory techniques, methods of extraction, assay procedures (e.g. acidification of samples gives a two fold increase in values), specificity of antisera and dietary sodium status. The range of normal human plasma samples in different laboratories using the same extraction methods varies between , 19 - 185 pg/ml (Marumo and Ando, 1990) and 231 ± 37 pg/ml (Inagami *et al.*, 1987; Mulrow and Schrier, 1987). Anderson and Bloom (1985) review the variations of ANP results in different laboratories.

Equine values are lower than values of rat plasma ANP at 101 ± 10 pg/ml (Inagami *et al.*, 1987) but higher than equine results of 18 ± 1 pg/ml in Standardbreds and 15 ± 2 pg/ml in Finnhorses (McKeever *et al.*, 1991 a, b; Kokkonen, 1993; Kokkonen, 1995). Equine atrial natriuretic peptide was determined in plasma by McKeever *et al.*, (1991a and b) and related to both volume loading and exercise (Kokkonen, 1993). The above studies used Peninsula antiserum without validation of the assay.

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Normal equine plasma ANP basal values for horses and ponies had to be established under controlled conditions. The previous reports (McKeever *et al.*, 1991 a, b; Kokkonen, 1993; Kokkonen, 1995) had not established normal values with reference to dietary and electrolyte status of animals but used animals as their own controls for physiology tests rather than establish normal values. The present study revealed seasonal differences in ANP; lower values in summer months when animals were kept at grass and values nearer usual human values when animals were stabled. Such seasonal variations were similar to those seen for AII (Chapter 6). The parallel relationship of AII and ANP is perhaps surprising since these hormones have been considered to be mutually antagonistic. Intuitively, if sodium is to be conserved then AII would be raised and ANP decreased whilst if sodium is to be excreted then AII would be low and ANP high. It is rather surprising to find that both hormones apparently increased and decreased in parallel (Figures 7.9 and 7.10 (ANP) and 7.11 (AII)). Full details of AII results are given in Chapter 6.

Figure 7.9 The seasonal relationship of mean equine plasma atrial natriuretic peptide (ANP) of normal ponies.

Seasonal variation of plasma atrial natriuretic peptide (ANP) of normal ponies

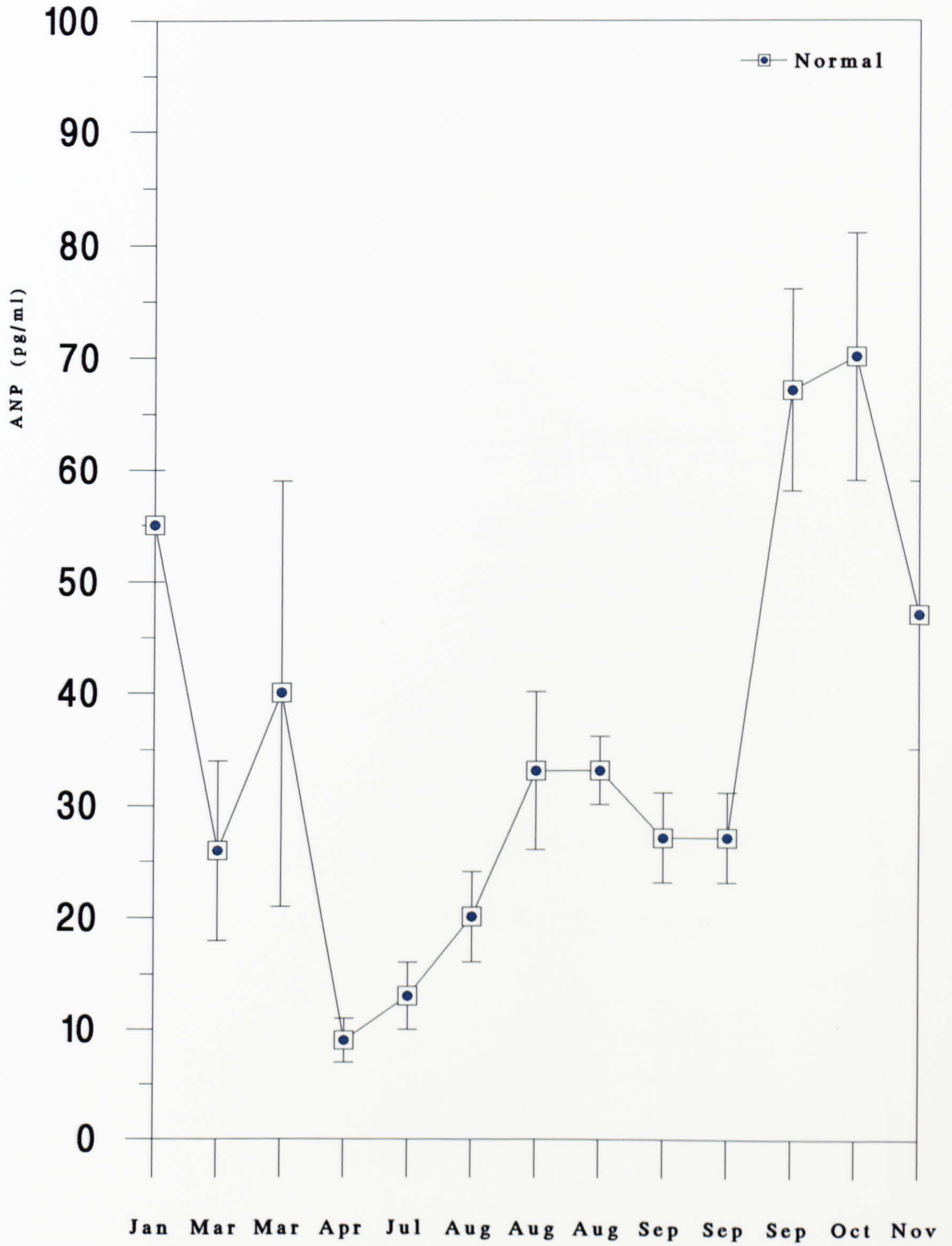


Figure 7.10 The seasonal relationship of mean quine plasma and atrial natriuretic peptide (ANP) of chronically laminitic ponies.

Seasonal variation in plasma atrial natriuretic peptide (ANP) of chronically laminitic ponies

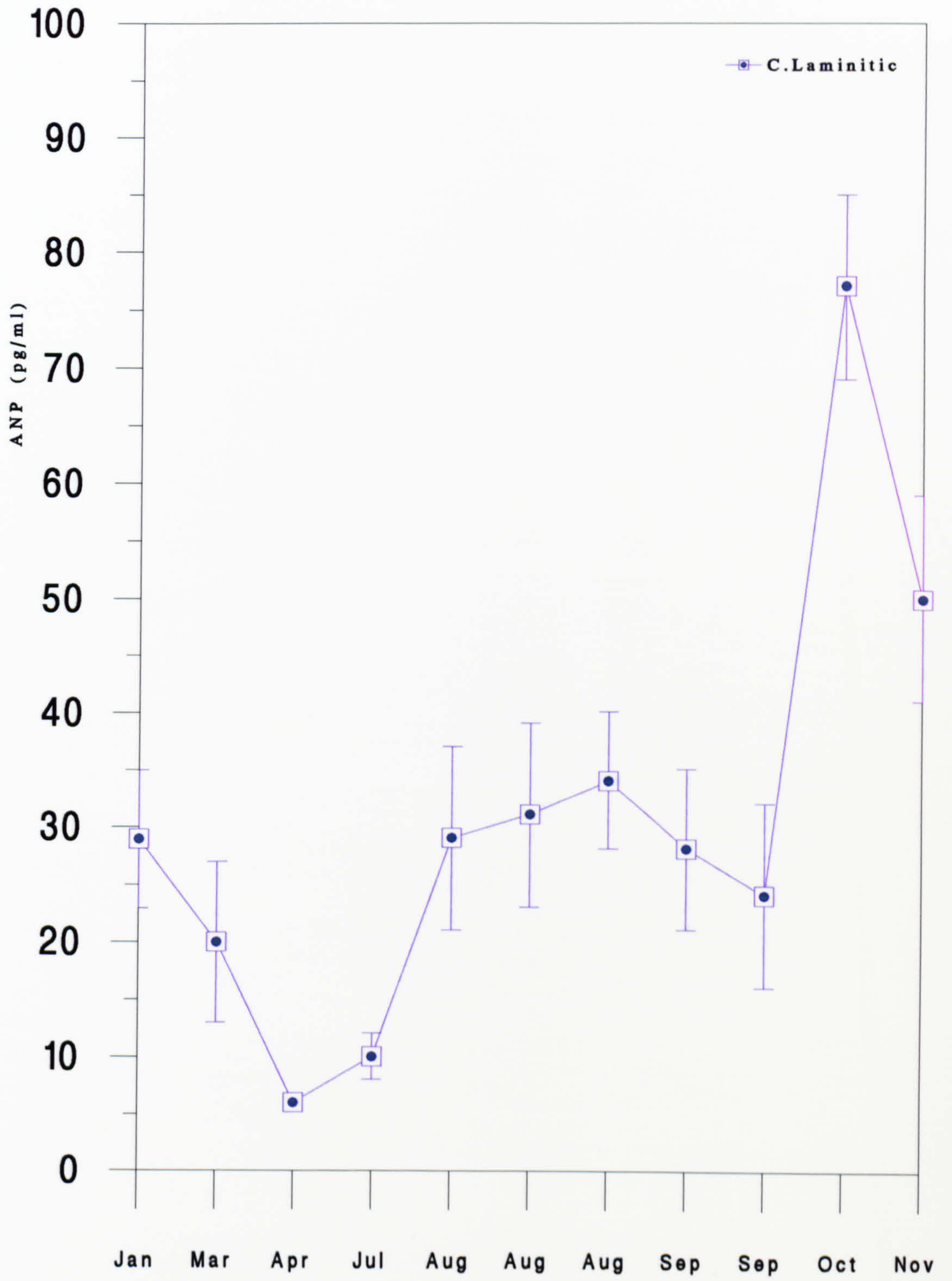
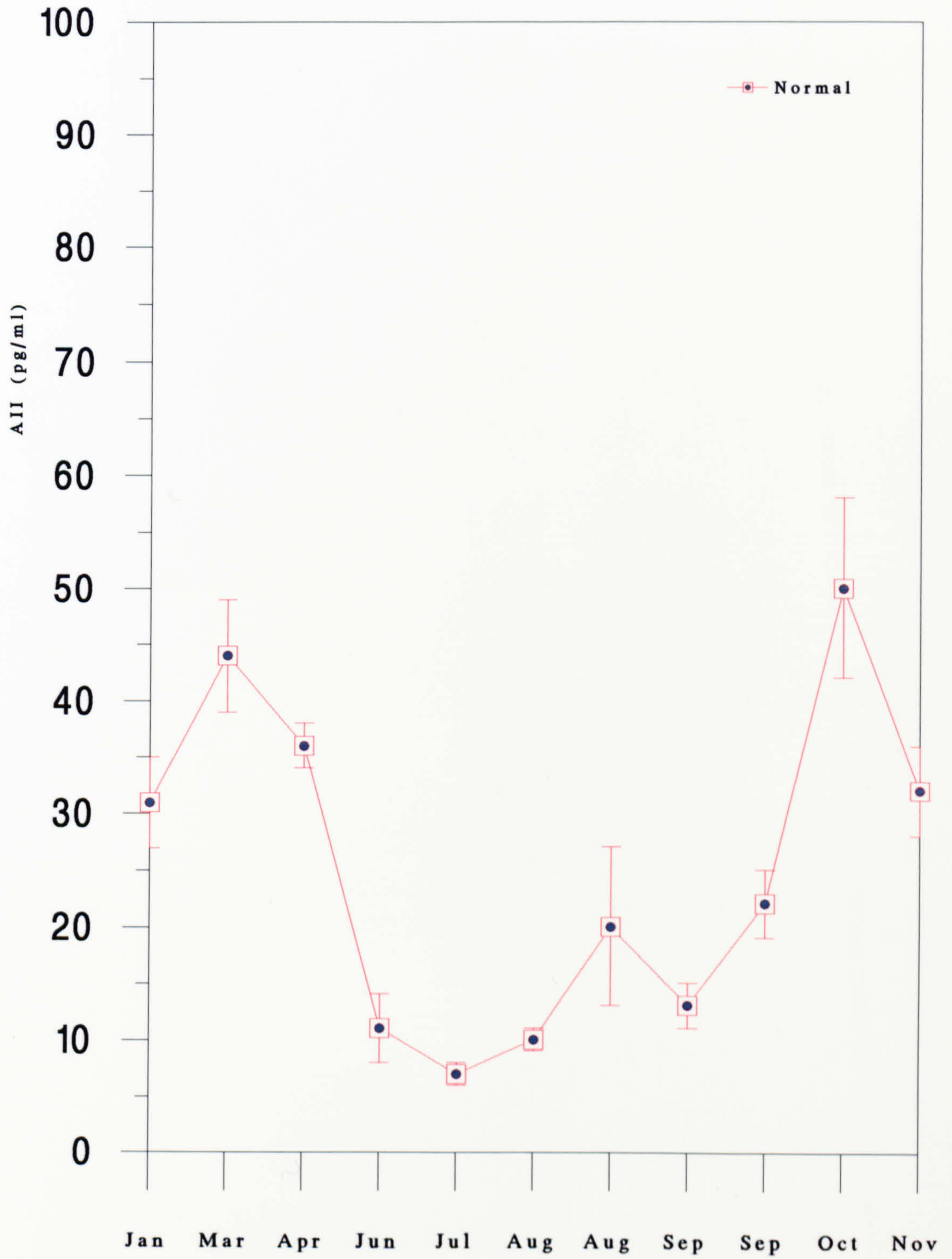


Figure 7.11 The seasonal relationship of mean equine plasma angiotensin II (AII) of normal ponies for comparison with previous two figures, 7.9 and 7.10.

Seasonal variation in plasma angiotensin II of normal ponies



An initial observation is to suspect that the handling of the samples was different in summer months and that there had been some degradation of peptide hormones in the samples during warm weather. However, all samples were meticulously kept on ice at all times and samples that were taken as 'individual' collections showed no difference in results. Similarly, there was no difference between the first animals to be sampled and the last. It is remotely possible, but unlikely, that reduced activity of the inhibitor solutions was responsible for reduced hormone levels but two different batches of Aprotinin were used in the summer period so this is also unlikely

Having rejected technical reasons for seasonal changes, then other causes must be considered. There are many putative factors that could regulate ANP and there are numerous reasons for lower circulating ANP. Synthesis of ANP could be reduced by low blood pressure or by humoral factors like glucocorticoids (Tonolo *et al.*, 1988; Kenyon and Morton 1994). If ANP synthesis is normal, low circulating levels of ANP could reflect either increased binding to receptors, especially ANP_C; increased clearance and/or upregulation of enzymatic breakdown by endopeptidases such as NEP (Connell and Jardine, 1990). Such mechanisms are at present beyond the scope of this study and more obvious regulatory factors should be considered first.

Regression analyses of plasma ANP and AII in normal and laminitic groups suggests that there is a relationship between the two hormones but that they do not oppose each other in the way one would have expected from data on infusion of the respective hormones (Wilkins, 1990; Pratt and Dzau 1993). It appears that when plasma AII increases, ANP is also increased. This is presumably a normal physiological response to external stimuli. There appears to be more variation in the regression analysis of the chronically laminitic group, suggesting perhaps that their regulatory mechanisms to counter external stimuli are not as tightly controlled as the normal group.

Figure 7.12 Regression analysis of plasma ANP and AII in normal ponies

ANP/AII values for Normal Animals

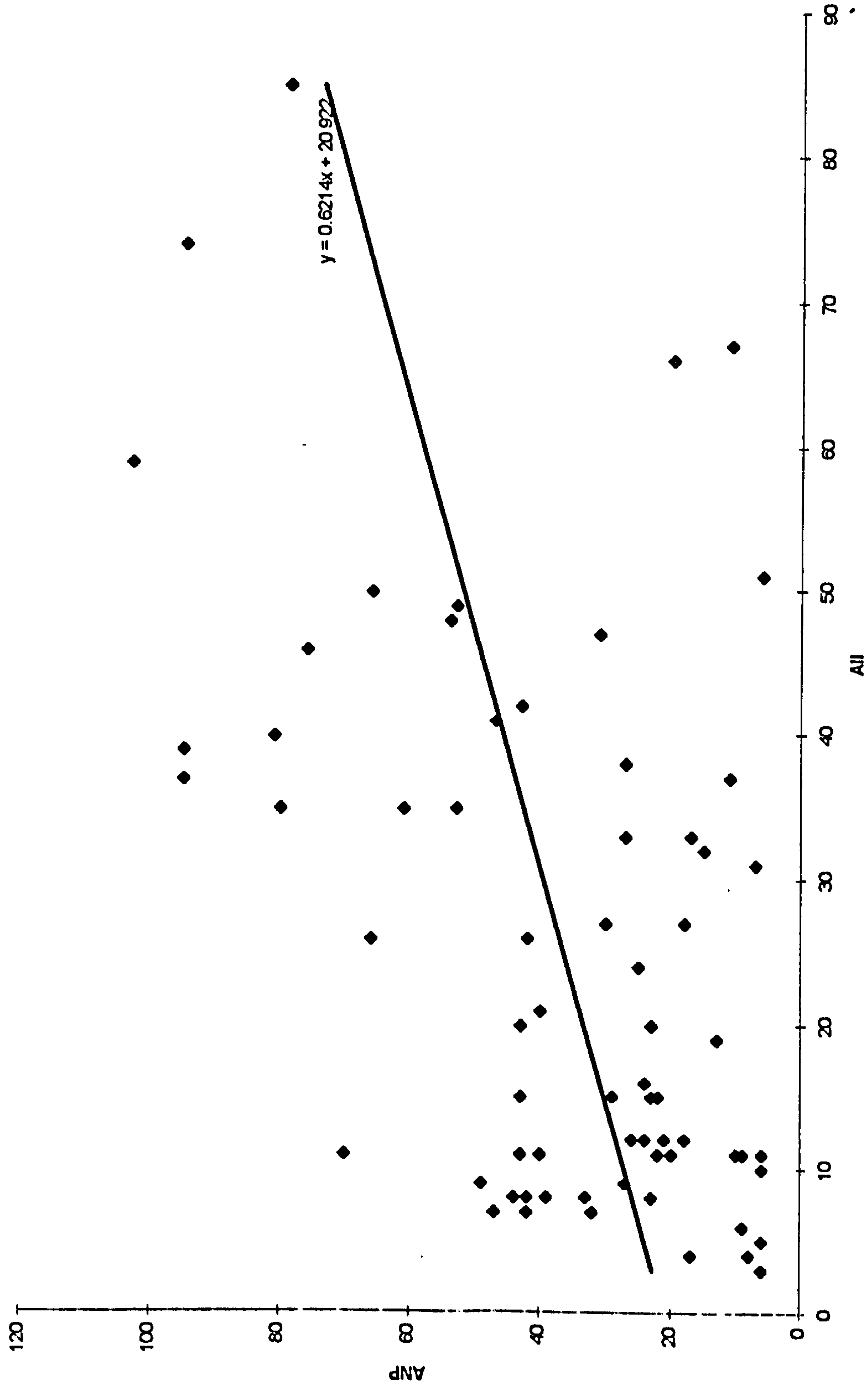
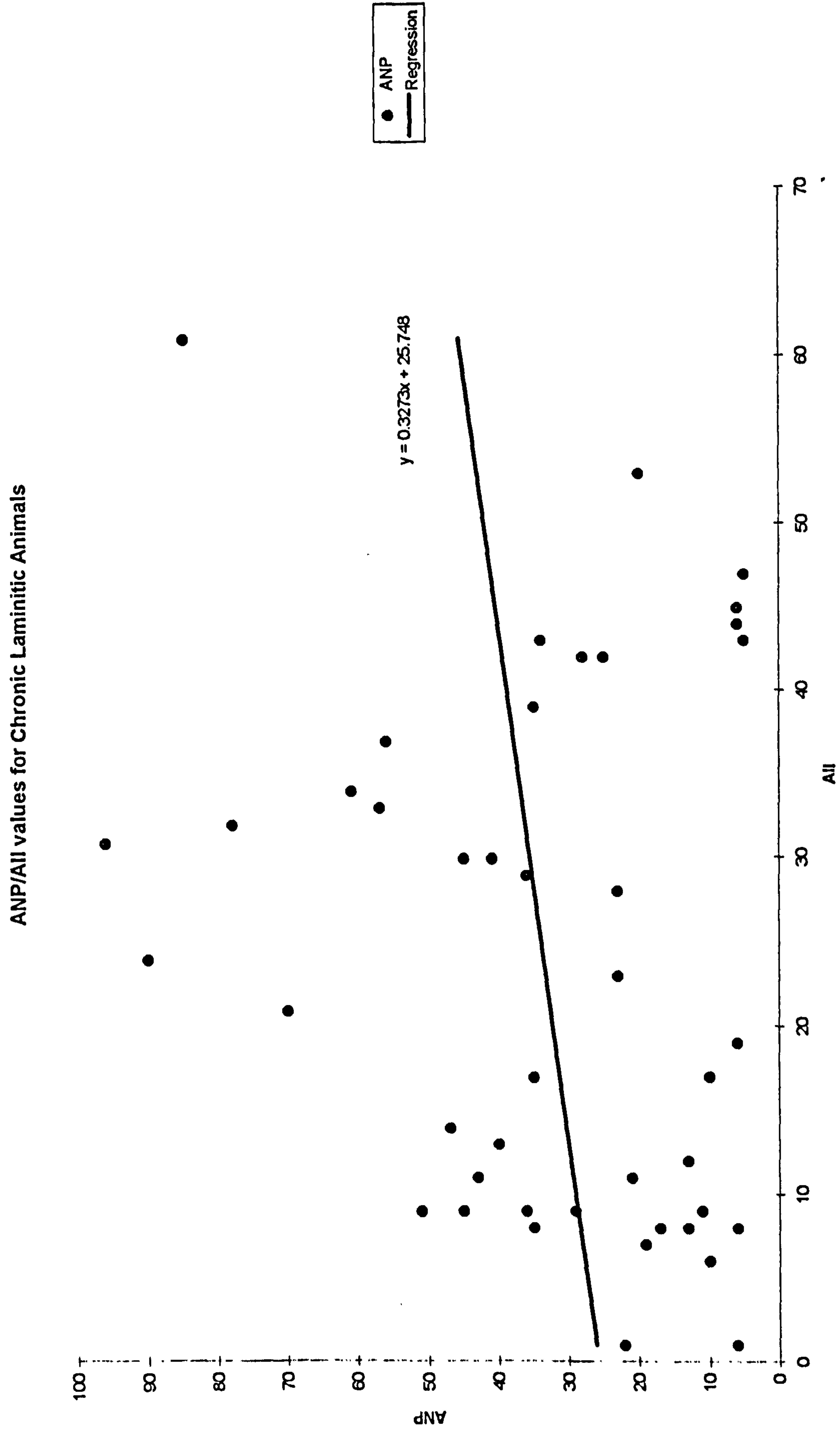


Figure 7.13 Regression analysis of plasma ANP and AII in chronically laminitic ponies



What could be suppressing both AII and ANP? The most obvious reason for seasonal changes is that both AII and ANP are related to diet, particularly dietary electrolytes which are known to regulate both AII and ANP (Corvol and Ménard, 1990). Dietary ingestion of sodium / potassium promotes alterations in hormones responsible of the maintenance of plasma levels of electrolytes. Low dietary sodium suppresses ANP mRNA levels in atrial tissue (Takayanagi, 1985). Expression of ANP is regulated at mRNA level by mineralocorticoids, glucocorticoids, thyroid hormone, α and β agonists, cholinergic agonists and unidentified pituitary factors (Zamir *et al.*, 1987). Herbivores ingest large amounts of potassium in grass *vide supra* (Chapter 6) alongside small amounts of sodium. On average, the horse at grass ingests ten times more elemental potassium than a human each day (Clarke *et al.*, 1988). High levels of ingested potassium would inhibit renin release and activity, therefore promoting excretion of both sodium and potassium. Aldosterone secretion would increase to conserve sodium. ANP dependent natriuresis may not occur under these circumstances. Plasma sodium variation can be tolerated to a greater degree than potassium variation so excretion of excess potassium is crucial - these excretory processes 'drive' the physiological system. Excess potassium would be excreted by the direct action of aldosterone. Aldosterone is known to increase during both sodium deficiency and potassium loading *in vitro* and *in vivo* (Blair West *et al.*, 1963; Ganong *et al.*, 1966; Kenyon *et al.*, 1978 a; 1978 b; Connell *et al.*, 1988), and there is a particularly high production of aldosterone during sodium deficiency and potassium loading. It is likely therefore that the horse could be producing seasonally different plasma aldosterone levels in response to varying dietary intake of electrolytes. Direct activation of aldosterone, independent of the renin angiotensin system, would act to conserve available sodium and upregulate AII receptors thereby increasing the sensitivity of the adrenal cortex and the kidney to circulatory AII. As infused ANP totally abolishes the aldosterone response to AII, it is unlikely that horses would maintain high plasma ANP since this would inhibit aldosterone and then excess potassium would not be excreted.

This presentation of low renin/ AII but possibly higher levels of aldosterone is akin to Conn's syndrome in which adrenocortical mineralocorticoids are secreted abnormally in excess (Conn, 1955). Clinically, this usually results in hypertension because sodium is inappropriately conserved. However, in the case of herbivores, who ingest little sodium when at grass, the adrenal mechanism of potassium loss and sodium conservation is ideal. Aldosterone synthesis is regulated primarily by the renin angiotensin system but is independently regulated by potassium; only a 0.2 mEq increase in plasma potassium briskly increases aldosterone secretion (Corvol and Ménard, 1990). There is a direct relationship between potassium load and aldosterone secretion in both man (Corvol and Ménard, 1990) and the horse (Clarke, 1982). It has been suggested that a potassium load can directly inhibit the secretion of renin within the kidney. Plasma renin activity is known to be low in the horse - about 50 % of human kidney renin activity (Gutherie *et al.*, 1980) and, if the horse receives a sodium diet equivalent to man, then the plasma aldosterone ranges from 140 - 830 pmol/l. As Gutherie *et al.*, (1980) declare

"a precise comparison is difficult because the state of the sodium balance in the horse was unknown ", and of course it is a notoriously difficult physiological parameter to monitor free grazing animals. The same group assert that

"these levels of aldosterone, relatively high compared with the low plasma renin activities observed suggest that the horse adrenal might be sensitive to the trophic effects of Angiotensin II"

Equine aldosterone has been measured (Zolovick *et al.*, 1966; James *et al.*, 1970; Clarke *et al.*, 1982) but these studies relate to housed animals which were fed concentrate rations which contain more sodium and less potassium than in grass, and cannot be directly compared.

The mechanisms of action of potassium on the secretion of aldosterone are still unclear - does potassium work by affecting synthesis in the zona glomerulosa through the

cholesterol/pregenalone pathway or by alteration of the corticosterone/cortisol processing? Since AII is not involved, is the excretion of the potassium load mediated directly by the adrenal or could pituitary control be involved? If horses regulate their potassium load by direct adrenal action, then horses at grass have, in a sense, developed their non-pathological equivalent to Conn's syndrome as an evolutionary strategy to counter seasonal dietary electrolyte changes. Increased NEP activity is regulated through corticosteroids (Leckie, 1987) and if corticosteroids are seasonally increased then this mechanism could be responsible for the low summer values of ANP seen in this study.

Chronic laminitis cases are slightly hypotensive (Chapter 3) and left ventricular hypertrophy has been recorded in chronically laminitic ponies (Sprouse *et al.*, 1987) which is often indicative of ventricular recruitment of ANP synthesis (Kohno *et al.*, 1987). It was considered possible, at the outset of the study that ANP could be reducing blood pressure in chronic laminitics. The present study revealed no differences in plasma ANP between normal and chronically laminitic groups. No differences in blood pressure or AII were seen in the two groups. The slight seasonal increase in heart rate was unlikely to be sufficient to alter secretion of ANP. The lack of differences in plasma ANP is consistent with equivalent values for both groups in blood pressure and AII. These results suggest that any physiological differences between the groups are mechanical (within the hoof) or local (within the microvascular circulation) rather than systemic. It may be that the chronically laminitic ponies are normal under normal conditions but that their responses to any abnormal environmental or physiological challenge would be abnormal. They may have a narrow range of normal responses and be more prone to adverse effects of say vasoconstrictor hormones, or local hypoxia, than normal animals and so be more at risk of pathophysiologies. Chronic laminitics may seem normal when conditions are favourable but they may 'be walking a tight-rope' and they may fall into the acute stage with the mildest challenge. Mineralocorticoids potentiate the effect of vasoconstrictors (Kenyon and Morton, 1994) including paracrine vasopressor hormones like endothelin (Doherty 1992). If glucocorticoids are also elevated, then these potentiate the effect of noradrenaline and inhibit the actions of vasodilatory hormones such as prostacyclin and nitric oxide (Kenyon and Morton, 1994). If this is so then horses at grass are 'primed' to react to vasoconstriction and are highly susceptible to react to any factors that they encounter.

During renal disease α -ANP varies between 80 - 380 pg/ml and in coronary heart failure (CHF) ANP can rise to 155 pg/ml (Cody *et al.*, 1986). Gross changes, akin to those seen in CHF or renal failure were not observed in acute laminitis cases. This suggests that renal and cardiac functions were relatively normal during disease. During untreated

acute laminitis plasma ANP was elevated and this was consistent with the observed increases in blood pressure and heart rate. It is likely that ANP is released in response to atrial stretch therefore. The increases were slight and were not sufficient to increase haematocrit / PCV (see Chapter 9). Increased ANP was also observed during pain after accidental injury (Misty - Table 7.11). This suggests that pain and CNS mediated increases in blood pressure stimulated the release of ANP. The highest reading taken from Beau was during acute laminitis when he also received intravenous therapy of l-arginine in 0.9% saline. In this instance, not only was the pony experiencing pain and increased blood pressure but also had increased plasma volume which is known to increase plasma ANP (Yamaji *et al.*, 1985; Lang, 1985) The *post partum* case of laminitis (Table 7.12) was hypertensive for weeks afterwards but did not show particularly elevated levels of ANP. Of course, this mare was treated with considerable doses of α -agonists which are known to suppress ANP, and PBZ which will alleviate pain responses and lower blood pressure (Struthers, 1990).

Clinical cases of acute laminitis show lower values for ANP than is normal for the time of year (Table 7.13). Of course, these animals were receiving veterinary treatment of ACP and PBZ before the samples were taken so this again will have influenced the plasma ANP . In clinical circumstances, it is impossible to know the sodium or another dietary factors with any certainty. Most clinical cases of acute laminitis are stabled and given a sodium/calcium supplement (Methisal, Univet, Banbury, Oxon) as routine. Such management changes would restore sodium/potassium ratios to winter levels. Stabling alone will be sufficient to increase sodium and reduce potassium load and such management is beneficial. This may explain why Beau had values of ANP during summer months, when he was recovering form repeated acute attacks, which are comparable with winter results. Conversely, some increases are seen when ponies are turned out to grass after laminitis treatment. It may be that this increase reflects the end of the glyceryl trinitrate treatment and restoration of blood pressure to normal levels and is a brief compensatory mechanism mediated by ANP.

It seems that there are not major changes in ANP during acute laminitis but minor alterations in response to blood pressure variation. The seasonal variation in ANP and All are intriguing and warrant further investigation. It will be most interesting to analyse circulating aldosterone from animals kept under controlled conditions and to see if there is seasonal variation in this hormone. Similarly, development of a BNP radioimmunoassay may reveal greater changes than are evident in the ANP analyses.

Chapter 8 Endothelin

		Page
8.1	Introduction	358
8.2	Materials and Methods	364
8.3	Results	369
8.4	Discussion	372

"Snakes, in my heart-blood warm'd, that sting the heart !"

W. Shakespeare , Richard II

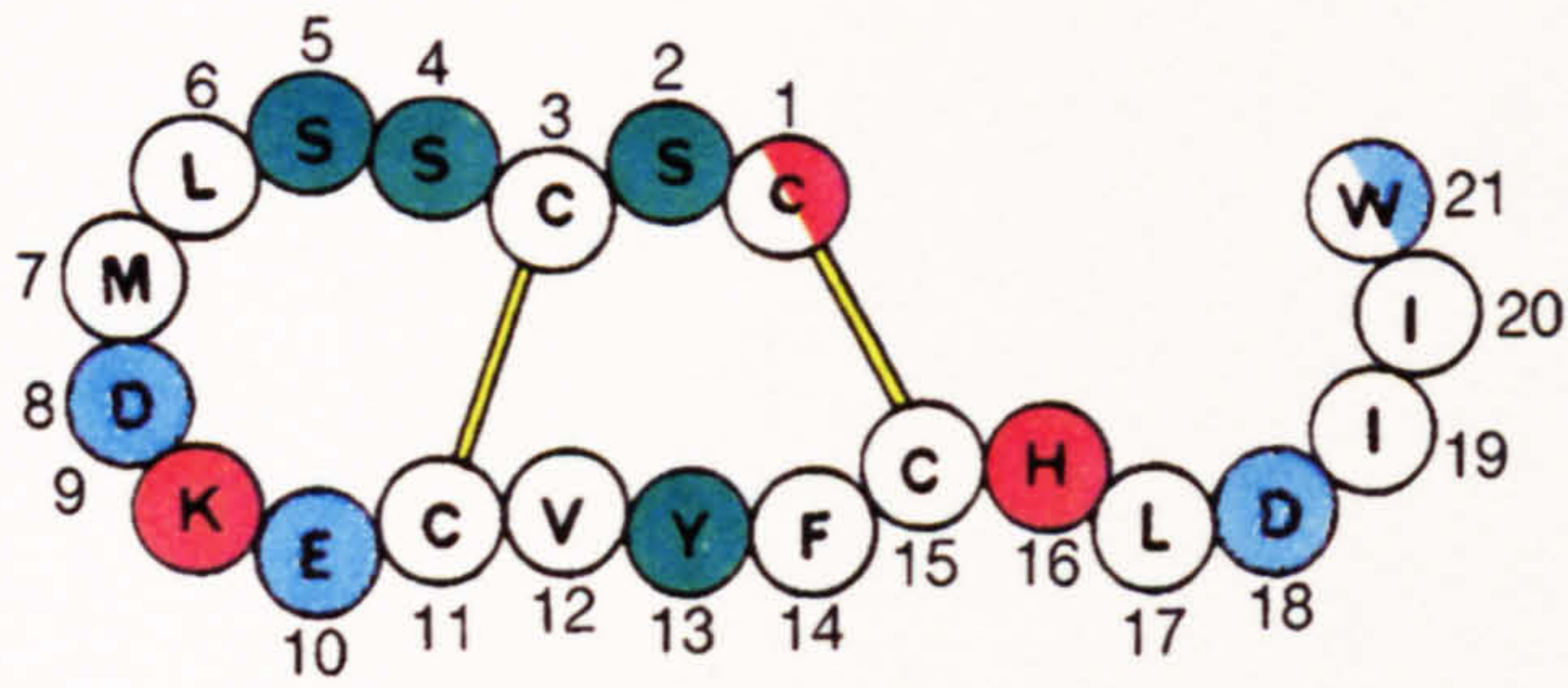
8.1 Introduction

The discovery that the vascular endothelium in addition to generating NO, was a source of systemic and paracrine regulatory peptide hormones (Ånggård, 1990; Moncada *et al.*, 1991), generated intense investigations into their respective roles in many pathophysiologies. The recently identified paracrine hormones of endothelial origin function alongside the classical systemic hormones such as angiotensin, vasopressin, and catecholamines.

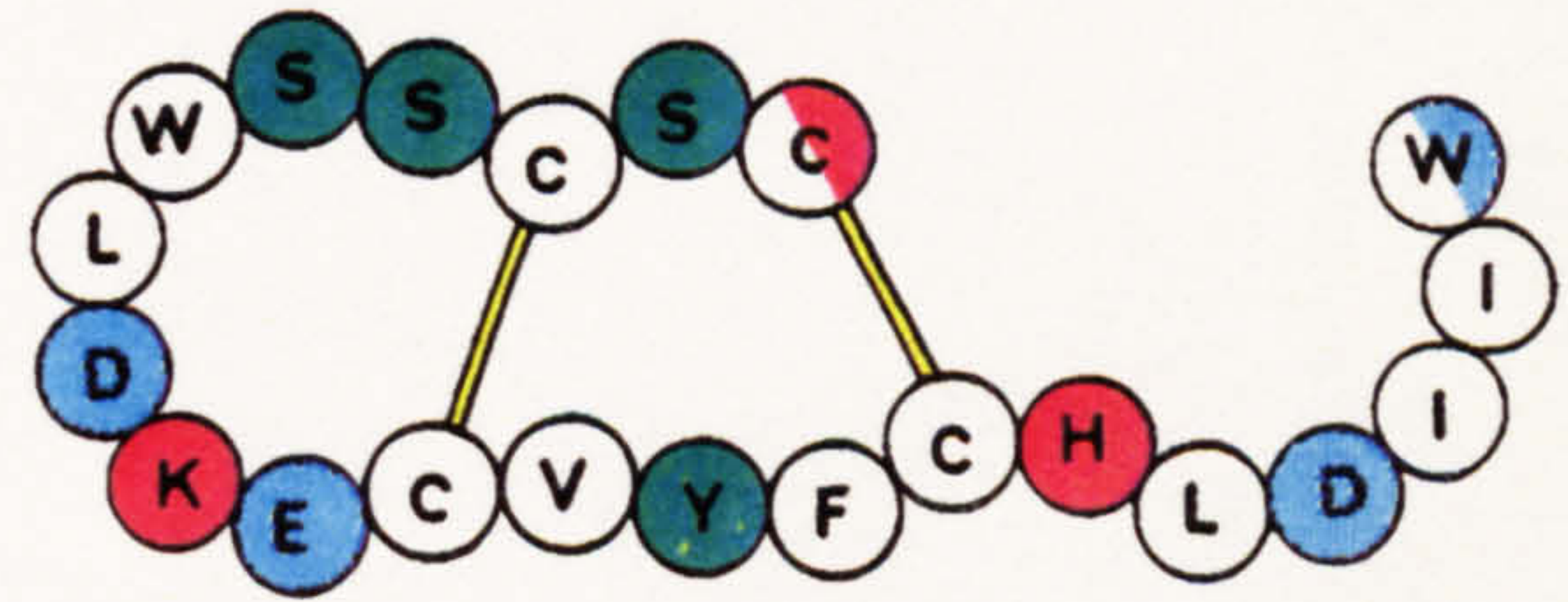
An endothelial derived contracting factor (EDCF) was first noticed and identified by Hickey *et al.*, (1985) who named it endotensin. The hormone was isolated, characterised as a peptide hormone, and later sequenced by Yanagisawa *et al.*, (1988) who gave the hormone its name - "Endothelin". Endothelin is the most potent vasoconstrictor peptide known, being ten times more potent than angiotensin II or vasopressin (Munger and Badr, 1992).

The family of active Endothelins comprise: Endothelin -1 (originally called porcine or human endothelin), Endothelin -2, Endothelin -3 (also known as rat endothelin). There are three isomers of endothelin, expressed by three different genes. The active endothelin peptide consists of twenty one amino acids, held together by two disulphide bridges. The molecules have very similar structures (see Figure 8.1) and bear a striking similarity to the sarafotoxins found in the venom of the Israeli burrowing asp *A. engaddensis* (Doherty, 1992) and to scorpion and bee venoms (Miyazaki *et al.*, 1992). Endothelin is also closely related, differing by only three amino acids, to endothelin- β or vasoactive intestinal contractor (VIC) (Saida *et al.*, 1989).

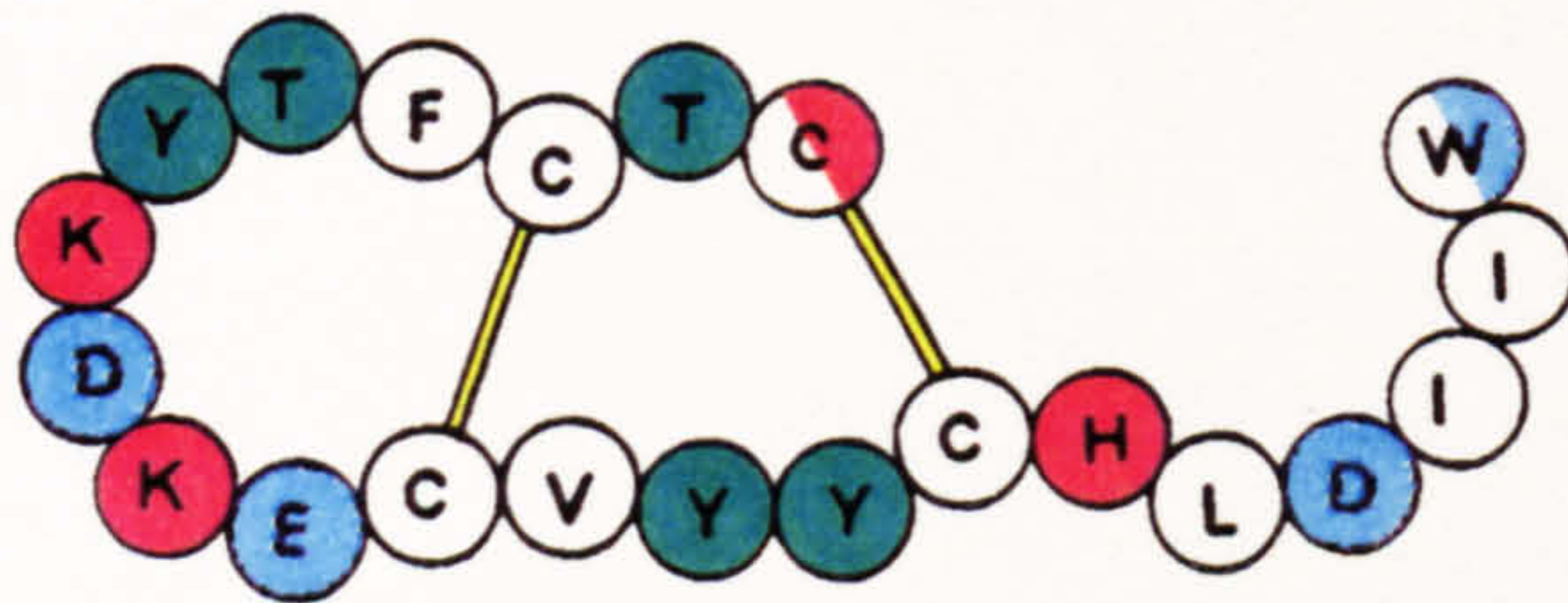
Figure 8.1 Amino acid sequences of the ET peptide family (Erhardt, 1992)



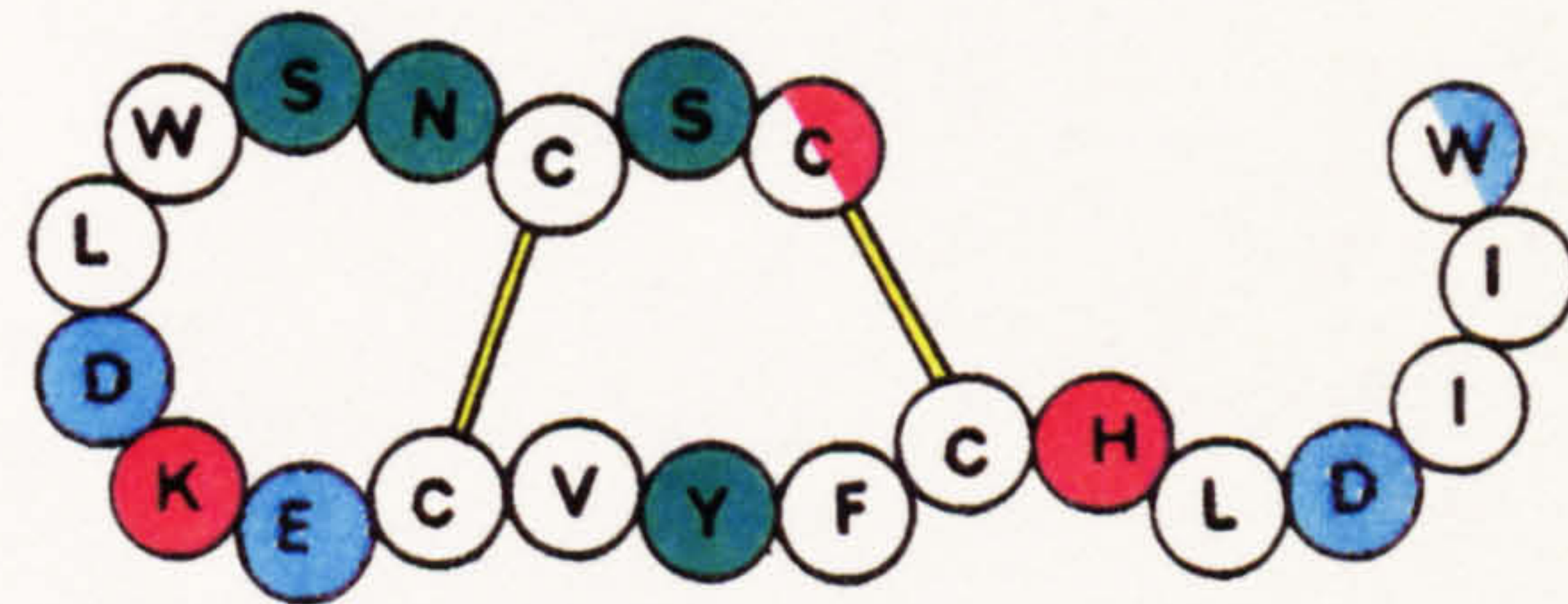
Endothelin-1



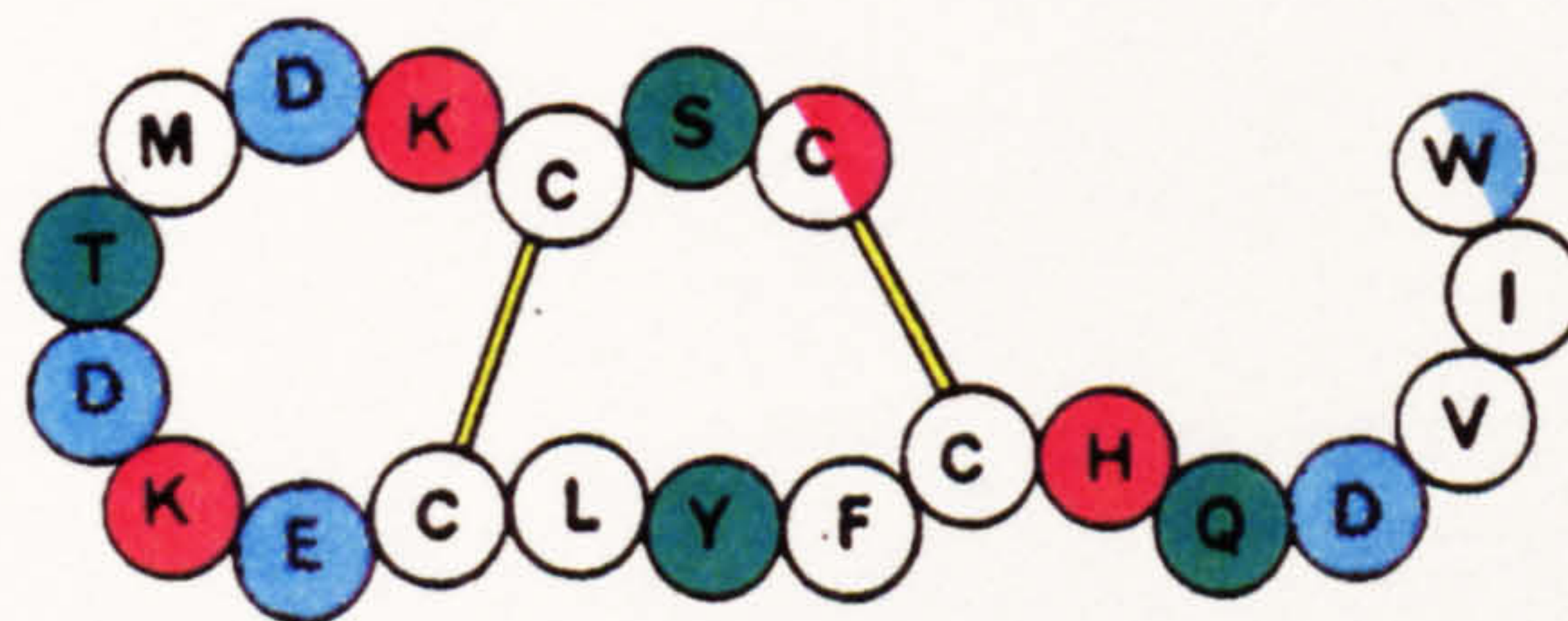
Endothelin-2



Endothelin-3



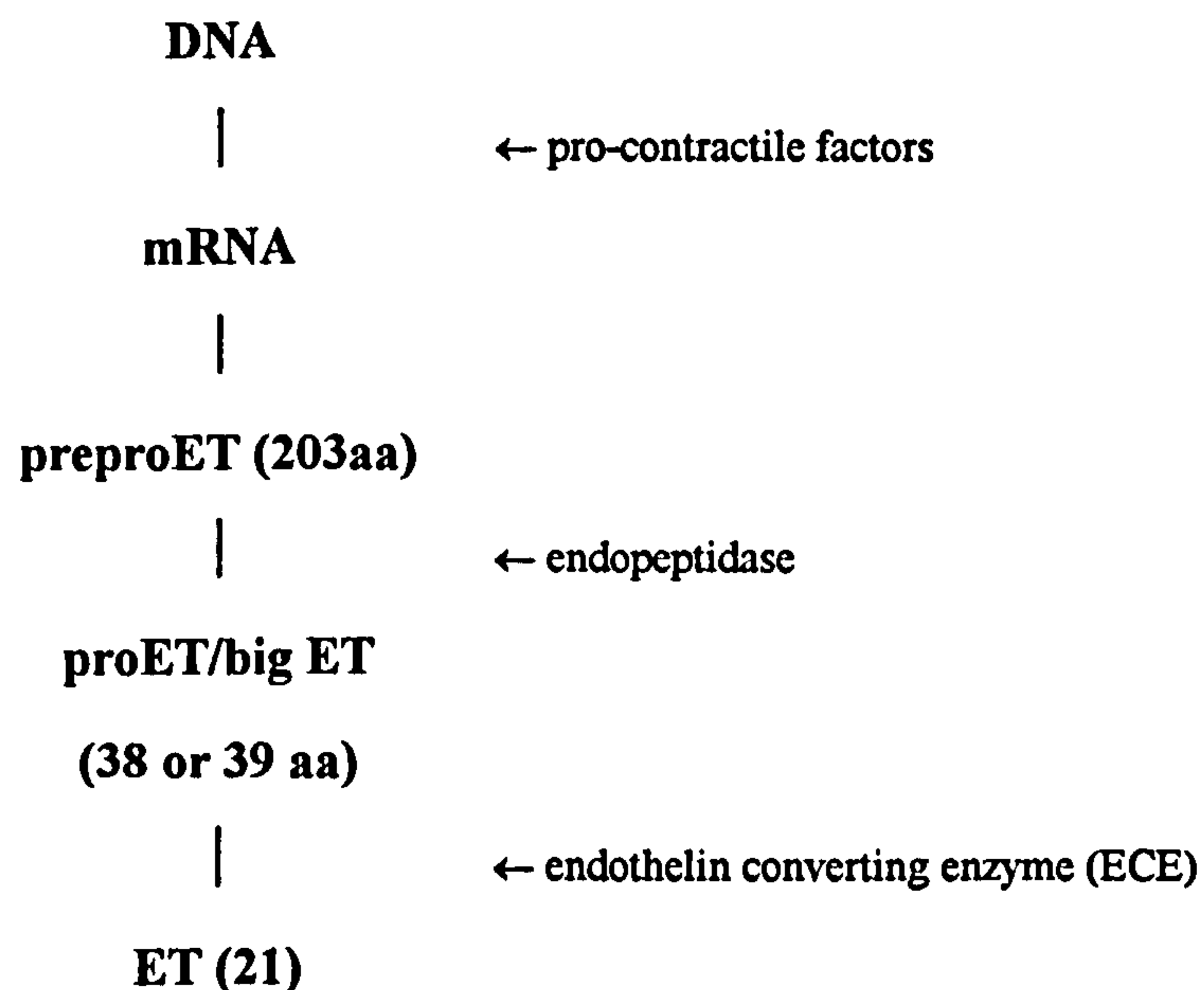
Endothelin-β



Sarafotoxin-S6b

Endothelins are synthesised, in response to stimuli, by gene expression at DNA level. A prepropeptide of 208 amino acids is cleaved to the prohormone, big endothelin. Big endothelin is 38 or 39 amino acids long and is cleaved by endothelin converting enzyme (ECE) to form active endothelins 1 - 3 (Figure 8.2).

Figure 8.2 Biosynthesis of endothelin.



Endothelins are synthesised in the vascular endothelium and not stored; the synthesis is regulated at mRNA level. Expression of the preproendothelin gene is stimulated by thrombin, TGF- β , adrenaline, vasopressin, bradykinin (Munger and Badr, 1992), oxyhaemoglobin (Doherty, 1992) phorbol esters and the calcium ionophore A23187 (Doherty, 1992). Endotoxins, elevated glucose levels and hypoxia (Doherty, 1992) and insulin (Hu *et al.*, 1993; Wolpert *et al.*, 1993) all increase endothelin synthesis. Endothelins causes vasoconstriction by binding to specific ET receptors.

There are two types of ET receptor ET_A which is specific for ET -1 and ET_B which binds most endothelins. Endothelin and angiotensin II act synergistically to maintain vasoconstriction, while nitric oxide and atrial natriuretic peptide oppose the vasoconstriction and rise in blood pressure caused by endothelin infusions (Vierhapper, 1995). Endothelin isomers are expressed in vascular tissues but are widespread in many other tissues (Parker Botelho *et al.*, 1992; Vierhapper, 1995) and endothelins may, for example, regulate release of pituitary hormones and influence adrenal function (Vierhapper, 1995), wound healing, control of menstruation, and regulation of blood pressure (Doherty, 1992). Endothelins are also implicated in disease (Haynes and Webb, 1994).

In addition to chemical stimuli, biomechanical factors initiate synthesis of endothelin by stretching of endothelial cells. Endothelial cells recognise stretching perhaps through activation of potassium or other ion channels in the plasma membrane and this is independent of protein-kinase-C and cAMP (Daniel and Ives, 1989). These represent part of the paracrine mechanisms for local vascular control: pressure stretch (acting in a perpendicular fashion to the cell surface) results in the activation of endothelin; conversely shear stress (or increased flow past the cells surface) results in the production of nitric oxide (Davies, 1989). Certainly this process is observed clinically and during surgery (Pollock *et al.*, 1993; Nomura *et al.*, 1994). *In vivo* administration of endothelins causes a transient vasodepression, seen prior to the typical long lasting vasoconstrictor action of endothelin (Botting and Vane, 1992). The transient vasodepressor response to endothelin is eliminated by concurrent administration of l-arginine analogues (such as L-NAME) which also result in a more potent response to endothelin so is held to be homeostatic compensation by nitric oxide .

Different receptor sub-types are also held to be responsible for the transient vasodepression seen before the onset of vasoconstriction (Lüscher, 1994; Pollock *et al.*, 1995). Endothelin receptor antagonists reduce or eliminate vasoconstriction caused by endothelin; ECE inhibitors have the same effect. 'Negative feedback' controls endothelin production and synthesis is by interactions at cellular and molecular levels (Figure 8.3).

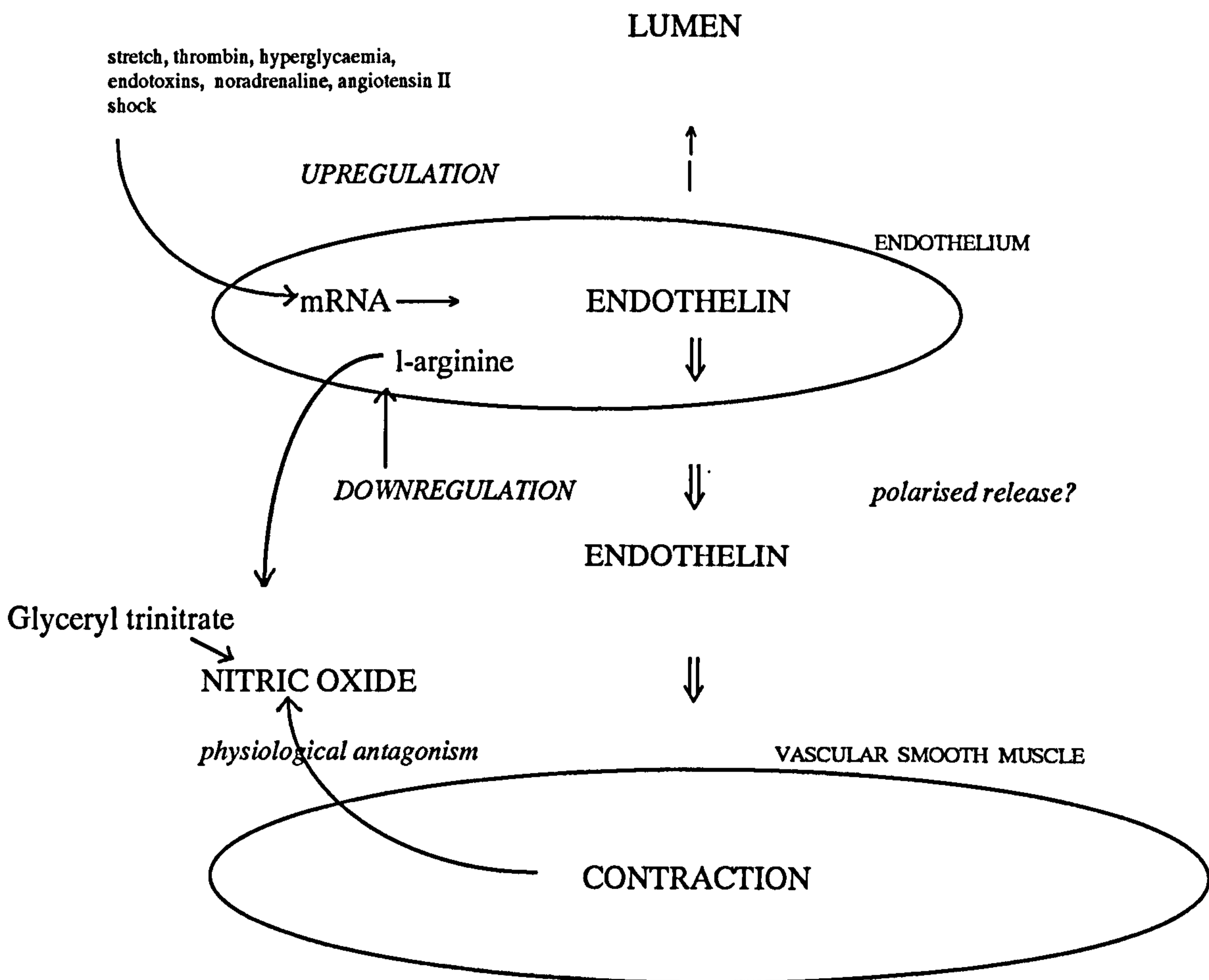


Figure 8.3. Mechanisms of vascular regulation by nitric oxide and endothelin (after Lüscher *et al.*, 1992).

Endothelin has a long lasting vasoconstriction effect both *in vitro* and *in vivo* (Rubanyi, 1992). Binding to the receptor last several hours and irreversible binding has been observed (Kurihara *et al.*, 1989; Parker Botello, 1992). Endothelin is cleared by the lungs where 50% is cleared on the first circulation (Munger and Badr, 1992); the peptide therefore has a very short half-life in the circulation, but a very long period of action since it exhibits prolonged binding to the receptors. Endothelin behaves differently to angiotensin II which circulates as precursors angiotensinogen and angiotensin I. Angiotensin II is cleared from its receptors much more quickly than endothelin.

Full accounts of tissue specificity, receptors, cellular actions and clinical considerations are well described by Rubanyi (1992) and Vierhapper (1995) and the detailed pharmacology of endothelin *in vivo* is by Doherty (1992) and Lüscher (1994). The complex interaction of endothelin with other paracrine agents within the vascular endothelium is outlined in Figure 5.1 (Vanhoutte *et al.*, 1993) and Figure 5.4 (Lüscher, 1994) in Chapter 5. Endothelins have been implicated in many disease states, particularly those involved with vascular pathologies including septic and endotoxic shock, hypertension and renal failure, all of which have been observed in equine laminitis (Table 8.1).

Table 8.1 Human diseases with increased levels of endothelins (after Rubanyi, 1992).

Shock (cardiogenic, septic, endotoxin)
Acute and chronic renal failure
Systemic hypertension
Pulmonary hypertension
Myocardial infarction
Congestive heart failure (severe)
Subarachnoid haemorrhage
Major surgery
Physiological stress

It has been suggested that endothelin has a role in vascular disorders that seem to be clinically equivalent to equine laminitis, namely Raynauds disease, Buergers disease and diabetic complications (Doherty, 1992). As equine laminitis is a vascular pathophysiology and the clinical biochemistry of affected animals (hyperglycaemia, endotoxaemia, thrombi) is likely to initiate synthesis and release of endothelin. It is speculated that endothelins could be involved in the aetiology of equine laminitis. Plasma endothelins have never been measured in equids, therefore normal values were established and compared with values in animals with diseases of different aetiologies.

8.2 Materials and Methods

Animals Sixteen horses and ponies, 3 geldings and 13 mares, aged from 4 -20 years and weighing 250 - 650 Kg bodyweight were studied. 5 mares were in the last trimester of pregnancy at the time of sampling. All animals were stabled and fed home grown hay; some were supplied with a proprietary feed mix ('Stud Diet', Dodson and Horrell, Northants, UK). All animals had been kept on the same tightly controlled management for at least 6 months before the samples were collected; many had been bred on the premises.

Acute laminitis (Two case studies). Two animals kept under controlled management developed laminitis spontaneously. A cross bred pony (250 kg b.wt.) developed acute laminitis whilst at grass. A Registered Irish Draught mare (600 kg b.wt.) developed acute laminitis after dystocia, retained placental membranes and endotoxaemia. The cases exhibited Obel Grades of lameness 2 and 4 respectively.

Treatment of acute laminitis. Both cases of acute laminitis were treated with transdermal glyceryl trinitrate (GTN) (Percutol Paste, Cusi, Welwyn Garden City, UK) applied to the clipped palmar surfaces of the pasterns once daily as nitric oxide donors are known to be therapeutic for acute laminitis (Hinckley *et al.*, 1994; Hinckley *et al.*, 1996). The *post partum* case received adjunct therapy of analgesics/ cyclooxygenase inhibitors - i.v. flunixin meglumine (FX) (Finadyne, Schering -Plough Animal Health, Suffolk, UK); i.v. non steroidal anti inflammatory agents - Phenylbutazone/ Ramifenazone (PBZ) (Intervet UK Ltd., Cambridge, UK) twice daily i.v.; and α -adrenergic blockade- acepromazine maleate (ACP, C-Vet, Bury St. Edmunds, UK) four times daily. Systemic antibiotics of penicillin /streptomycin (AB1) (Streptopen, Pitman Moore, Cheshire, UK) and later gentamycin (Gentaject 10%, Franklin Pharmaceuticals, Trim, Co Meath, Eire) (AB2) were given i.m. twice daily. Uterine irrigation (UI) of 5L 0.9% sterile saline (Baxter, UK), followed by twice daily treatment of 6mls each of penicillin (Penillin, Univet, Oxford, UK) and

framomycin (15% Framomycin, C-Vet, Bury St. Edmonds, UK) solutions respectively. 25 i.u. oxytocin (OX) i.v. (Leo Animal Health, Princes Risborough, Bucks) was given four times daily. Grass induced laminitis was treated with GTN and oral PBZ (Equipalazone, Arnolds Veterinary Products, Shrewsbury, UK) alone. Treatments are detailed in Tables 8.2 and 8.3 below and in Appendix IV.

Table 8.2 Treatment of *post partum* acute laminitis, see text for abbreviations.

	GTN (mg/day)	PBZ (mls/day)	FX (mls/day)	ACP (mls/day)	Other
Day 1	100	-	12	-	AB 1
Day 2 (7.00 hr)	-	-	10	4	AB 1
Day 2 (11.00 hr)	56	20	10	7	AB 2, UI, OX
Day 2 (19.00 hr)	-	-	10	7	AB 2, OX
Day 2 (23.00 hr)	-	20	10	7	OX
Day 3	56	20	40	28	AB 2, OX
Day 4	56	20	40	28	AB2, UI, OX
Day 5	56	-	40	28	AB2, OX
Day 6	56	-	40	28	AB2, OX
Day 7	56	-	40	28	AB2, OX
Day 8	56	-	40	28	AB2, OX
Day 9	56	-	40	28	-
Day 10	56	-	40	28	-
Day 11	56	-	40	28	-
Day 12	24	-	40	28	-
Day 13	24	-	40	28	-
Day 14	24	-	40	28	-
Day 15	24	-	40	28	-
Day 16	24	-	40	28	-
Day 17	24	-	40	28	-
Day 18	24	-	40	28	-
Day 19	24	-	40	28	-

Table 8.3 Treatment of grass induced acute laminitis

	GTN (mg/day)	PBZ (g/day)	FX	ACP	Other
Day 1	80	1	-	-	-
Day 2	40	1	-	-	-
Day 3	40	0	-	-	-
Day 4	20	-	-	-	-
Day 5	0	-	-	-	-

Blood Samples Samples from normal horses were collected between 10.30 am and 12.30 pm on selected days of sampling and the animals were standing quietly for at least 20 minutes before the sample was collected. The animals were accustomed to the procedures and were not stressed during the sampling. Blood samples were taken by jugular venepuncture into glass vacuum tubes containing 0.12 ml 0.34M ethylene diamine tetracetic acid (EDTA) (Vacutainer, Becton Dickinson). The samples were then mixed with inhibitor solution -comprising : 250 mM EDTA, 0.05 M 1,10 Phenanthroline (Sigma) in ethanol, and 1000 KIU/ml Aprotinin (Sigma) - in the ratio of 50 μ l/1ml blood and kept on ice until centrifuged at 600g for 12 minutes. The plasma samples were stored in polypropylene tubes at -20° C until analysis

Extraction. Samples were extracted following protocols outlined in the Nichols RIA Kit (Nichols Institute Diagnostics BV, The Netherlands). Samples (2mls) were mixed with 3 mls of a solution of 4% glacial acetic acid (96%) (Solvent A). Sep pak C18 plus extraction columns (Waters, Millipore, USA) were placed on a Vac -Elut SPS 24 station and prepared by passing 5mls methanol through the column under 5mmHg vacuum pressure. The columns were then washed with 5 mls distilled water, and pretreated with 5 mls Solvent A. The sample solutions were placed on the cartridges and taken through under minimum pressure, the sample tubes were rinsed with 5 mls of distilled water. The columns were

rinsed with 3 mls 25 % ethanol in distilled water, then eluted in 2 mls of a solution of : 25 % solvent A in a solution of 86 % ethanol in distilled water. Eluted samples were dried under vacuum at 37° C (Univap) and reconstituted in 0.5 mls assay buffer comprising borate buffer pH 8.4. The samples were four fold concentrated . The samples were frozen at -22 ° C until assayed.

Radioimmunoassay. A radioimmunoassay was prepared within the range 0 -190 pg/ml according to the guidelines outlined in the Nichols RIA kit. Duplicate tubes for total counts (TC) and non-specific binding (NSB) were prepared. 300 µl Assay buffer (borate buffer) were added to NSB. The standard curve was prepared by pipetting 200 µl of either each standard solution given in the kit, and from other dilutions of the standards, so the standards for the curve were 0, 1.75, 3.5, 6.0, 11, 19, 42, 97 and 182 pg/ml respectively. 200 µl of samples were placed in assay tubes. 100 µl I-¹²⁵-Endothelin (Rabbit, Nichols Institute Diagnostics BV, The Netherlands) were added to all tubes. 100 µl of anti-endothelin antiserum (Rabbit, Nichols Institute Diagnostics BV, The Netherlands) were added to all tubes except TC and NSB. Tubes were vortexed and incubated for 18 hours at 4 °C. Anti-Rabbit precipitant was added to the tubes except TC and they were vortexed and left for 20 -30 minutes at room temperature. Afterwards, 1.0 ml of distilled water was added to all except TC, they were vortexed and then centrifuged for 15 minutes at 1000g. The supernatant was aspirated and the pellet in each tube counted in a gamma counter (Packard) for 2 minutes. Values for samples were calculated from the standard curve - %B/Bo. Recovery was between 91 -93 %. The values for the samples were adjusted for recovery and concentration. Samples were concentrated 4-fold before assay and at these concentrations intra-assay and inter-assay variations were 6.8% and 18% respectively. Lowest detectable limit was 0.1 pg/ml.

Cross reactivities.

Cross reactivities with other endothelin isomers at 50% B/Bo are stated to be :-

	% cross reactivity
Endothelin -1	100
Endothelin -2	67
Endothelin -3	84
Big -Endothelin -1	2.6
Big -Endothelin -2	5.3
Big -Endothelin -3	0.2

(Nichols Institute Diagnostics BV, The Netherlands)

At low concentrations, where %B is greater than 50 % it must be assumed that all active isomers are bound. Therefore the assay is not specific for Endothelin -1 at these concentrations and measured values reflect total active endothelins in plasma.

Statistics.

Statistical evaluation employed Microsoft Excel (Microsoft). Values are expressed as Means \pm SE. A Student's t-test was used to assess statistical significance and differences were considered significant if $p < 0.05$.

8.3 Results

Inter assay variation was 6.8% and inter assay variation was 4.5% at values of 14 pg/ml but higher at 30 % at very low values. Human control samples of ET in lyophilised sera were provided in the Nichols RIA kits with stated values of 8.8 - 14.4 pg/ml gave RIA values of 9.4 pg/ml and 12.6 pg/ml respectively

Normal horses.

The mean plasma ET \pm SE of normal horses (n = 16) was 1.78 ± 0.2 pg/ml (range 0.45 - 3.1pg/ml). These data are shown in Table 8.4

Table 8.4 Plasma endothelins (ET) of normal horses.

Name of horse	Date of collection	ET (pg/ml)
Albert	16.10.95	1.2
Amanda	19.4.95	0.5
Biscuit	21.1.95	0.8
Biscuit	19.4.95	1
Blossom	19.4.95	3.1
Bryn	19.4.95	2.4
Cracker II	19.4.95	2.7
Dolly	19.4.95	2.3
Emily	19.4.95	2.1
Poppy	19.4.95	2
Rosieto	19.4.95	2.1
Maree Gray	19.4.95	2.4
Jasper	19.4.95	0.45
Selluci	19.4.95	2.4
Plasma Pool 93	1993	1.8
Plasma Pool 94	1994	1.3
	Mean	1.78
	SE	0.20
	Range	(0.45 - 3.1)
		n = 16

Grass induced acute laminitis. The mean value of plasma ET during treated spontaneous grass induced acute laminitis was 0.734 pg/ml which was significantly lower than the mean value for normal horses (1.79 ± 0.2 pg/ml) ($p < 0.001$), although within the normal range.

Name of horse	Acute laminitis	ET (pg/ml)
<i>Beau</i>	Day 1	0.65
	Day 2	0.78
	Day 3	0.67
	Day 4	0.64
	Day 5	0.93
	Mean	0.73
	SE	0.05

Post partum acute laminitis. When acute laminitis followed endotoxaemia, plasma levels were lower at the onset of the disease than results obtained for the animal in previous months. On Day 2 when the disease was at its worst, the plasma ET were below detectable limits (4 samples < 0.1 pg/ml); normal values (> 0.42 pg/ml) were restored on day 3 and were maintained for several weeks (Table 8.5). There seemed to be no relationship between plasma ET and blood pressure in acute cases.

Table 8.5 Plasma endothelins during *post partum* laminitis.

Name of horse	Clinical condition	Date/ time of collection	ET pg/ml
<i>Biscuit</i>	Normal	Day 0	0.8
	Normal	Day 0	1.05
	Onset (Obel 2)	Day 1	0.75
	Acute (Obel 4)	Day 2 (7.0 am)	0.18
	„	Day 2 (11.0 am)	<0.1
	„	Day 2 (7.0 pm)	<0.1
	„	Day 2 (11.0 pm)	<0.1
		Day 3	<0.1
	improvement	Day 4	0.37
		Day 5	0.42
		Day 6	0.63
		Day 7	0.86
	„	Day 8	0.67
	„	Day 9	1.09
	„	Day 10	0.87
	„	Day 11	0.62
	„	Day 12	0.95
	„	Day 13	0.99
	„	Day 14	0.88
	„	Day 15	0.59
	„	Day 16	1.42
	„	Day 17	1.02
	„	Day 18	0.72
	„	Day 19	0.35

8.4 Discussion

Endothelins are present in all mammalian species (Inoue *et al.*, 1989) and seem likely to be widespread in the animal kingdom (see Hasegawa and Kobayasi, 1990). The possible physiological actions of endothelins and their effects on cardiovascular homeostasis have been examined in rat, mouse, dog, cat, guinea-pig, non-human primates and humans (Botting and Vane, 1992). The extensive distribution of ET, both in species and in tissues suggests that they play an important normal physiological roles. An increasing volume of research results show that ET functions in both normal and pathological vascular function. It is eminently reasonable to assess the potential participation of ET in equine laminitis.

There is considerable variation in published values for human plasma ET determined by RIA (Saito *et al.*, 1992). Extraction methods probably make an enormous difference to the types of ET that are collected in the columns. In addition, the specificity of the anti-serum used is important. Studies that report higher values also record higher cross reactivity with other ET molecules (Saito *et al.*, 1992). Using Sep -Pak C18 cartridges, identical protocols to this study, the human values were in the region of 0.29 -2.0 pg/ml (Saito *et al.*, 1992) and 1.59 ± 0.32 pg/ml (Suzuki *et al.*, 1989). Equine results in this study were 1.8 ± 2 pg/ml. Equine plasma values of ET compared very well with the published values of human ET. Comparisons were made with other species (porcine, rat and sheep) and, apart from the dog which has higher circulating levels than other species, the equine ET values were very similar (Parker Botello *et al.*, 1992).

Several investigators have suggested that the measurement of plasma ET may not be worthwhile (Lüscher, 1994). There are several reasons for this assertion. The abluminal and paracrine actions of ET may not be reflected in plasma values unless there is huge physiological challenge (Lüscher, 1994).

ET are essentially paracrine or autocrine hormones; they are locally produced and locally bound. ET are synthesised abluminally i.e. towards the smooth muscle from the endothelial source, and the ET are never really circulating in plasma like other more familiar hormones. This being so, systemic levels are unlikely to change when endothelin is synthesised. Similarly, a transient peak of synthesis may be missed since the ET are either quickly bound or cleared in the lungs. The times that plasma ET are observed to change are either at times of massive synthesis or after endothelial damage or surgery where, in both cases, they 'spill over' into the circulation (Kurihara *et al.*, 1989; Nomura *et al.*, 1994). Notwithstanding this argument, it is still valuable to ascertain the normal circulating values of ET as plasma levels may still alter during dysfunctions.

This preliminary study suggests lower concentrations during acute laminitis and these preliminary findings have been published (Hinckley and Henderson, 1995). There are several possible reasons for this observation. First, the increased plasma proteins in the diseased samples increased viscosity and reduced recovery for individual samples. Secondly, a transient increase in plasma endothelins may have been missed. Thirdly, respiratory rate is increased during the worse stage of the disease as the animal is in pain. Reduced plasma ET may indicate increased clearance of normal plasma levels of ET although synthesis is unchanged. Fourthly, increased receptor activity and /or clearance may account for reduced concentrations during the disease. Finally, synthesis of ET may have been inhibited by the therapeutics administered, especially nitric oxide donors. Nitric oxide is known to inhibit the synthesis of ET (Luscher *et al.*, 1992) and ET vasoconstriction, whereas cyclooxygenase inhibitors do not (Botting and Vane, 1992).

ET synthesis is regulated by the relationship of ET and nitric oxide at endothelial level (Figures 5.4 and 8.3 *vide supra*). Reduced plasma endothelin levels may relate to the amount of glyceryl trinitrate given transdermally. When the GTN dose is at a maximum,

plasma ET are at a minimum and, as the GTN is withdrawn, ET levels return to normal. If nitric oxide is responsible for the inhibition of ET mediated vasoconstriction during acute laminitis, then administration of exogenous nitric oxide will reduce ischaemia and also have additional beneficial anti-proliferative and anti-platelet aggregating actions.

Clearly, the investigation of the role of endothelins in the pathogenesis of equine laminitis is preliminary. Although plasma levels were reduced there is no indication that they are involved in the aetiology. Elevated levels were expected because of endotoxaemia initiating ET synthesis, but if this occurred (and it may not have done) then it is possible that it is a local response, rather than a systemic one. As the value of measuring plasma levels of ET has been criticised (Lüscher, 1994), then future studies should concentrate on tissue biopsy and immunohistochemistry/binding studies *in vitro*. Immunohistochemistry of tissue taken from pedal vascular beds may provide a clearer picture of the possible involvement of ET in the pathogenesis of equine laminitis, not least because of ET's paracrine action. In this way prolonged binding locally, which is probably specific to the tissues concerned, would be recognised.

Only when such studies are done in conjunction with studies of plasma levels of endothelins, can the role of endothelin in acute equine laminitis properly assessed.

Chapter 9

Serum electrolytes and observations on selected urinary metabolites in normal and laminitic horses

		Page
9.1	Fluid electrolytes and haematocrit	376
	9.1.1 Introduction	376
	9.1.2 Materials and Methods	379
	9.1.3 Results	380
	9.1.4 Discussion	386
9.2	Urinary content of organic acids	390
	9.2.1 Introduction	390
	9.2.2 Materials and Methods	392
	9.2.3 Results	394
	9.2.4 Discussion	413

9.1 Fluid electrolytes and haematocrit

9.1.1. Introduction

This chapter is concerned with changes in certain selected components of urine and blood during the course of laminitis. Observations on these two body fluids must take account the general principles first expounded by Bernard (1856) and extended by Cannon (1929), Homer Smith (1959) and others, that chemical analyses of the blood and urine are excellent indices of an animal's homeostatic state.

Water, but not its dissolved solutes, is evenly distributed throughout the body. Water, moreover, is the solvent in which all metabolic reactions take place - it is essential for life and within the body contains the essential elements that permit molecular, cellular, organ and whole animal interactions to take place.

Water comprises at least 60% of the body weight and is evenly distributed between intra- and extra cellular compartments in an approximate ration of 4 : 1. Characteristically, intracellular fluid is rich in potassium ions and protein whilst the extracellular fluid is rich in sodium and chloride and has relatively low levels of protein. Clearly, active metabolic processes sustain these gradients between the two compartments. Extracellular fluid, include lymph, lachrymal secretions, cerebospinal and ocular fluids, saline, sweat and urine. The composition of the latter reflects a primary function of the kidney to maintain a homeostatic balance that ensures *la fixité milieu intérieur*.

Blood itself consists of cellular and extracellular elements - plasma, erythrocytes, leukocytes, thrombocytes etc. It is becoming increasingly clear that the cellular parts of blood contribute enormously to regulatory mechanisms. The cellular content of blood obviously donates a viscosity; determination of the percentage contributed by red blood

cells (PCV) is an important feature of local haemodynamics and endothelial reactions to the changes in shear stress imposed by reduced or raised haematocrit.

Extracellular components of blood, are precisely balanced with regard to solute concentration and volume. The vital mechanism of osmoregulation maintains plasma concentrations of necessary electrolytes within a narrow range in all species. Cellular and extracellular fluids consist of a solution of salts (many as chlorides or phosphates) and organic solutes, including proteins, vitamins, enzymes and hormones. Osmoregulation is based on the permeability of cell membranes to various molecules (including water) which travel along concentration gradients and on the active transport of molecules and ions across cell membranes. One active transport system is the 'sodium pump' which exchanges three molecules of intracellular sodium (Na^+) for two molecules of extracellular potassium (K^+) using the enzyme Na^+ , K^+ ATPase (Balment and Henderson, 1987). A range of physiological mechanisms and hormones are involved in osmoregulation and control of plasma volume. Renal function maintains plasma volume under usual conditions and glomerular filtration rate is a key influence of water excretion and reabsorption under normal conditions. Similarly, the kidney is central to the excretion of excess solutes. When plasma volume is decreased hormonal influences like arginine vasopressin and renin -angiotensin-aldosterone system inhibit diuresis and natriuresis to maintain blood pressure. Aldosterone enhances sodium retention but inappropriate retention of sodium can lead to hypertension (Grobbee, 1994). As blood pressure is increased then diuresis is enhanced by the action of atrial natriuretic peptide. It follows therefore that any investigation of hormones that alter plasma electrolytes must be accompanied by biochemical analysis of principle solutes such as sodium and chloride. Some solutes, such as calcium, are essential for intracellular activity and are cofactors for enzymes like nitric oxide synthase. Deficiencies may activate other paracrine hormones such as endothelin. In addition, packed cell volume(PCV) indicates possible dehydration (if the proportion of red blood cells is raised) or hypertension based on increased plasma volume (if the proportion of red blood cells is decreased). Such analyses provide greater

understanding of the mechanisms involved in regulation of the vascular system and blood pressure in health and disease.

The purposes of the studies in the present chapter were to relate the findings of Chapters 6 and 7 to electrolyte concentrations and packed cell volume (PCV).

9.1.2 Materials and Methods

Animals. Animals were kept as a controlled group as described in earlier Chapters. Normal and chronically laminitic horses and ponies, and acute laminitics of grass induced or *post partum* aetiology were studied.

Blood samples. Blood was collected for serum on the same occasions as that taken for plasma sample analyses in previous chapters. Blood samples (10mls) were taken by jugular venepuncture into plain sterile glass vacuum tubes (Vacutainer, Becton Dickinson, UK) and allowed to clot at ambient temperature for up to 2 hours. They were then left for 24 hours at 4°C. Serum supernatant was removed and stored in polypropylene tubes at -20°C until analysis. Plasma samples were collected as described in Chapters 6 and 7.

Packed cell volume (PCV). Plasma samples were centrifuged at 600g for 15 minutes. PCV was calculated by measuring the proportions of the red cell pellet and the proportion of plasma. This was expressed as a percentage of the total volume and compared with known ranges for the horse.

Analysis. Electrolyte analysis was by an automatic analyser at Grange Laboratories, Wetherby, Yorkshire. Samples were analysed for concentrations in $\mu\text{mol/L}$ of sodium (Na), Chloride (Cl), Potassium (K), Calcium (Ca), Phosphorous (P), Magnesium (Mg) and Sodium : Potassium ratio (Na : K) respectively. Normal ranges are those specified by the laboratory which are established for equine samples.

Statistics. Group mean values for a specified date were calculated using Microsoft Excel (Microsoft, USA) and are expressed as Mean \pm SE. During the acute stage of the disease animals acted as their own controls; differences were analysed using a student's t-test with unequal probability. Results were considered significant if $p < 0.05$.

9.1.3 Results

Data are for samples collected at the same time and date of those analysed by radioimmunoassay for angiotensin, atrial natriuretic hormone and endothelin (Chapters 6, 7, and 8). Mean values for horses and ponies in the normal and chronically laminitic groups are shown in Tables 9.1 and 9.2 respectively. Individual data and standard errors of mean values are shown in Appendix III. Values for all electrolytes tended to be within the usual ranges for both and chronically diseased animals during winter months with the exception of inorganic phosphorous which was higher than normal in both normal and chronic laminitic groups.

Both normal and chronically laminitic groups displayed increased serum sodium and potassium concentrations when at grass at the end of April. Although the marginally decreased to normal in June, high plasma sodium and potassium were evident during summer months until September. There appeared to be no difference in PCV of the chronically laminitic group and although there was some decrease in the PCV of the normal group during summer months, the values were however well within normal range. Increases in inorganic phosphates are usually the result of contamination by red blood cells or slight haemolysis on sampling (L. Roberts, personal communication) and are probably of no consequence. It is possible that the increases in many electrolytes seen is the consequence of evaporation during summer months as the serum samples are not normally placed on ice and are only refrigerated after clotting has occurred - usually on the following day. However, the vacutainer tubes used for collection are sealed and have a vacuum so that evaporation would be minimal. These tubes are routinely used for veterinary clinical samples sent by post so evaporation, although possible, is unlikely.

Serum electrolytes of normal horses and ponies

Table 9.1 Mean values of serum concentrations of sodium (Na), potassium (K), chloride, phosphorous (P), calcium, magnesium and packed cell volumes (PCV), Na: K ratio are also presented. Values outside normal ranges are shown in bold. Individual data are given in Appendix III

Date collected	Sodium mmol/l	Potassium mmol/l	Na:K Ratio	Chloride mmol/l	Inorganic P mmol/l	Calcium mmol/l	Magnesium mmol/l	PCV %
(normal range)	(129-140)	(3.90-4.80)	(27.71-34.62)	(98.0-109)	(0.55-0.88)	(3.09-3.35)	(0.64-0.84)	(32-53)
Normal								
17.04.94	136	4.45	30.61	100.5	1.12	3.17	0.81	45
30.04.94	150	5.17	29.52	112.5	1.17	3.66	0.85	48
12.06.94	131	4.32	30.55	98.56	1.06	3.11	0.75	43
17.07.94	169	5.81	29.26	127.56	1.62	4.14	1.00	39
14.08.94	156	5.09	30.98	117.60	1.49	3.71	0.90	39
21.08.94	155	5.24	29.93	117.89	1.37	3.67	0.94	40
28.08.94	155	5.78	27.43	117.40	1.39	3.74	0.85	42
04.09.94	132	4.42	31.38	99.50	1.09	3.21	0.74	42
11.09.94	136	4.21	32.45	101.40	1.11	3.06	0.73	41
16.10.94	136	4.75	28.86	100.70	1.21	3.14	0.68	43
19.11.94	136	5.91	23.58	102.50	1.00	3.12	0.66	45
21.01.95	134	4.20	26.26	103.19	0.90	3.13	0.68	42
08.03.95	135	4.41	30.84	105.08	0.73	3.15	0.72	41

Serum electrolytes for chronically laminitic horses and ponies

Table 9.2 Mean values for the herd of serum concentrations of sodium (Na), potassium (K), sodium: potassium ratio, chloride, phosphorous (P), calcium, magnesium and packed cell volumes (PCV), Na : K ratio are also presented. Abnormal values are shown in bold. Individual data are shown in Appendix III.

Date collected	Sodium mmol/l	Potassium mmol/l	Na:K Ratio	Chloride mmol/l	Inorganic P mmol/l	Calcium mmol/l	Magnesium mmol/l	PCV %
(normal range)	(129-140)	(3.90-4.80)	(27.71-34.62)	(98.0-109)	(0.55-0.88)	(3.09-3.35)	(0.64-0.84)	(32-53)
Chronic Laminitic								
17.04.94	135	3.84	35.17	101.00	1.09	3.04	0.75	39
30.04.94	180	5.70	31.80	139.00	1.29	4.41	1.09	45
12.06.94	132	3.76	35.54	100.20	0.91	3.12	0.69	37
17.07.94	163	4.82	34.99	124.20	1.31	3.91	0.94	39
14.08.94	172	5.42	31.97	133.20	1.28	4.18	0.93	42
21.08.94	160	6.02	26.73	123.40	1.10	3.80	0.89	45
28.08.94	177	6.04	29.26	139.60	1.15	4.34	1.02	40
04.09.94	131	4.06	35.00	100.80	0.90	3.16	0.75	40
11.09.94	136	4.14	33.16	103.20	0.96	3.06	0.72	42
16.10.94	135	4.54	29.99	103.40	1.01	3.11	0.72	41
19.11.94	134	4.74	29.49	103.40	0.86	3.08	0.70	38
21.01.95	135	4.60	31.03	103.00	0.74	3.15	0.77	42
08.03.95	134	4.50	29.78	104.00	0.78	3.10	0.70	ND

Serum electrolytes during acute laminitis

Table 9.3 Changes in serum electrolytes and packed cell volume during grass induced acute laminitis (Beau - Case Report 1).

Beau	Sodium mmol/l	Potassium mmol/l	Na : K Ratio	Chloride mmol/l	Inorganic P mmol/l	Calcium mmol/l	Magnesium mmol/l	PCV %	Comments
16/03/94	158.00	4.50	35.11	116.00	1.36	3.67	0.96	42	At grass
17/04/94	135	3.90	34.62	103.00	1.04	3.15	0.90	45	Stabled
30/04/94	181.00	5.80	31.21	142.00	1.27	4.66	1.07	45	Stabled
15/05/94	130.00	4.50	28.89	94.00	1.80	3.02	0.85	43	To grass
20/05/94	132.00	3.90	33.85	96.00	1.10	3.14	0.78	ND	Hyperlipidaemia
22/05/94	134.00	4.10	32.68	98.00	1.04	2.99	0.77	45	Lush grazing
03/06/94	103.00	4.70	21.91	76.00	0.81	2.35	0.65	34	Lush grazing
04/06/94	92.00	4.70	19.57	72.00	0.71	1.94	0.41	42	Acute laminitis
05/06/94	134.00	3.90	34.36	103.00	1.06	3.03	0.75	42	Stabled
12/06/94	133.00	4.10	32.44	99.00	0.75	3.25	0.75	44	Stabled
19/06/94	152.00	4.80	31.67	113.00	0.88	3.54	1.06	46	low BP 97/53
24/06/94	180.00	5.10	35.29	136.00	0.96	4.04	1.09	46	At grass
03/07/94	160.00	4.40	36.36	119.00	1.31	3.65	1.13	ND	At grass
09/07/94	126.00	3.60	35.00	91.00	1.06	2.89	0.75	40	At grass
17/07/94	154.00	3.50	44.00	118.00	0.84	3.57	0.96	38	At grass
24/07/94	148.00	4.20	35.24	109.00	1.31	3.49	0.96	45	At grass
26/07/94	145.00	3.60	40.28	107.00	1.49	3.32	0.91	38	At grass
29/07/94	144.00	4.70	30.64	108.00	1.14	3.29	0.82	36	Acute laminitis
31/07/94	127.00	3.80	33.42	94.00	0.71	2.84	0.76	40	Stabled
07/08/94	159.00	5.00	31.80	117.00	1.43	3.81	1.05	42	Stabled
14/08/94	162.00	5.20	31.15	123.00	1.46	3.97	0.95	44	Stabled
21/08/94	171.00	6.30	27.14	132.00	1.39	3.98	0.84	44	Stabled
28/08/94	190.00	6.20	30.65	149.00	0.98	4.91	1.16	36	Stabled
04/09/94	132.00	4.50	29.33	101.00	0.82	3.18	0.82	38	Salt appetite
11/09/94	137.00	4.00	34.25	107.00	0.80	3.22	0.78	44	Normal
16/10/94	135.00	4.00	33.75	103.00	1.22	2.96	0.73	40	At grass
19/11/94	136.00	3.70	36.76	102.00	0.85	2.97	0.74	37	At grass
14/03/95	139.00	4.10	33.90	105.00	0.91	3.06	0.81	ND	At grass
Mean	147.81	4.43	33.98	111.56	1.11	3.44	0.89	41	
Standard Error	4.34	0.22	1.04	3.64	0.07	0.13	0.03	0.7	
Range	(92 - 190)	(3.50 - 6.30)	(19.57 - 44.00)	(72.00 - 149.00)	(0.71 - 1.80)	(1.94 - 4.91)	(0.41 - 1.16)	(34 - 46)	
Normal Range	(132 - 152)	(3.30 - 5.40)	(28.00 - 40.00)	(89.00 - 108.00)	(0.90 - 1.80)	(2.50 - 3.60)	(0.60 - 1.00)	(32 - 53)	
No. of Tests	28	28	28	28	28	28	28	25	

Table 9.4 Changes in serum electrolytes and packed cell volume during grass induced acute laminitis (Misty - Case Report 2).

Misty	Sodium mmol/l	Potassium mmol/l	Na : K Ratio	Chloride mmol/l	Inorganic P mmol/l	Calcium mmol/l	Magnesium mmol/l	PCV %	Comments
Normal Range	(132 - 146)	(3.30 - 5.40)	(28.00 - 40.00)	(89.00 - 108.00)	(0.90 - 1.80)	(2.50 - 3.60)	(0.60 - 1.00)	(32 - 53)	
15/05/94	136.00	4.80	28.33	103.00	1.27	3.04	0.80	39	Normal
22/05/94	136.00	4.50	30.22	104.00	1.08	3.00	0.66	46	At grass
30/05/94	134.00	4.30	31.16	100.00	0.95	2.95	0.66	37	Collision
05/06/94	136.00	4.20	32.38	103.00	1.28	3.04	0.66	40	Stabled
12/06/94	136.00	4.30	31.63	105.00	1.01	3.03	0.64	37	Stabled
19/06/94	150.00	5.10	29.41	115.00	1.21	3.42	0.73	42	At grass
24/06/94	178.00	4.80	37.08	137.00	1.10	3.83	0.75	40	Acute laminitis
03/07/94	159.00	4.90	32.45	120.00	1.28	3.63	0.84	40	Stabled
10/07/94	141.00	4.20	33.57	110.00	1.39	3.02	0.61	ND	
11/07/94	148.00	5.70	25.96	116.00	1.27	3.38	0.68	ND	
12/07/94	137.00	4.70	29.15	105.00	1.07	3.07	0.61	ND	
13/07/94	117.00	3.80	30.79	89.00	0.95	2.58	0.50	ND	
14/07/94	128.00	3.60	35.56	96.00	1.01	2.84	0.59	34	
17/07/94	132.00	4.20	31.43	102.00	0.88	2.94	0.63	37	to grass
24/07/94	152.00	4.60	33.04	116.00	1.33	3.42	0.83	40	mild laminitis
26/07/94	140.00	4.20	33.33	108.00	1.12	3.12	0.65	39	stabled
29/07/94	127.00	4.30	29.53	99.00	0.79	2.77	0.59	38	
31/07/94	147.00	4.50	32.67	115.00	0.88	3.25	0.65	38	
07/08/94	153.00	5.40	28.33	116.00	1.19	3.35	0.91	42	to grass
21/08/94	178.00	6.80	26.18	140.00	1.07	3.88	0.90	42	stabled
28/08/94	118.00	3.90	30.26	92.00	0.76	2.46	0.50	40	
04/09/94	143.00	5.40	26.48	110.00	0.91	3.17	0.80	40	
11/09/94	121.00	4.30	28.14	90.00	0.95	2.65	0.57	43	
19/11/94	140.00	4.10	34.15	109.00	0.97	3.16	0.65	40	
Mean	141.13	4.61	30.88	108.33	1.07	3.13	0.68	38	
Standard Error	3.19	0.14	0.59	2.57	0.04	0.07	0.02	0.6	
Range	118 - 178	3.8-6.8	25 - 37	89 - 140	0.76-1.33	2.5-3.8	0.5-0.9	37-46	
No. of Tests	24	24	24	24	24	24	24	24	

Table 9.5 Changes in serum electrolytes during *post partum* acute equine laminitis (Biscuit).

Biscuit	Sodium mmol/l	Potassium mmol/l	Na : K	Chloride mmol/l	Inorganic P mmol/l	Calcium mmol/l	Maenesium mmol/l	PCV %	Comments
Normal	(132 -	(3.30 - 5.40)	(28.00 -	(89.00-	(0.90 - 1.80)	(2.50 -	(0.60 - 1.00)	(32-53)	
08/03/94	136.00	4.40	30.91	105.00	0.74	3.10	0.75	43	Normal basal
23/05/95	130.00	4.60	28.26	104.00	0.64	2.74	0.43	37	Acute onset mild Obel 2
23/05/95	130.00	4.60	28.26	104.00	0.64	2.74	0.43	37	Acute severe Obel 4
24/05/95	152.00	6.60	23.03	120.00	1.28	3.53	0.56	33	Some improvement Salt appetite
24/05/95	137.00	4.90	27.96	107.00	1.05	3.10	0.47	38	Some improvement Salt appetite
24/05/95	158.00	5.60	28.21	125.00	1.01	3.49	0.57	33	Still Obel 4 Salt appetite
24/05/95	158.00	5.60	28.21	126.00	1.08	3.66	0.60	35	Salt appetite oedema
25/05/95	164.00	4.60	35.65	128.00	1.17	3.75	0.68	36	Salt appetite
26/05/95	153.00	5.50	27.82	120.00	1.46	3.56	0.74	36	Salt appetite
27/05/95	144.00	5.10	28.24	114.00	1.25	3.28	0.66	36	Salt appetite
28/05/95	155.00	6.60	23.48	123.00	1.14	3.54	0.76	36	Salt appetite
29/05/95	132.00	5.00	26.40	103.00	1.26	3.02	0.61	36	Some improvement Obel 3
30/05/95	135.00	4.30	31.40	107.00	1.13	2.86	0.55	36	No salt appetite
28/05/95	57.00	122.00	0.47	188.00	0.74		32.40	36	Some improvement Obel 3
31/05/95	138.00	5.90	23.39	110.00	1.05	3.00	0.84	36	
01/06/95	146.00	5.10	28.63	115.00	1.06	3.27	0.65	36	
02/06/95	177.00	5.70	31.05	141.00	1.39	4.01	0.83	36	
03/06/95	157.00	7.50	20.93	125.00	1.33	3.52	0.79	39	
04/06/95	187.00	10.30	18.16	146.00	1.53	4.66	1.24	35	
12/06/95	186.00	5.80	32.07	151.00	1.30	3.73	1.03	41	Obel 4 'Sinking'
14/06/95	134.00	7.00	19.14	97.00	1.35	2.70	0.67	45	Some improvement ventral oedema

1

9.1.4 Discussion

Horses have a seasonal variation in ingested electrolytes in that, during spring and summer months, they have a diet of grass that is very low in sodium and very high in potassium (Chapter 7). Evolution has ensured that horses are equipped to compensate for these seasonal changes in electrolytes and water intake (grass has a lower dry matter content than winter fodder). During winter months potassium intake is much lower and dietary sodium is increased see Table 6.15. Chapters 6 and 7 show seasonal changes in certain water and electrolyte regulatory hormones; both plasma AII and ANP decrease during summer months when animals are at grass. ANP would not be activated if sodium was to be conserved during dietary deficiency; AII levels would be inhibited, despite incipient sodium deficiency, by ingested potassium. It was suggested that high ingested potassium activates aldosterone, promoting excretion of potassium, and that aldosterone would enhance sodium retention, independently of the renin - angiotensin system. In other words, horses may have adopted an unusual mechanism to deal with what is a major dietary variation.

The dietary factors seem to be evident in the normal range of electrolyte values for sodium, potassium and other electrolytes during winter months. There was an increase in sodium, chloride and potassium particularly when both normal and laminitic animals went to grass. In normal circumstance, the sodium : potassium ratio would reflect increased aldosterone activity by reduced levels of potassium. In the equine samples, the sodium : potassium ratio did not show aldosterone involvement but this may have been masked by huge increases in ingested potassium. Sodium retention was evident in all animals at grass and this too fits the theory of aldosterone association. Unfortunately, aldosterone assays were not performed but this is an interesting avenue to explore in future work. Investigations of aldosterone levels in the horse have always used animals that have been housed and on dry fodder and / or additional feed which contains increased sodium (Clarke, 1982).

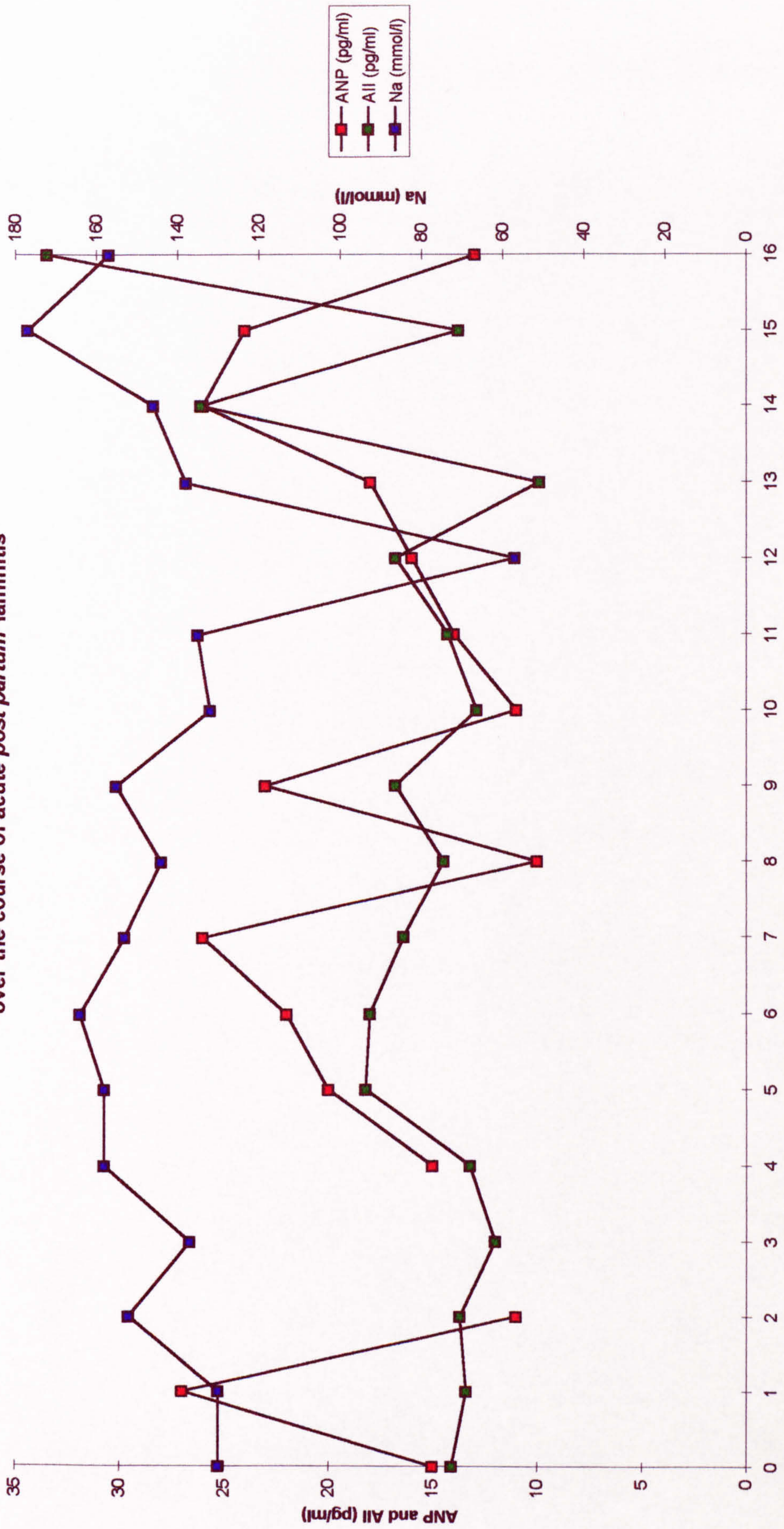
Packed cell volume did not exceed normal ranges although perhaps some decrease in mean PCV was seen in the normal and chronically laminitic groups when turned out to grass. This would be indicative of increased plasma volume. Blood pressure for both groups did not increase (Chapter 3), nor did plasma ANP despite the sodium retention. ANP is inhibited by aldosterone (Kenyon and Morton, 1994) and vasopressin (Jard, 1990). Vasopressin is normally activated by low blood pressure but high plasma osmolality also activates this hormone which prevents diuresis, and restores plasma osmolality by water retention (Jard, 1990). Endothelin would be activated by aldosterone and it may be that the involvement of these pressor hormones has a part to play in the predisposition of the aetiology of equine laminitis at grass (see Figure 10.-Chapter 10).

The cases of acute laminitis showed transient hyponatraemia prior to, or concurrent with, the onset of the disease (see Beau 3/6/94). These findings are consistent with those of Moore *et al.*, (1981) who noted slight hyponatraemia with the onset of diarrhoea after induced laminitis. Both Beau and Biscuit displayed a salt appetite during acute and refractory stages of laminitis which roughly correlated with hyponatraemia. Such a salt appetite had a rapid onset at acute stage and was dramatically revealed by animals avidly licking all surfaces; if salt was given to the animal in the form of a salt 'block' then the salt was eaten with relish. A whole block could be consumed each day. This response may be mediated in the first instance through angiotensin, rather than aldosterone, because of the fast timing of the response, although aldosterone increases immediately after acute phase of laminitis (Moore *et al.*, 1981). Plasma angiotensins did not appear to be grossly elevated. The reasons for this are unknown but it is possible that a transient increase in AII could have been missed. The relationship between AII, ANP and plasma sodium in a post partum case of acute and refractory laminitis is shown in Figure 9.1. These analyses are of single samples taken over the course of the disease. Although AII does not increase enormously, it is possible that the AII receptor was

upregulated during the acute stage by prolonged pain induced activation of renin - angiotensin system. This would explain the increased plasma sodium despite seemingly normal values of AII for stabled horses.

Figure 9.1 Relationship between plasma atrial natriuretic peptide (ANP), angiotensin II (AII) and plasma sodium (Na) over the course of acute equine laminitis.

Changes in plasma All and ANP compared with sodium concentration over the course of acute post partum laminitis



Abnormally high plasma sodium was related to high blood pressure in this case -Biscuit -(see Chapter 3) but not in other cases. This picture of sodium induced hypertension is not uncommon (Padfield and Edwards, 1992) but it is surprising that higher levels of ANP were not seen. Perhaps aldosterone suppressed ANP, as ANP has an inverse relationship with plasma renin and aldosterone (Richards and Nicholls, 1992), or administered nitric oxide donors opposed ANP synthesis. Unfortunately it is not possible to do more than speculate on the available data but more studies in this area are required.

Veterinary practitioners have assumed in the past (in the absence of a radioimmunoassay for AII) that if animals are first hyponatraemic, then hypernatraemic, AII would be responsible for sodium retention. Angiotensin converting enzyme (ACE) inhibitors were suggested as therapy for acute laminitis, based on this assumption. As plasma AII is relatively low in normal and acutely diseased horses, it is not surprising that ACE inhibitors have a profound effect on the blood pressure of laminitic horses (Stashak, 1987). However, the fact that ACE inhibitors are never used clinically (despite trials) supports the evidence in the present study that AII is not a primary factor. The regulatory physiology of fluid and electrolyte homeostasis is far from simple.

9.2 Urinary content of organic acids

9.2.1 Introduction

Recent discoveries in cardiovascular physiology have led to awareness of the importance of certain amino acids as substrates for the synthesis of nitric oxide (NO) (Moncada *et al.*, 1991) *vide supra*. Changes in urinary nitrates which, like urinary cyclic guanosine monophosphate cGMP, indicate production of nitric oxide are considered in Chapter 5 in the context of nitric oxide.

Although the involvement of the l-arginine -nitric oxide related pathways in the pathogenesis of acute equine laminitis has been investigated, several substrates, metabolites and end products have been focused upon including orotic acid and hippuric acid which potentially have close relationships with the l-arginine - NO pathway (Alonso and Rubio, 1989). Although l-arginine is part of the urea cycle, a deficiency of this amino acid may alter urea cycle components and be reflected in the urinary content of a variety of metabolites. Certainly, deficiency of l-arginine results in orotic aciduria in rats (Alonso and Rubio, 1989). Orotic acid is closely related to hippuric acid. Hippuric acid was first identified in equine urine, hence its name (Lewis, 1914). It is freely excreted by the kidney independent of urine volume (Scheinberg and Myers, 1948) suggesting transport processes of considerable avidity, independent of glomerular filtration, at least in the dog. Hippuric acid is a glycine -benzoate conjugate (Rodwell, 1990). Sodium benzoate is used in paediatric medicine to treat enzyme insufficiencies in those children that have urea cycle deficiencies and cannot synthesise arginine (Snodgrass, 1981; Brusilow, 1992).

The metabolism of l-arginine is complex. Horses ingest l-arginine (in grass) or synthesise their own using symbiotic bacteria in the digestive tract. Symbiotic bacteria are likely to produce the substrates for the synthesis of hippuric acid - glycine (Smith, 1951) and sodium benzoate (synthesised from phenols or related compounds, such as cresol, or

fatty acids), (Professor E. Haslem personal communication). Intestinal micro-organisms are key to equine nutrition. Since digestive disturbance has been associated with the pathogenesis of acute equine laminitis (Garner *et al.*, 1975; Harkema *et al.*, 1978) and phenols have been observed in the urine of cases of acute laminitis (Urmas, 1968). It seems probable that disturbance of intestinal microflora is part of the pathogenesis of the disease.

The purposes of this study were to determine:

(i) whether the urinary content of the postulated end products and intermediates, differed in acute laminitis and normal horses.

(ii) if intestinal micro-organisms were responsible for the changes.

Organic acids, such as orotic acid, hippuric acid, together with phenols/cresols were investigated in samples of urine from normal horses, clinical cases of acute laminitis and from a horse given oral antibiotics.

9.2.2 Materials and Methods

Normal horses

A group of 8 clinically normal horses were kept together at grass under controlled management. Spontaneously voided urine was collected and frozen at - 20° C until analysis.

Clinical cases of acute laminitis

Local veterinary surgeons collected clinical samples of spontaneously voided urine from horses diagnosed as having acute laminitis. These samples were kept at - 20° C until needed.

a) Alteration of intestinal microflora.

One of the group, a three year old mare, was given oral antibiotics - 4g oxytetracycline (Terramycin, Pfizer Ltd, Kent); metronidazole (RMB Animal Health Ltd. Kent) and penicillin/ streptomycin powder in a small feed. A small quantity of finely chopped yellow sponge was also added to the feed to assess transit time when seen in the droppings. Urine was collected through a bladder catheter beforehand and at daily intervals. Samples were frozen at - 20° C.

b) Administration of sodium benzoate and restoration of intestinal flora.

A solution of 40g Sodium benzoate (Sigma, Poole, Dorset) was prepared in 1L 0.9% saline, 20 % dextrose solution (Baxter, Thetford, Norfolk, U.K.) pH 7.6. This solution was sterilised by passing through a 0.2µm filter (Nalgene Filterware, Nalco Co., New York, U.S.A.) in sterile conditions in a laminar air flow hood. When the transit time markers were seen in the faeces two days later, this solution was given intravenously through an indwelling catheter. Intestinal microflora were reseeded the following day by administration of faeces from another horse suspended in a 1 L solution of electrolytes and glucose (Lectade, SmithKline Beecham Animal Health, Surrey, UK) through a

nasogastric tube. Twenty four hours later a sterile solution of 8g sodium benzoate in 40 mls of 0.9% saline (Huddersfield Royal Infirmary Pharmacy) was given intravenously.

Analysis

Analysis of organic acids was by gas chromatography mass spectrometry (GCMS) using standard methods of extraction and evaluation. Organic acids are extracted from acidified salt saturated urine. The extracts are evaporated to dryness under nitrogen and trimethylsilyl (TMS) derivatives formed using BSTFA and pyridine. TMS derivatives are identified using gas chromatography mass spectrometry GCMS (electron impact). Derivatisation is necessary to produce compounds that are thermally stable, chemically inert and volatile below 300°C so that separation can be achieved by GC. Compounds are partitioned between a moving inert carrier gas (He) and a stationary phase (non-volatile liquid) coated directly onto the inner surface of the capillary column, and elute at characteristic times (retention time). The eluant from the GC enters the electron ionisation chamber of the mass spectrometer where the molecules are ionised and fragmented by collision with an electron beam giving a unique fragmentation pattern (mass spectrum). A compound is identified on the basis of its retention time and mass spectrum. (Dr Melanie Downing, personal communication)

9.2.3 Results

Urinary orotic acid could not be assessed as hippuric acid is chemically similar and present in large quantities; the peaks of hippuric acid are labelled on Figure 9.2.

Urinary profiles of normal horses

Normal horses showed an abundance of hippuric acid and a much smaller, variable peak of p-cresol. All normal horses (n=7) showed a similar pattern. GCMS urinary profiles of normal horses are shown in Figures 9.2- 9.8.

Urinary profiles of horses suffering acute laminitis.

Laminitic horses showed markedly different profiles, having reduced amounts of hippuric acid alongside increased amounts of p-cresol. The urinary profiles of these clinical cases (n=2) are shown in Figure 9.9 and 9.10.

Figure 9.2 GCMS profiles of urinary organic acids (hippuric acid and cresols) of a normal horse showing hippuric acid peaks and small quantities of cresols

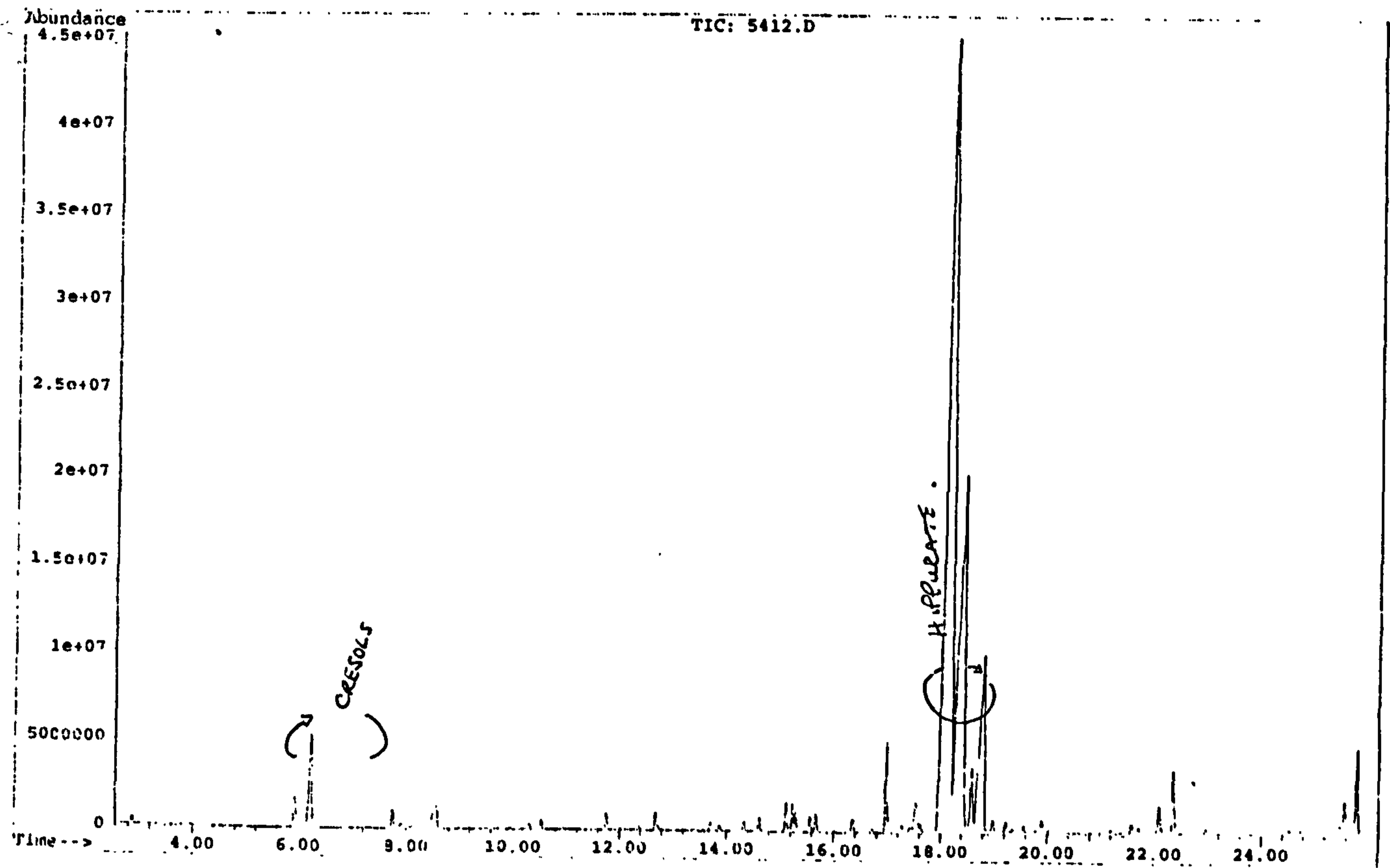


Figure 9.3 GCMS profiles of urinary organic acids (hippuric acid and cresols) of a normal horse showing hippuric acid peaks and small quantities of cresols.

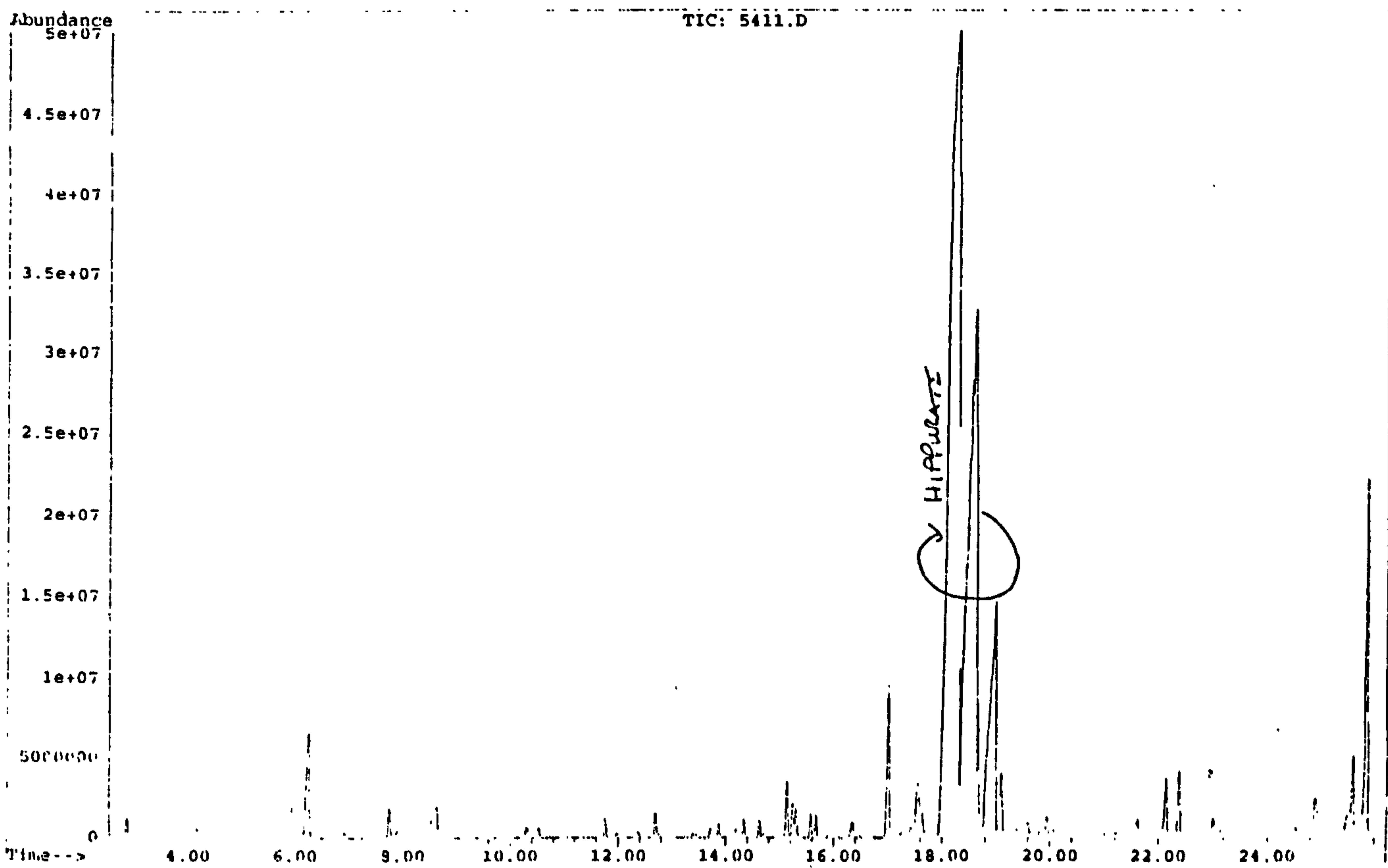


Figure 9.4 GCMS profiles of urinary organic acids (hippuric acid and cresols) of a normal horse showing hippuric acid peaks and small quantities of cresols.

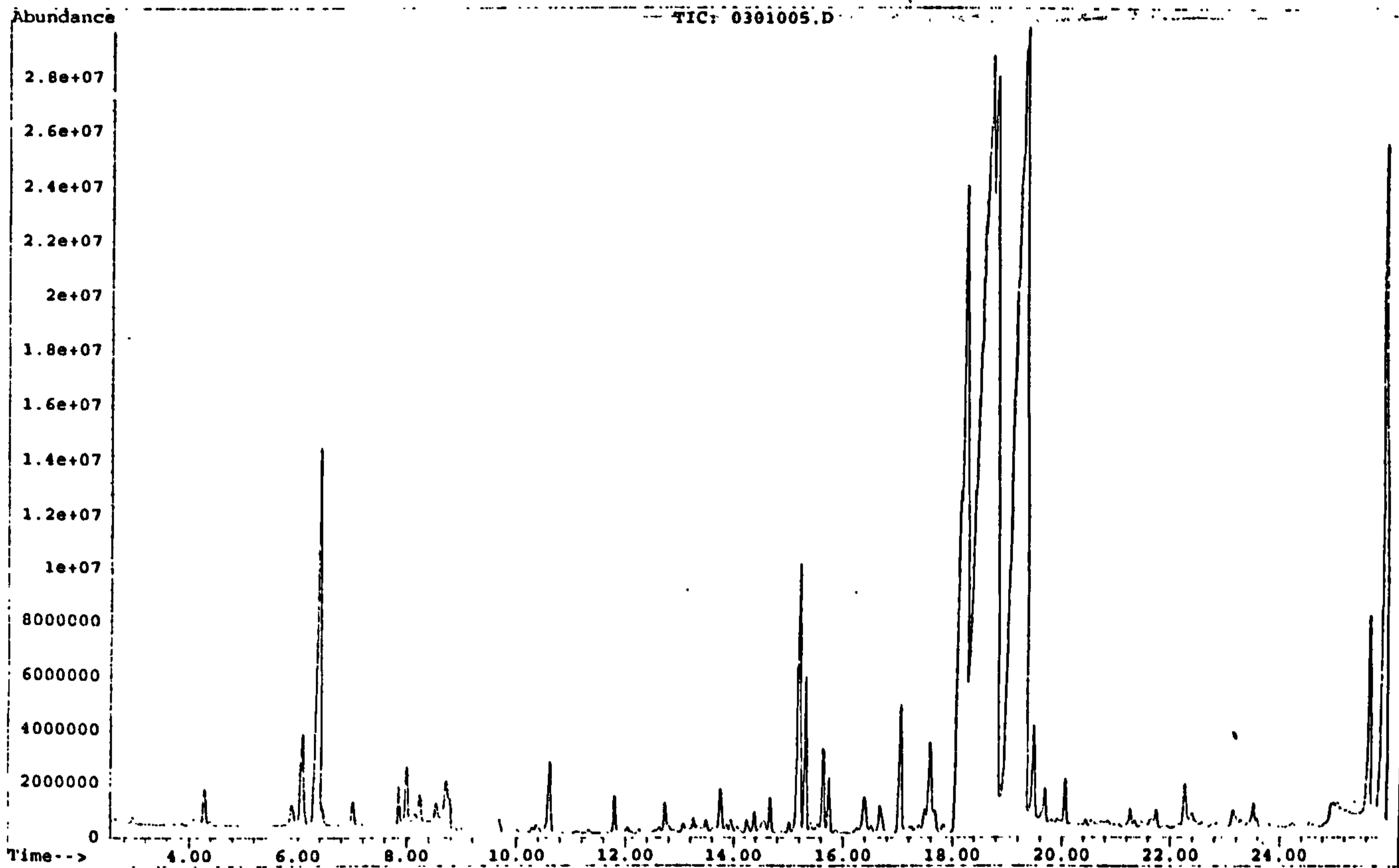


Figure 9.5 GCMS profiles of urinary organic acids (hippuric acid and cresols) in a normal horse showing hippuric acid peaks and small quantities of cresols

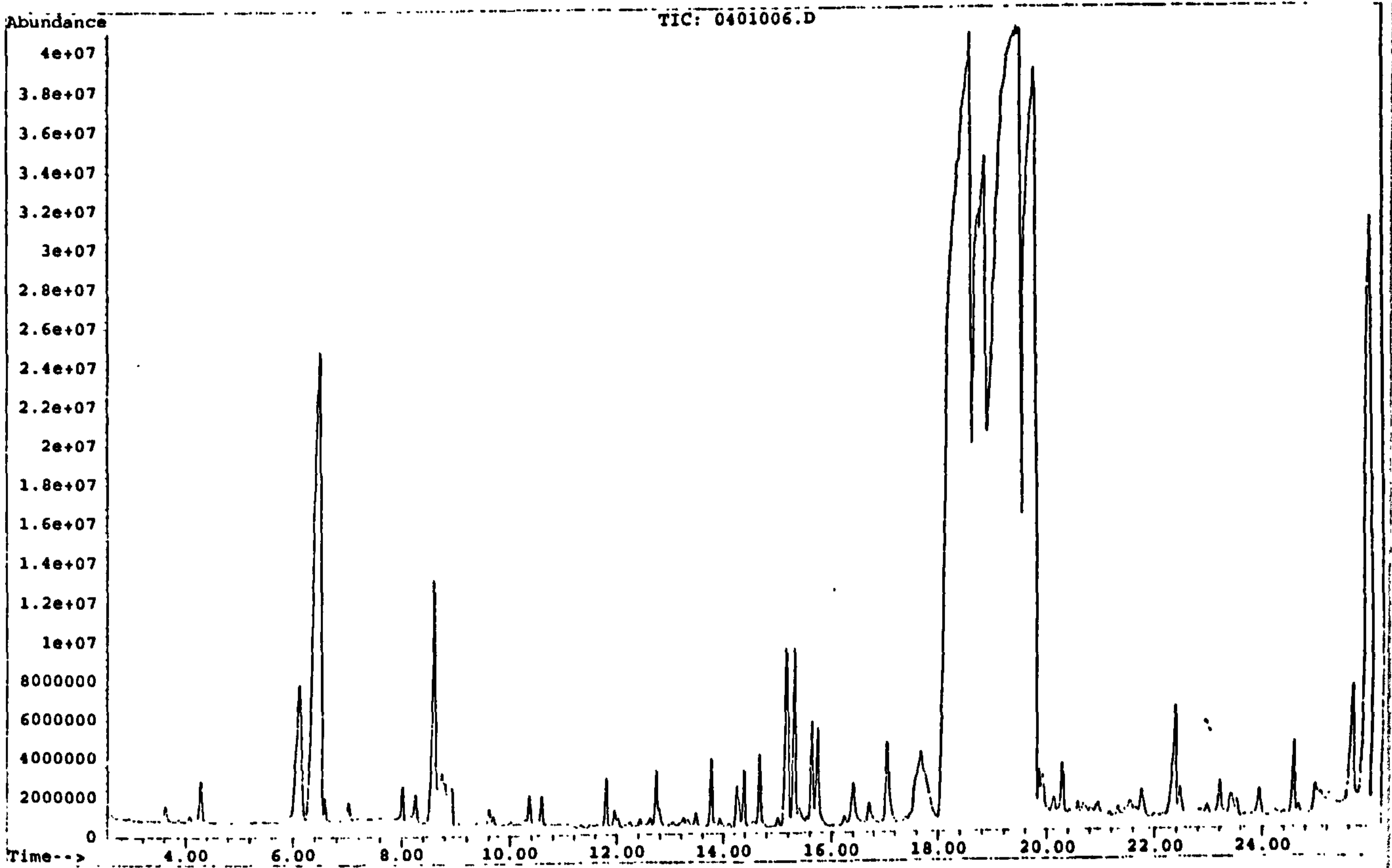


Figure 9.6 GCMS profiles of urinary organic acids (hippuric acid and cresols) of a normal horse showing hippuric acid peaks and small quantities of cresols.

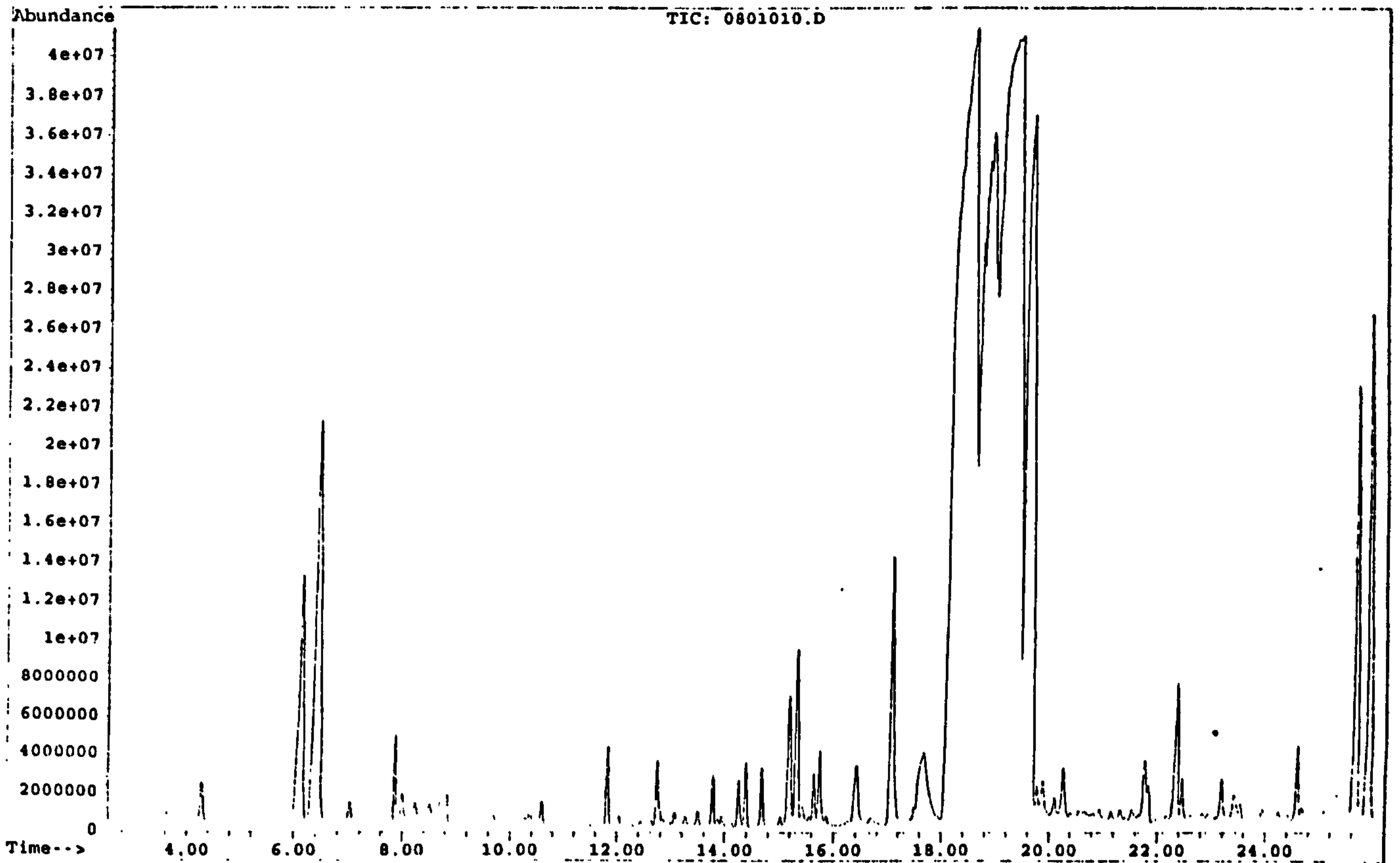


Figure 9.7 GCMS profiles of urinary organic acids (hippuric acid and cresols) of a normal horse showing hippuric acid peaks and small quantities of cresols

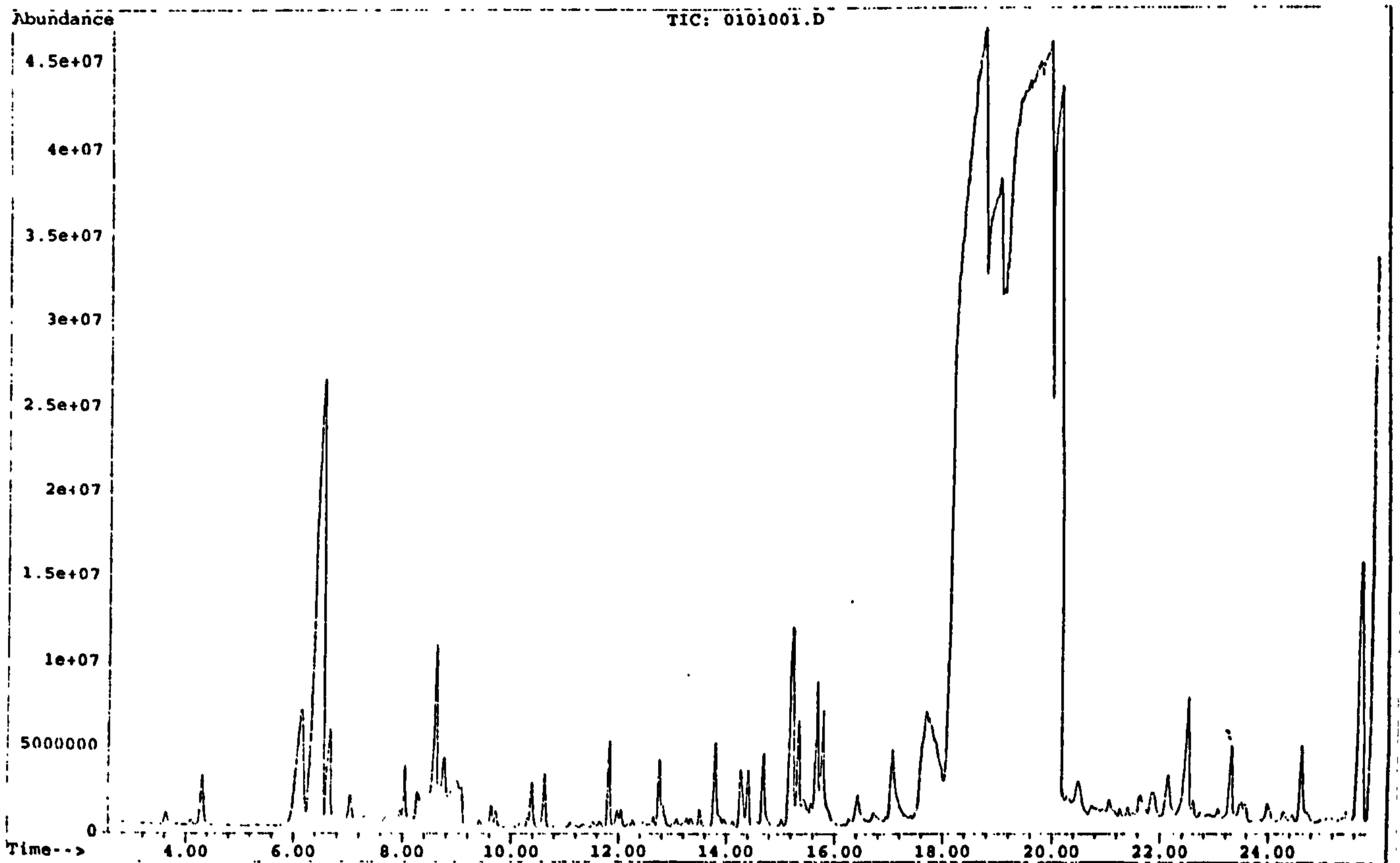


Figure 9.8 GCMS profiles of urinary organic acids (hippuric acid and cresols) of a normal horse showing hippuric acid peaks and small quantities of cresols

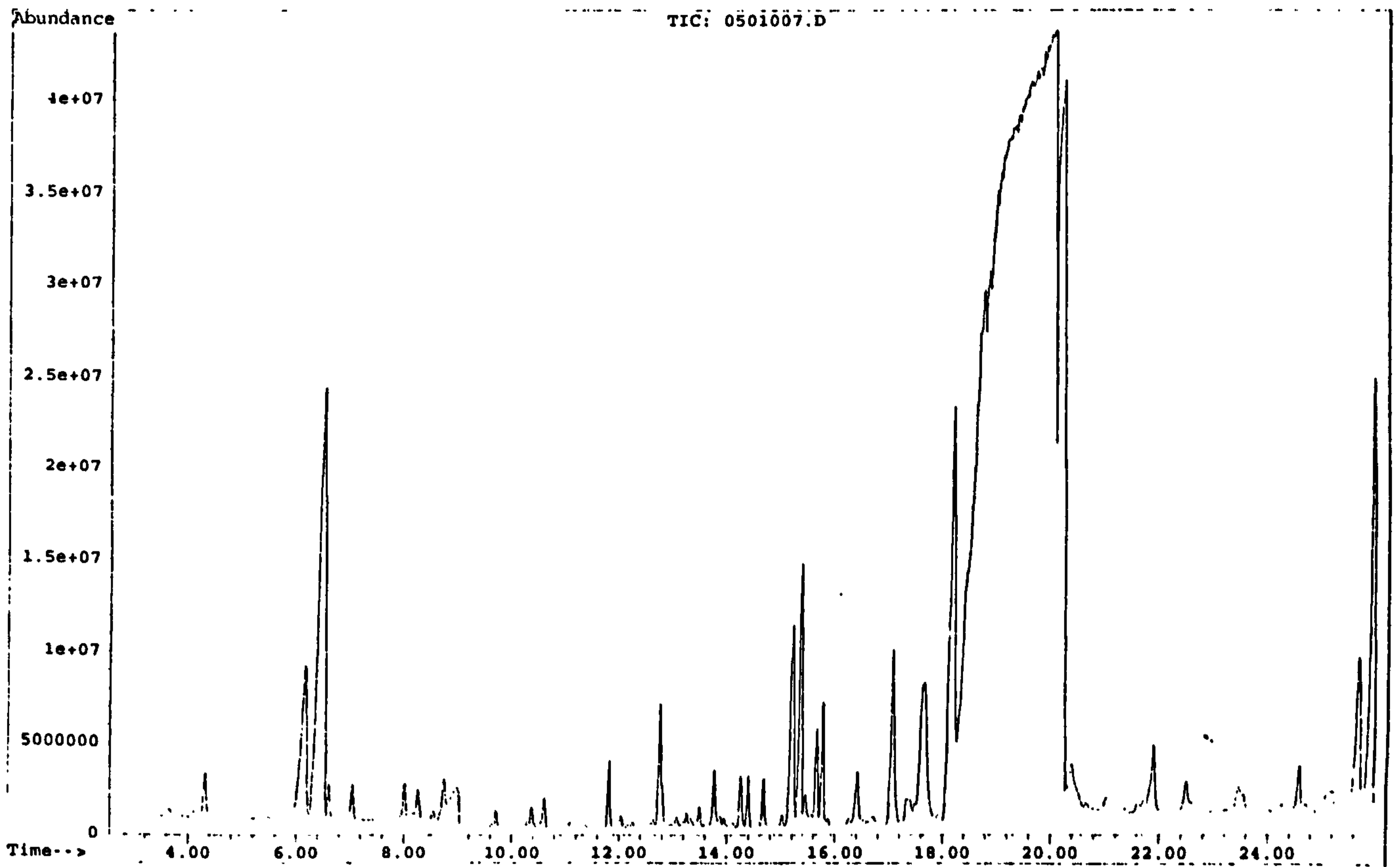


Figure 9.9 GCMS profiles of urinary organic acids (hippuric acid and cresols) of a horse with acute laminitis showing disappearance of the hippuric acid peaks and increase in cresol peaks

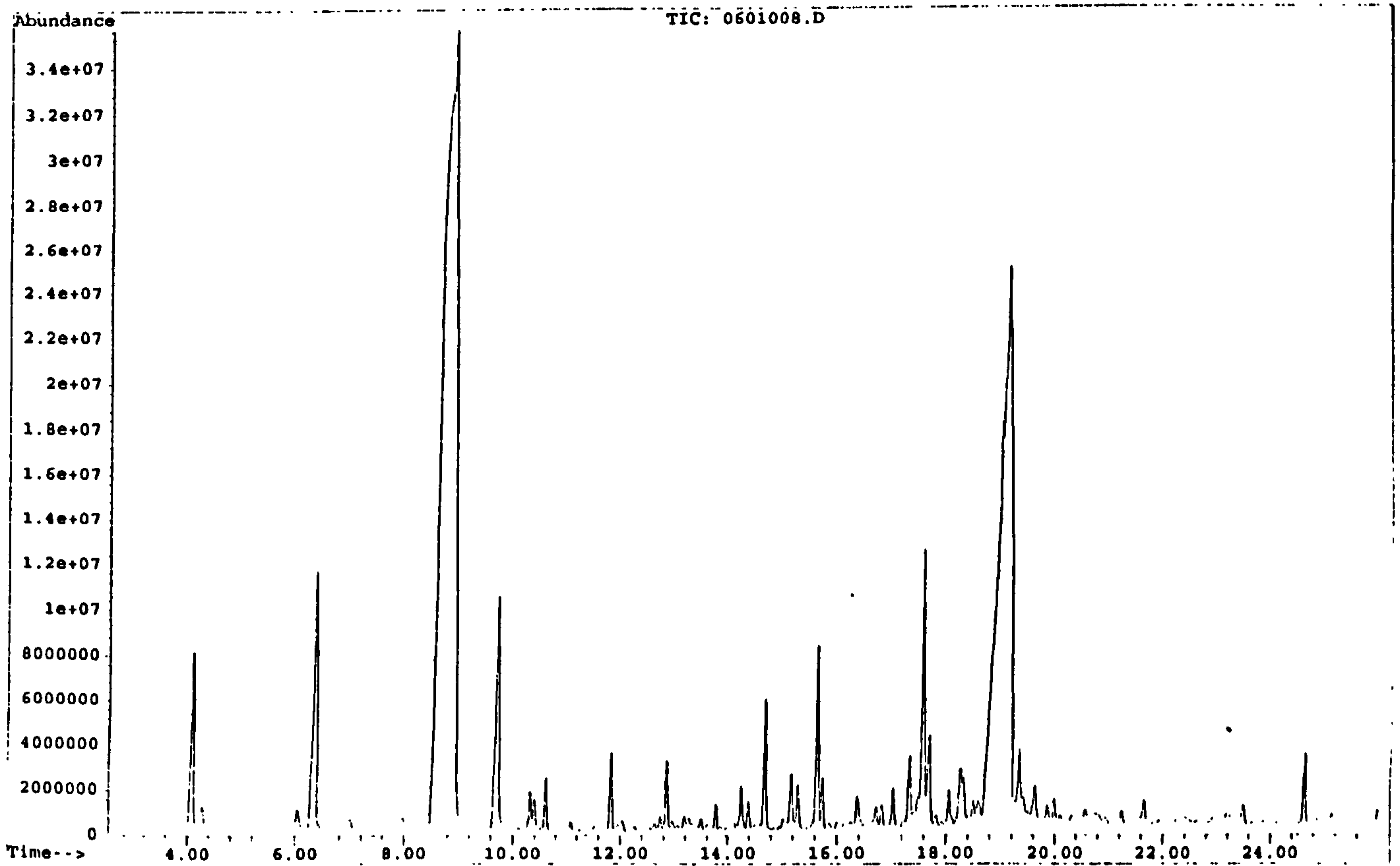
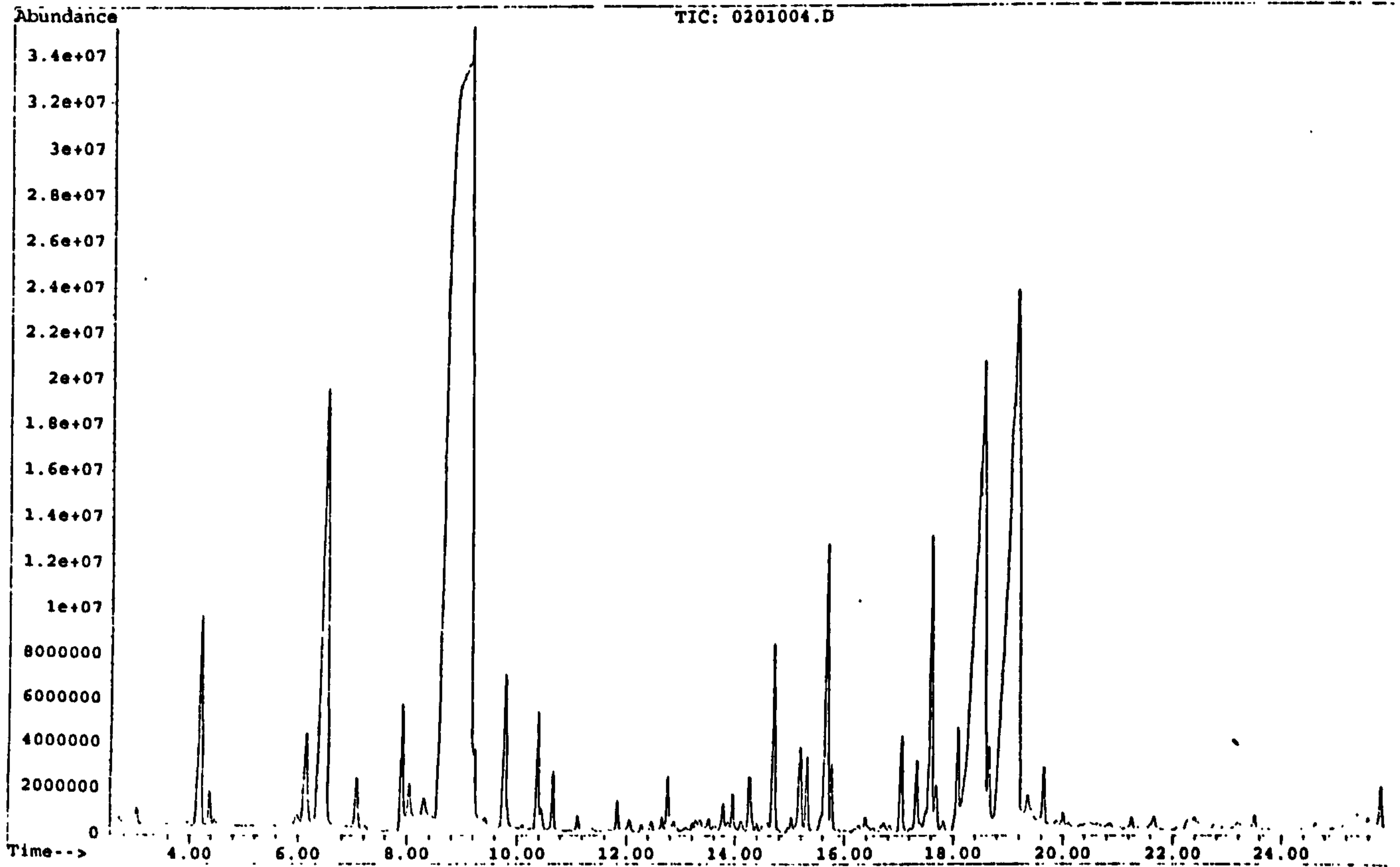


Figure 9.10 GCMS profiles of urinary organic acids (hippuric acid and cresols) of a horse with acute laminitis showing disappearance of the hippuric acid peaks and increase in cresol peaks



Urinary profile before, during and after administration of oral antibiotics to a normal horse.

The urinary profile before the administration of oral antibiotics is the same as those of normal horses - large peaks of hippuric acid and modest amounts of p-cresol. This profile is seen in Figure 9.11 The normal profiles continued for two days Figure 9.12 and 9.13 .

The urinary profiles changed at the same time as the transit markers appeared in the faeces 2 days later; the peaks of hippuric acid and p-cresol disappeared. (Figures 9.14, 9.15 and 9.16). Peaks of hippurate did not reappear until after the administration of sodium benzoate (Figures 9.17 and 9.18) and the peak of p-cresol reappeared. These events are summarised in Table 9.6

Clinically, the mare developed diarrhoea concurrent with the appearance of the transit marker; the diarrhoea was treated with standard methods of fluid therapy and ameliorative agents (Stat Liquid, Intervet, Cambridge), but laminitis did not develop even though digestion was disturbed.

Figure 9. 11 GCMS of urinary organic acids of a normal horse before oral antibiotics (normal profiles)

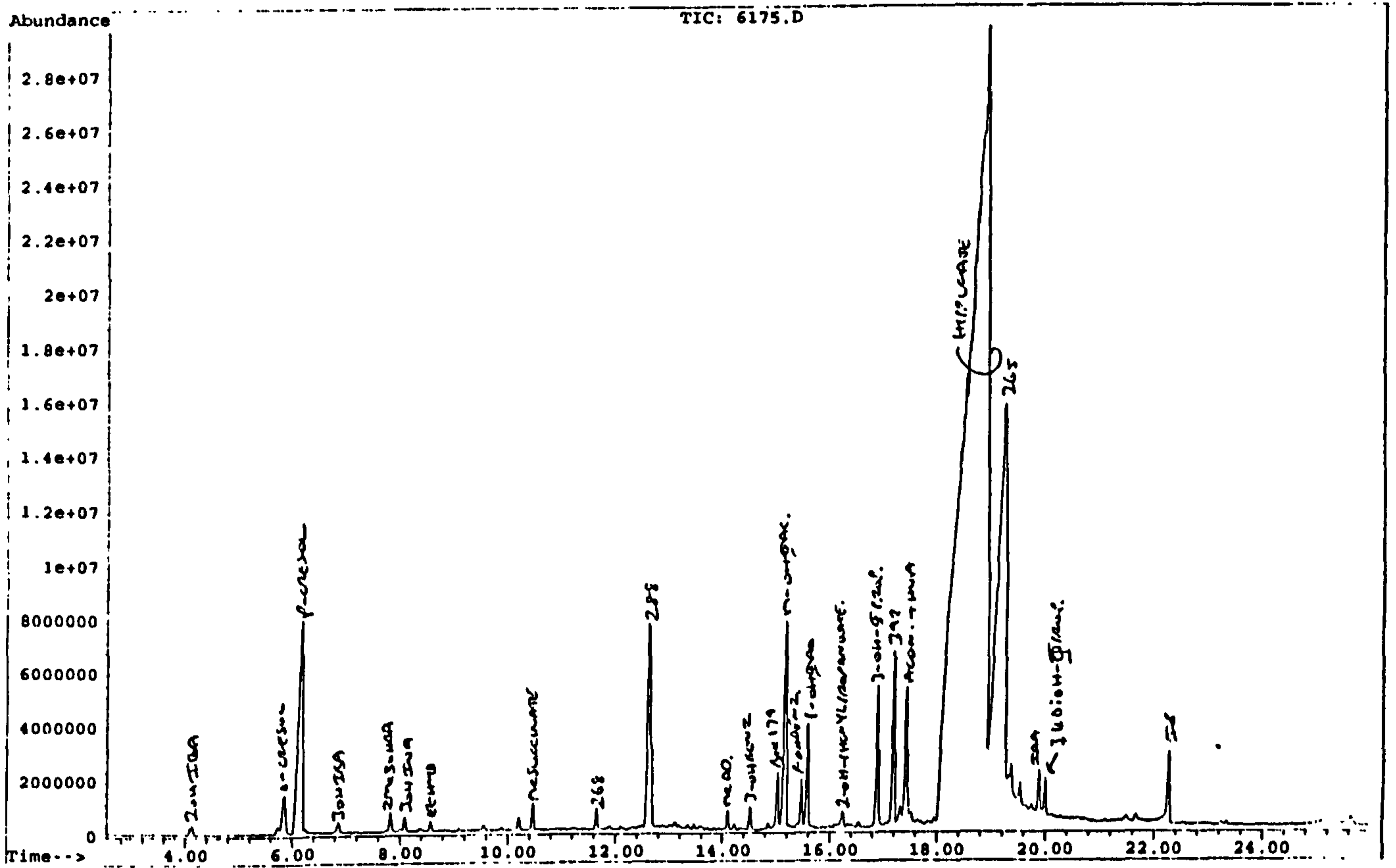


Figure 9. 12 GCMS of urinary organic acids of a normal horse 24 hours after administration of oral antibiotics (normal profiles).

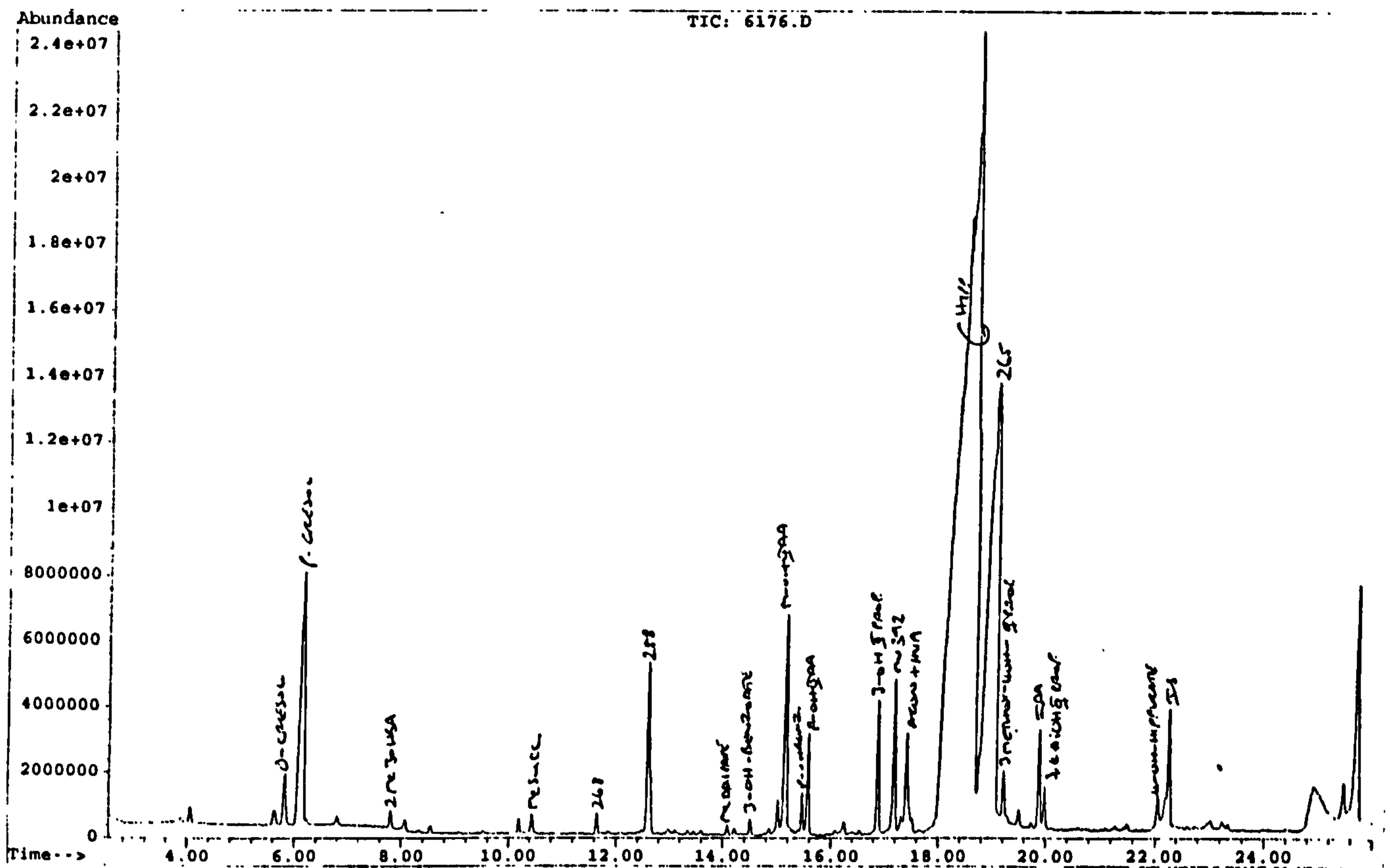


Figure 9.13 GCMS of urinary organic acids of a normal horse 42 hours after administration of oral antibiotics (normal profiles)

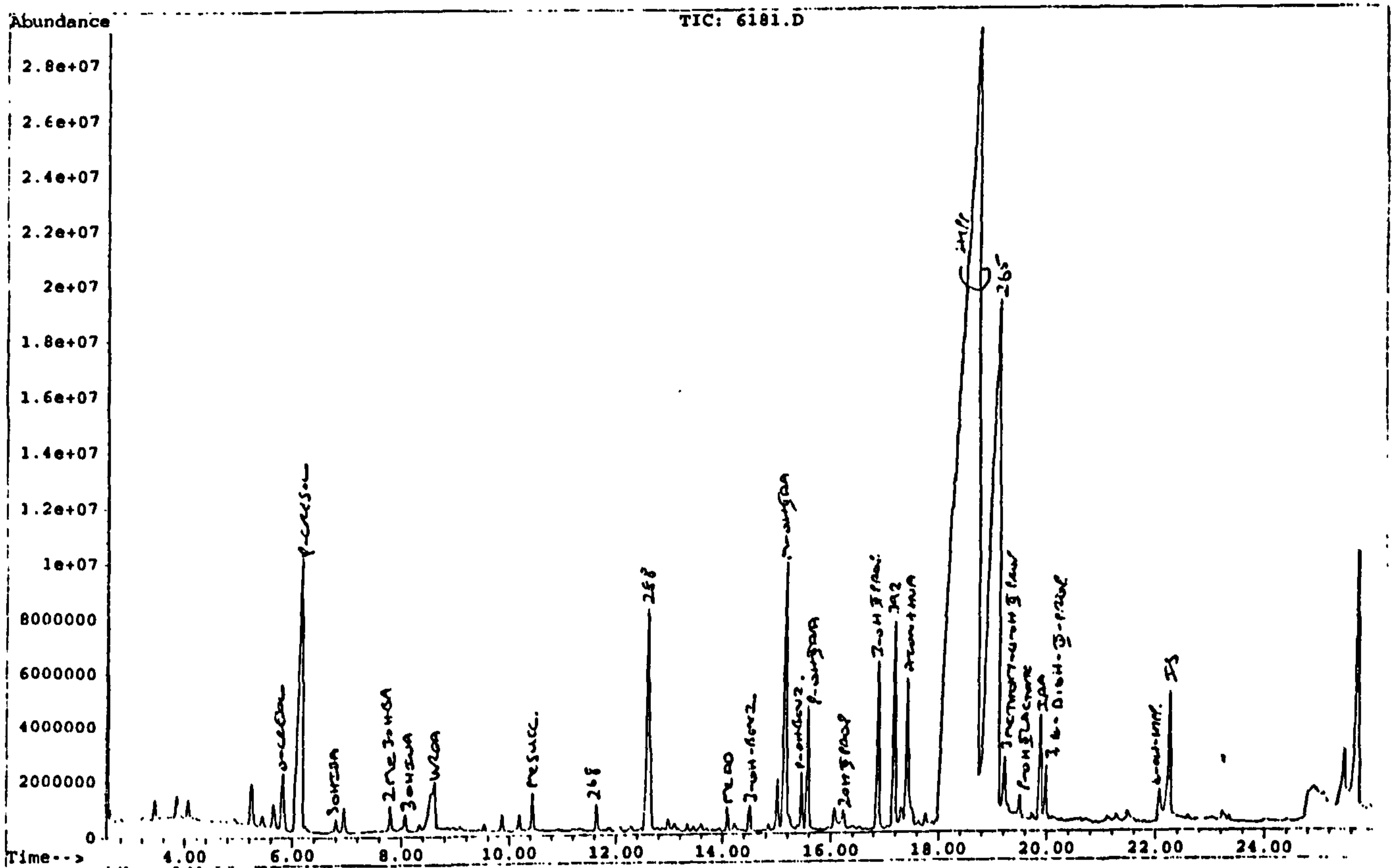
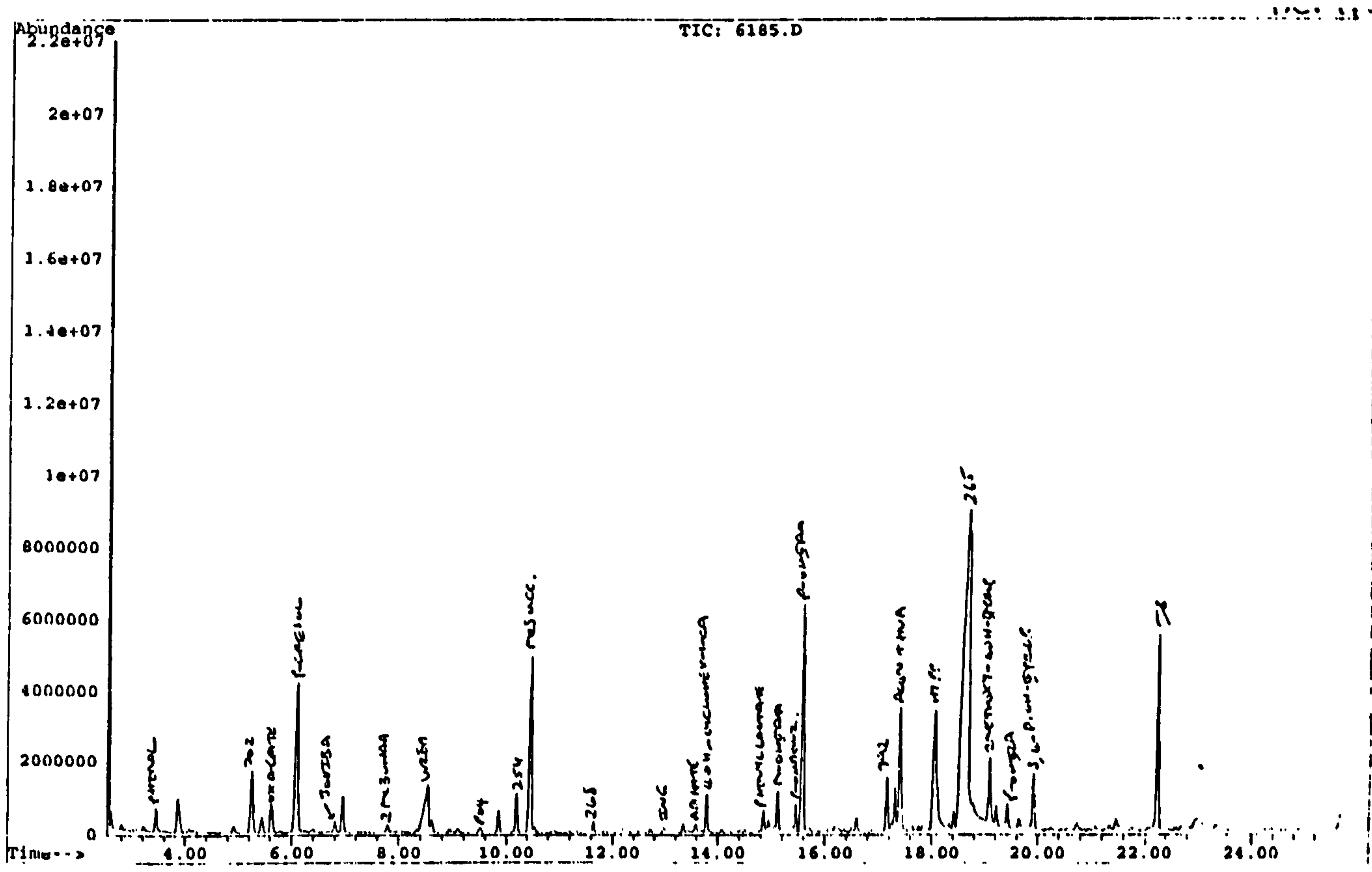


Figure 9.16 GCMS of urinary organic acids of a normal horse 108 hours after administration of oral antibiotics; hippuric acid concentration is low.



PAGE

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Table 9.6 Comparison of organic acids (hippurate and cresol) in equine urine by GCMS with opacity of urine during alteration of intestinal microflora with oral antibiotics.

		<i>Hippurate present</i>	<i>Cresol present</i>	<i>Urinary Appearance</i>
<i>Normal horses</i>		yes	yes	turbid
<i>Acute laminitis cases</i>		no	increased	less turbid
<i>Alteration of microflora</i>				
Fig 9.11	Before antibiotics	yes	yes	turbid
Fig 9.12	24 hours	yes	yes	turbid
Fig 9.13	42 hours	yes	yes	turbid
Fig 9.14	56 hours	no	no	clear
	transit marker appears			
	v. 60 hours	no	no	clear
	74 hours, begin sodium benzoate therapy			
Fig 9.15	84 hours	no	no	clear
Fig 9.16	108 hours	yes	slight	turbid
Fig 9.17	132 hours	yes	yes	turbid
Fig 9.18	156 hours	yes	yes	turbid

9.2.4 Discussion

Normal horses have an abundance of urinary hippuric acid and moderate levels of p-cresol. The urinary profiles of clinical cases of laminitis markedly differed. Clinical cases of laminitis have minimal levels of urinary hippuric acid and clearly increased peaks of urinary p-cresol.

The reasons for these changes are unknown but are possibly related to digestive disturbances of microflora concurrent with the disease. One of the clinical cases of laminitis had some digestive disturbance after ingesting huge amounts of feed following entry to the feed room. The other case developed laminitis whilst at grass during a period of rapid Spring growth. Alterations in intestinal microflora were likely to be responsible for the altered urinary profiles of these clinical cases.

When a mixture of oral antibiotics and antimicrobial agents were given to a horse, urinary hippurate disappeared. After sodium benzoate was administered and the intestine was reseeded with microorganisms, urinary output of hippurate was restored. It is uncertain whether sodium benzoate was responsible for the restoration of normal profiles or whether reseeded of the intestinal tract by opportunistic microorganisms restored normal synthesis of hippurate. It is difficult to separate endogenous and exogenous factors.

Bunge and Schiedeberg (1876) outlined the synthesis of hippuric acid in the human kidney from glycine and benzoate. Hippuric acid is also formed from glycine and benzoic acid in the liver (Chantrenne 1951; Brusilow 1991). It is said that the maximal formation of hippurate is only limited by the supply of glycine (Smith 1951). It is however extremely difficult to separate supplies of glycine arising from ingestion, microorganisms, or metabolic protein breakdown (Shoenheimer 1942).

The reduction in quantities of hippuric acid seen in the urine of clinical cases of laminitis may arise because :

(i) of inhibition, or blockade, of enzymatic pathways that participate in the production of benzoate.

(ii) the synthesis of hippurate is limited by substrate availability of a) cresols (themselves the substrate for benzoate production) or b) reduced glycine availability.

(iii) of degradation of hippurate to sodium benzoate and glycine. Although hippuric acid is usually excreted, Schmiedeberg (1881) found that extracts of animal tissues hydrolyse hippuric acid to benzoic acid and glycine by an enzyme which is probably similar to a carboxypeptidase (Fruton and Simmonds, 1953). Inappropriate upregulation of this enzyme to reduce plasma levels of urinary hippurate is possible, but this is unlikely to be a mechanism involved in laminitis.

(iv) competition for the renal tubular transport carrier for hippuric acid.

Hippuric acid depresses creatinine clearance ratios (Smith, 1951). Alteration in creatinine phosphokinase (CPK) has been reported in acute laminitis (Coffman *et al.*, 1972) and the altered urinary hippurate may explain this phenomenon. The renal secretory mechanisms for hippuric acid and p-cresols are highly complex and largely unknown for the horse.

The substrate for hippurate, benzoic acid is formed from m-cresol which is one of the phenol family (Professor E. Haslem, personal communication). A close relationship exists between phenols and their derivatives and the formation of benzoic acid, and in turn hippuric acid. Hippuric acid is freely excreted by the kidney but free phenols and other substances are not (Smith *et al.*, 1945). Hippuric acid depresses excretion of

phenol red and phenolic glucuronides (Smith 1951): whether such applies to the renal function of the horse which produces such relatively huge quantities is unknown. In laminitic horses, an absence of hippuric acid, a known inhibitor of cresol excreting mechanisms, is removed.

Little is known about the supply of cresols or phenols that are the potential substrates for benzoate synthesis. Some phenols are found in plants (Van Sumere, 1986) and the precursor of m-cresol is salicylic acid (Professor Haslem, personal communication) Salicylic acid is found in willow tree bark (*Salix spp*). However, plants are unlikely to be the only sources. It is likely that the major source of phenols, or cresols, is by degradation of fatty acids (Voet and Voet, 1990) either by metabolic breakdown or through synthesis by intestinal micro-organisms (Karrer, 1950).

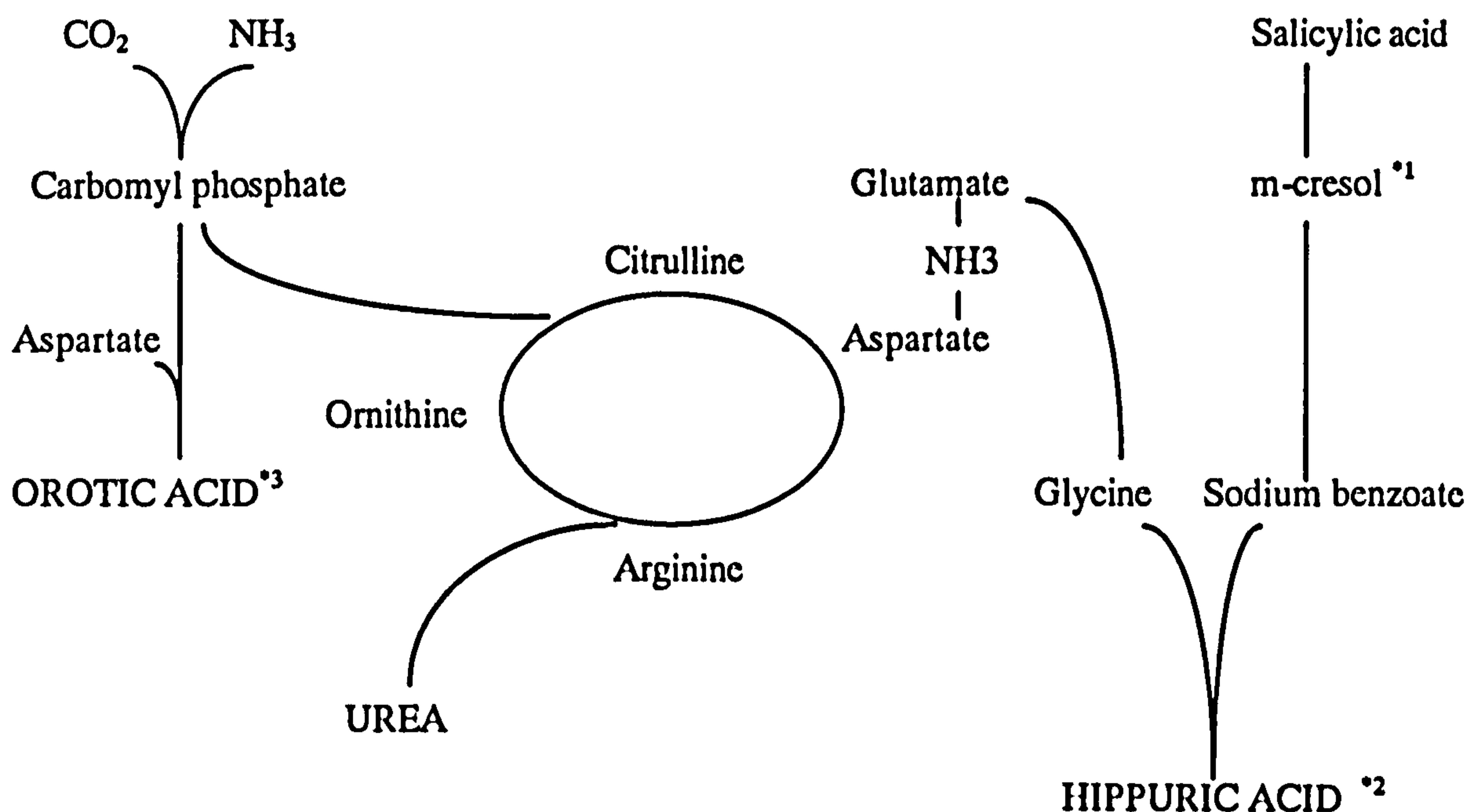
Removal of intestinal micro-organisms by oral antibiotics reduced the urinary content of both hippuric acid and p-cresol. This suggests that gut flora synthesise both hippurate and cresols, since if cresols remained in plasma they would have been excreted in the absence of hippurate; indeed, they would have increased. Cresols reappeared after the appearance of hippurate, suggesting that intestinal reseedling was the source of cresol or that there had been a breakdown of fatty acids concurrent with acute laminitis.

Digestive disturbance of intestinal microflora, or alteration of the microbial population, will disrupt the supply of the glycine/benzoate conjugate and urinary excretion of hippuric acid. Administration of intravenous glycine (included in one proprietary brand of vitamins) to horses suffering laminitis brings improvement whereas administration of similar vitamin therapies, containing other amino acids but no glycine, do not bring improvement (K.A.Hinckley, unpublished observations). Glycine may be an essential substrate for hippurate synthesis in the horse. Anecdotaly, willow branches are a 'horseman's cure' for laminitis (B. Payne, personal communication) and contain salicylic acid which is the precursor of sodium benzoate. Salicylic acid is better known as aspirin,

with analgesic and anti-platelet aggregating qualities(see Chapter 1). Glycine, salicylic acid, and possibly sodium benzoate, may be beneficial to horses prone to laminitis. It is undoubtedly beneficial to reseed the intestinal microflora and probiotics may be a worthwhile therapy (Galer, 1991). Probiotics would be especially beneficial after digestive disturbance or antibiotic therapy.

Nitrogenous waste can be incorporated into hippuric acid, as well as urea and ammonium, hence providing another mechanism for the removal of waste ammonia and to maintain acid base balance (Lewis, 1914). Sodium benzoate is used in paediatric medicine as a therapy for arginine deficiency, and hyperammonaemia, caused by inherited urea cycle enzyme insufficiency (Brusilow, 1991), where it binds to glycine and aids removal of ammonia. The relationships between arginine, orotic acid and hippuric acid are shown in Figure 9.17.

Figure 9.17 The physiological relationship between arginine, glycine, sodium benzoate, hippurate and orotic acid(after Milner, 1985; Brusilow, 1991).



*1 (Professor Haslem); *2 (Brusilow, 1991) *3 (Milne, 1985)

Hippuric acid is closely related to orotic acid seen in the urine of rats deprived or deficient in l-arginine (Alonso and Rubio, 1989). However, orotic acid is not seen in urine taken from humans deprived of dietary arginine (Carey *et al.*, 1987). Orotic acid is reported as having a protective cardiovascular effect (Williams, 1992). It is possible that there is an as yet unknown mechanism whereby the closely related hippurate affects equine vasculature functions. Equines do not suffer from arteriosclerosis, coronary heart disease or hypertension. This may be because of dietary factors or because of inherent difference in metabolism. Whether hippuric acid plays a role in cardiovascular regulation remains to be investigated.

Chapter 10

Discussion - Speculation, Conclusions and a Field Theory of the aetiology of laminitis.

*" You will eat, bye and bye,
In that glorious land above the sky;
Work and pray, live on hay "*

Joe Hill (1879 - 1925)

The horse evolved from small, padded toed animals that browsed bushes and boughs of pre-historical forests to fleet-footed hoofed beasts that, despite increased size, could out-gallop its predators on the open grassy plains. Contemporary equines, that can be viewed in roadside fields during any car journey, have essentially similar physiology to that of their ancestors several thousand years ago. Unfortunately, although the purpose and function of the horse has hardly changed (it is still galloping , but on the racecourse rather than grass steppes), environmental changes render it less well adapted to contemporary environments.

The contented image of horse and ponies quietly grazing green pastures disguises the truth - horses are ill equipped to cope with modern grazing. Stock rearing or dairy farming aim to produce large quantities of high quality silage as cattle fodder and to this end new varieties of grass have been developed to enhance growth, protein and water soluble carbohydrate contents. The composition of grass is entirely different to that of 50 years ago. Artificial feeding of carbohydrates is associated with the pathogenesis of laminitis in cattle :-

"A diet rich in carbohydrate is the most important contributing factor in [bovine] laminitis" (Greenhough, 1990).

Bovine laminitis has a high incidence affecting an estimated 25% of dairy cows nationally (Kirby, 1993) and is acknowledged to be a serious problem of the dairy industry (Greenhough and Vermunt, 1995; Logue, 1995).

Equine laminitis has a lower incidence than bovine laminitis and is also linked to ingestion of grass but the mechanisms involved have not been established. Ingestion of grass is known to cause most cases of equine laminitis and there is a seasonal incidence (see Chapter 2). The months when laminitis occur are Spring and Autumn, when growing conditions for grass are optimal. Surprisingly, the results of the surveys (Chapter 2) did not show a difference between North and South in terms of the seasonality of laminitis. It might have been expected that the 'laminitis season' would have started in the South of the country and spread northwards, concurrent with increases in soil and air temperature. This was not apparently the case. Anecdotally, laminitis affects animals throughout the country at the same time - animals at grass will contract laminitis from Dorset to Cumbria literally in one weekend (C.M.Colles, personal communication). Similarly, in the U.S.A. animals at grass will contract laminitis during periods of high barometric pressure (R.Redden, personal communication). Observations on the experimental herd of ponies confirm these anecdotal accounts. During warm weather, providing rainfall is adequate, grass grows steadily and is lush but animals are unaffected by laminitis; if there is a hard overnight frost or drought, then laminitis occurs. In contrast, ponies kept on nearly bare 'starvation' paddocks still contract laminitis despite reduced ingestion of grass; even a very small quantity of grass can cause an acute attack of the disease in vulnerable candidates (Eustace, 1992). What are the substances in grass that cause these effects ?

Most horses spend all or part of their time "at grass". It is obvious, even to the casual observer, that grass grows at different rates throughout the year. It appears more vividly green in colour in the Spring but yellow or brown during summer or winter. Such changes reflect adaptive physiological / biochemical responses of the plant to environmental changes, but there are less obvious, perhaps more relevant, responses that are just as important. Seasonal variations in the soluble carbohydrate levels in grass species are well documented (Pollock, 1991) and of commercial importance to farmers. Fluctuations may be diurnal, seasonal or long term. Changes in day length, rainfall,

temperature and nutrient availability produce short , medium or long term responses. Other factors such as intensive grazing pressure, fertilisers, pollution, carbon dioxide levels, and even 'global warming' are all relevant. Unlike animals, plants cannot maintain their own environmental conditions within a constant range by homeostasis nor can they move to enjoy a more favourable environment ; they must respond as best they can and the result is considerable physiological variation (Pollock, 1991).

Even when horses are kept on farm land there can be such a variation in plant responses that agricultural data can only be used to draw broad conclusions on matters such as geographical distribution, seasonal and species variations. A separate study would be needed to assess the range of particular variations, field to field, day to day, hour to hour. However, a brief review of the botanical and agricultural publications reveals some general features of grazing land, and hence dietary variations of horses at grass. Of particular importance are water soluble carbohydrates (WSC), amino acids and fructans, the principle ingredients of modern varieties of grass.

Fructans, or fructoligosaccharides, are soluble carbohydrates; long chain polymers of fructose (see Waterhouse and Chatterton, 1993); these are stored principally in the vacuoles of photosynthetic tissue. Very broadly, plant families fall into two groups - those which use fructans (about 15%) and the rest (85%) that use starch, a polymer of glucose, as a storage mechanism (Pollock, 1990). Plants such as onions, Jerusalem Artichokes and leeks utilise fructans and store large quantities in the bulbs (Suzuki and Chatterton, 1993). All grasses use fructans for storage but clovers do not.

Traditionally, fructans were thought to have three physiological functions: a major or reserve carbohydrate store; an osmoregulator; a protective device to improve the freeze, or chill, tolerance of the plant. The first function is undisputed, as 15% of angiosperms store fructan as a principle reserve carbohydrate, but plant scientists are increasingly disputing the importance of the last two categories (Hendry, 1987). It is now suggested

that the distribution of fructan rich families in temperate and sub-tropical regions is associated with variation in rainfall rather than temperature (Hendry, 1993). In addition, low temperatures may restrict water availability and hence growth rates. Species that normally grow during early Spring may be challenged during cold spells. Growth during such variable conditions is by cell expansion rather than cell division. Plants utilising fructans in the vacuole carry carbohydrate reserves within a single cell and therefore need not rely on slow intercellular transport systems when rapid growth is required.

Obviously, such a mechanism for growth depends on high concentrations of fructans being stored when growth is inhibited, so that they can be used when compensatory rapid growth is required. Typically, this happens during periods of long day-length - which encourages photosynthesis - but when photosynthesis is inhibited by lack of water or cold temperatures. Fructans accumulate in grasses during Spring months when day length is increasing but when there are overnight frosts and/or periods of drought. Similarly, fructan concentrations will be greater in the Autumn when growth may be initiated by warm damp conditions then inhibited by the start of Winter frosts. The seasonal production of fructans occurs at exactly the months that ponies at grass contract laminitis. Ponies that are susceptible should avoid grass during months of high 'risk' (see Chapter 2). On a daily basis, concentrations of fructans are highest at dawn (G. Hendry, personal communication). If fructans are part of the pathophysiology of equine laminitis, then the daily ingestion of fructans by ponies could be minimised by turning out to grass later in the day rather than early in the morning. An association between laminitis and grass biochemistry is clearly present.

Fructan synthesis is a response to stress and plants may produce fructans when stressed by intensive grazing, damage by animals hooves, or if challenged by fungal infections, cold temperatures or water deprivation. Closely cropped pasture may produce more fructans than lush unstressed swards - this could explain why ponies on bare pastures still contract laminitis. Symbiotic fungal parasites of perennial ryegrass also induce fructan

synthesis. An epidemiological study has suggested a relationship between the incidence of acute laminitis and the distribution of endophyte infection in the U.S.A. (Rohrbach *et al.*, 1995). Fructans are therefore potential candidates in the pathogenesis of laminitis, especially as ingested fructans are known to alter intestinal microflora in man, rat and pig (Farnsworth, 1993). Ingested fructans enhance weight gain in pigs and it is possible that the 'fat laminitic pony' stems in part from the ingestion of fructans. The change in urinary profiles seen in Chapter 9 may also result from a digestive disturbance caused by fructan-induced changes in intestinal microflora. It remains to be seen whether ingested overload of fructans would have a similar effect to forced carbohydrate overload and induce laminitis and / or shock.

Constituents of grass vary in response to challenges during ideal growing conditions. If the plant is in any way challenged by environmental conditions during the growing season it will store large amounts of fructans in its herbage instead of synthesising amino acids (Hendry, 1993). Under ideal conditions, angiosperms (grass species) synthesise amino acids, particularly arginine (in the herbage) and lysine (in seeds). Dietary influences of arginine and lysine are of physiological importance as semi-essential and essential amino acids respectively. As arginine is the substrate for nitric oxide synthesis *in vivo* and is essential to the maintenance of basal vasodilatory tone, the ingestion and synthesis of arginine may be relevant to the pathophysiology of laminitis.

Arginine is an essential requirement for all mammals. It is part of the urea cycle and is the substrate for the production of nitric oxide which causes normal vasodilatation of blood vessels and is crucial for the maintenance of vascular health. Arginine is produced by grasses and stored in the leaves but, as mentioned above, plants sometimes produce larger amounts of fructans and lysine rather than arginine. How could such changes in diet, grazing, or concentrate rations be responsible for physiological changes or disease ?

Although the precise amino acid requirements of horses are unknown (Hintz, 1989), lysine is required by young animals for normal growth rates. However, studies on adult animals of other species suggest that excess dietary lysine leads to arginine deficiency since lysine inhibits hepatic arginase (Visek, 1983). Adult horses are routinely fed concentrate rations containing high levels of lysine (Hintz, 1989) but the precise effects of excess dietary lysine are undocumented in horses. Both horses and cattle receiving a high lysine diet are prone to laminitis and a connection is more than plausible. Excess dietary lysine results in acute or chronic arginine deficiency (Visek, 1983). Animals deficient in arginine are usually hyperglycaemic and hyperammonaemic (Morris, 1984) and there is usually a dramatic increase in hepatic lipids (Milner, 1985). An increase in hepatic lipids has been documented as routine in cases of laminitis (Colles and Jeffcott, 1977) and chronically laminitic ponies are hyperglycaemic and hyperinsulaemic (Freestone *et al.*, 1992). Excess dietary lysine may cause a short or long term deficiency of arginine, with either short or long term effects i.e. reduced vasodilatation and/or hyperglycaemia and eventually advanced glycosylated end products (AGE). Arginine deficiency is likely to cause a multitude of sub-clinical effects that may go unnoticed or produce ambiguous clinical signs that could be misinterpreted. Whilst a slight physiological deficiency of arginine may not be important under normal conditions, it may be a crucial part of a multifactorial aetiology.

It is hard to imagine how a deficit of l-arginine could occur when it is synthesised from other amino acids in the liver, kidney and small intestine and is a vital ingredient of the urea cycle. However, little if any supplies of arginine result from the urea cycle (Brosnan *et al.*, 1992). Arginine is synthesised efficiently in the kidney from the substrate citrulline and all arginine from this source is available to the circulation (Brosnan *et al.*, 1992). Much can be learnt about the mechanisms of arginine deficiency from cats. Cats have a hyperessential requirement for dietary arginine as they are unable to convert metabolic precursors. Indeed, eating only one meal deficient in arginine will kill a cat through the effects of hyperammonaemia (Rogers *et al.*, 1985). There are differences in feline

synthesis of arginine because of deficiency of the enzyme pyroline -5-carboxylate synthase which converts glutamate to pyroline-5-carboxylate (P-5-C). P-5-C is then processed to ornithine, which is then converted to citrulline - a substrate of arginine. The regulatory processes that felines utilise as compensation for intermittent dietary supplies of arginine are unknown.

Evidence in other species suggests that arginine is not supplied from the urea cycle (Brosnan *et al.*, 1992). Plasma arginine is normal in stabled horses not ingesting arginine; the explanation is that horses synthesise endogenous arginine from symbiotic microbial sources. The findings that plasma arginine increases when animals go to grass and are ingesting exogenous arginine support this assertion (Chapter 5). The pony at grass that suffered acute laminitis had lower than normal stabled values for one month prior to the attack, indicating a disturbance of the intestinal and hind gut organisms that normally synthesise arginine. The urinary profiles shown in Chapter 9 support the assertion that digestive processes are disturbed.

Spring grass is high in WSC (McDonald, 1991) which would be quickly taken up by those pony breeds predisposed to efficient metabolic processes to utilise periods of plenty. Ponies are four times more likely than horses to contract laminitis when at grass during high risk times of year (Colles, 1977). Genetic predisposition has been suggested but not substantiated in cattle and in some breeds of horse (Riber *et al.*, 1991; Hood, 1995, personal communication). One pure breed of horse in the USA apparently suffers pedal rotation without the usual vascular preliminaries (Hood, 1995, personal communication). However, factors that predispose certain breeds to laminitis are probably secondary to genetic metabolic and environmental differences. For example, hill ponies have evolved efficient intestinal transport upregulation (particularly in herbivores) and efficient feed to body weight conversion to overcome periods of hardship in much the same fashion as certain races of people are now predisposed to diseases of plenty

such as diabetes mellitus (Buddington *et al.*, 1987; Buddington and Diamond, 1992; Ferraris *et al.*, 1992).

Hyperglycaemia has been associated with peripheral vascular complications of diabetes mellitus by glycation of proteins (Brownlee *et al.*, 1986; Brownlee *et al.*, 1988; Williamson *et al.*, 1992) and reduced vascular elasticity resulting from permanent endothelial damage (Vlassara, 1992; Hsueh and Anderson, 1992). Glycation is defined as the non-enzymatic attachment of glucose molecules to amino acids (Brownlee *et al.*, 1986). All proteins can be glycated and the final product - advanced glycosylated end products (AGE) are very stable. AGE are known to “quench” nitric oxide and compromises vasodilatation (Bucala *et al.*, 1991). Similarly, fructose molecules readily attach to proteins producing very stable compounds (fructosamines) in a virtually irreversible fashion (Kennedy, 1992). Ingested fructose increases blood pressure, and promotes insulin resistance (Martinez *et al.*, 1994). Ingested fructose attenuates nitric oxide vasodilatation and increases sensitivity to inhibitors of nitric oxide synthesis (Martinez *et al.*, 1994). Ingested fructose or fructans are likely to be implicated in the pathogenesis of equine laminitis and will have a considerable physiological effect. A measure of exposure to ingested fructose/ fructans was sought. If horses are exposed to high levels of WSC and / or dietary fructans it is reasonable to assume that blood sugars would increase and that glycated haemoglobin and fructosamines would be produced. In some personal observations by the author hyperglycaemia, assessed with diabetic sugar testing devices (Accutrend and BM test 1-44 test strips, Boehringer Mannheim, Lewes, Sussex, UK) was not apparent in the clinical or controlled animals (3.0 $\mu\text{mol/L}$) contrary to reports that basal hyperglycaemia (5.0 $\mu\text{mol/L}$) is commonplace in laminitic cases (Jeffcott *et al.*, 1986 ; Freestone *et al.*, 1992).

Glycated haemoglobin and fructosamines are diagnostic tests for diabetes mellitus and reflect exposure of the individual to sugars over the previous 6 weeks or so (Johnson *et al.* 1982). Glycated haemoglobin and fructosamines were determined in normal horses

kept under controlled conditions. Glycated haemoglobins in the normal group (n = 5), kept under controlled conditions varied between 0.7% and 0.9% to 4.1% and 5.7 % which were within or below the normal human range of 3.5% - 6.8%. Glycated haemoglobin depends on an even rate of turnover of haemoglobin and, if this is not relatively constant, then the test is not reliable. Horses are subject to considerable endoparasitic challenges when at grass and although the experimental herd is subject to regular anthelmintic prophylaxis, haemoglobin turnover is not necessarily constant as the parasitic worm burden varies between a minimum and maximum. Glycated haemoglobin was not considered a reliable indicator to WSC in horses. Fructosamines are bound to plasma proteins, rather than haemoglobin, and therefore have a lower turnover than glycated haemoglobin so could be a better test of WSC intake.

In preliminary analyses, fructosamines were determined in clinical samples from laminitic horses by photometric determination (Boehringer Mannheim, Lewes, Sussex). Normal horses (n= 10), kept under controlled conditions had plasma fructosamine concentrations of $277 \pm 2 \mu\text{mol/L}$ (range 252 - 304 $\mu\text{mol/L}$). Normal human values are $< 300 \mu\text{mol/L}$. Samples from acute and chronic laminitic ponies (n = 33) had slightly higher levels of $288 \pm 5 \mu\text{mol/L}$ and a wider range (222 - 390) than normal animals kept under controlled conditions. Some clinical samples of normal ponies at grass had higher than normal values. It may be that these animals were about to develop acute laminitis; certainly some had noticeable 'bounding digital pulses' but were not overtly lame. However it was not possible to control their management or to follow the cases. Variations in plasma fructosamines were nonetheless within the normal human range and the huge variations seen in diabetic patients who do not control their sugar properly were not seen. However, clinical cases of acute laminitis might not be typical as they were assessed after the onset of the disease. Once the animal has acute laminitis, fructosamines may not be such good indicators of risk as plasma would be flooded with protein and immunoglobulins from endothelial leakage and as an immune response to lipopolysaccharides to give a false 'low' value.

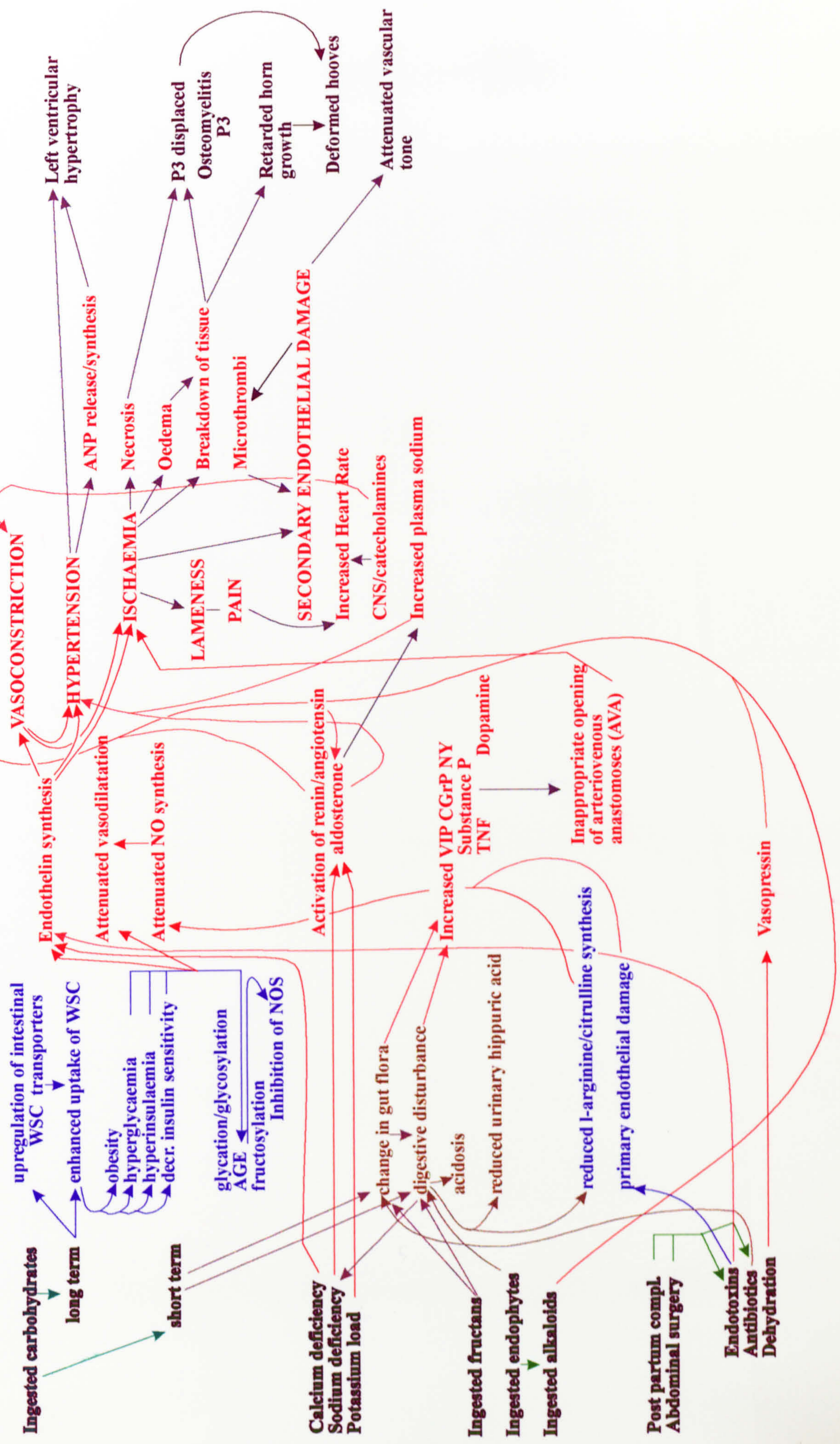
There was no monthly variation between March and October in either normal or laminitic horses (number of tests = 21 and 51 respectively). Responses to ingested glucose and insulin sensitivity are reported to be different in chronically laminitic groups; they maintain elevated blood glucose for longer periods than normal animals (Freestone *et al.*, 1992) but responses can be improved with diet and exercise. Potentially, fructosamines could therefore be used to assess the risk of laminitis. Fructosamines may indicate the risk of onset of acute grass laminitis and be part of the multifactorial pathogenesis outlined in Figure 10.1.

Figure 10.1 presents a scheme that attempts to describe the multifactorial aetiology of laminitis. Although complex it is by no means comprehensive and some of the connections are more tenuous than others; nevertheless the central hypotheses are all testable. Any one of the developmental factors shown in green on the left of the Figure 10.1, if sufficiently immense, will initiate acute laminitis on its own. In combination with other causative components a smaller insult is enough to instigate acute laminitis. Dietary factors are part, but by no means all, of the pathological picture, and the other physiological influences, and secondary effects, are shown in blue. Digestive aspects are shown in brown. The processes which occur during acute laminitis are outlined in red and long term chronic effects are shown in black. Clearly, the pathogenesis is very complex and the Figure is not intended to show all the processes involved but rather just to describe key relationships between some of the elements that have been addressed in this thesis.

DEVELOPMENTAL

ACUTE

CHRONIC



Dietary factors are implicated as a predisposing, or even causative factor, in equine laminitis and the physiological and metabolic connections are complex. Grass ingestion is only one of the factors involved in the aetiology of the disease and both carbohydrate overload and endotoxaemia are known to be causative factors in the disease. A high starch ration (Special Laminitis Diet, Theracon, Kansas) is used experimentally to induce laminitis (Trout, 1990), and laminitis is a frequent, if not inevitable, sequel to a horse gaining access to its feed shed. Ingestion of large quantities of carbohydrate has profound effects on intestinal microflora and production of amino acids, alters intraluminal pH and acidosis, diarrhoea and electrolyte loss.

There are such profound physiological effects that experimental induction of laminitis using carbohydrate overload must be regarded as an exceptionally blunt and imprecise approach. Many vasoactive peptides, such as vasoactive intestinal peptide (VIP), are synthesised under such circumstances, perhaps even by the micro-organisms themselves (Hood, 1995, personal communication), and have been proposed as prime agents in the induction of laminitis. Vasoactive peptide hormones of intestinal origin enter the circulation causing activation and opening of arteriovenous anastomoses in the digital microcirculation. This process may occur during grass induced laminitis. The events that occur during carbohydrate overload are probably a combination grass induced disease and endotoxaemia. Intestinal bacteria that are killed by the change in environment are lysed and the cell wall fragments absorbed by the circulation. The fragmented cell walls of Gram negative bacteria are known as lipopolysaccharides (LPS) or endotoxins.

Endotoxins can arise from other sources and under other conditions. For example, strangulating or non-strangulating infarctions of the small intestine result in necrosis of sections of intestine and absorption of endotoxins. This is why laminitis is often a sequel to colic surgery. Similarly, *post partum* complications (especially retained placental membranes) result in sepsis within the uterus and absorption into the circulation of huge

quantities of LPS which cause septicaemia and endotoxaemia. Once in the blood stream, LPS are responsible for profound inflammatory reactions and damage. The 'tail' of the LPS molecule binds to proteins including those on the endothelial surface. It destroys endothelial cells and some blood vessels are denuded of endothelium allowing platelet aggregation and microthrombi formation. LPS activate several inflammatory mediators, including macrophage mediated synthesis of tumour necrosis factor (TNF) and interleukin-1 (IL-1) which with phospholipase A2 (PLA2) also allows platelet aggregation and alteration of the endothelial cell surface (Elliot, 1995).

Similar histological changes are seen in the digital blood vessels of acute laminitic horses even before the onset of lameness (Hood *et al.*, 1993) and digital microthrombi are part of the pathology of laminitis (Weiss *et al.*, 1994). TNF is known to vasoconstrict equine digital arterial rings *in vitro* and *in vivo* (Baxter, 1994). TNF, IL-1 and AII downregulate NO synthase (Schini-Kerth and Vanhoutte, 1995). TNF is a powerful vasoconstrictor and it has been suggested to participate in endotoxic laminitis (Baxter, 1994; Molyneux *et al.*, 1994).

Many other humoral factors are also activated, including Hageman factor which activates plasminogen, resulting in fibrinolysis, the kallikrein-kinin and renin-angiotensin systems, catecholamines, β -endorphins, vasopressin and endothelin are released (Elliot, 1995). In addition, inflammatory mediators and cytokines, including prostaglandins, thromboxanes and leukotrienes, derived from arachidonic acid by cyclooxygenase and lipoxygenase, are likely to be involved. Most of the vasoactive agents (with the exception of nitric oxide and some metabolites of the arachidonic acid cascade) cause vasoconstriction, microcirculatory insufficiency and eventually end organ failure (Elliot, 1995). In other severe cases, disseminated intravascular coagulopathies (DIC) occurs with profuse bleeding and death. Human patients have a high mortality from DIC or septic shock, mediated by the inducible form of nitric oxide synthase in macrophages (*vide infra*). Cases of equine laminitis which have blood stained exudate at the coronary band have

the worst prognosis (Colles, 1991b) and such observations were made of the *post partum* case in this thesis. It is uncertain whether the bleeding was a direct effect of LPS mediated damage or whether it was secondary to the breakdown of laminal tissue. LPS have a considerable effect on the hoof growth making recovery difficult. LPS inhibit the differentiation of keratinocytes in that they divide but do not differentiate (Funquist, 1992) thereby inhibiting growth of horn tubules. This could explain why so many endotoxic cases survive the initial insult only to be destroyed 4 - 8 weeks after the acute attack when the digital structures collapse.

The many strategies to combat endotoxaemia include fluid therapy, antibiotics, non-steroidal anti-inflammatory drugs, cyclooxygenase and 5-lipoxygenase inhibition, glucocorticoids, thromboxane and platelet activation antibodies, oxygen free radical scavengers; such an exhaustive list indicates that none is truly effective ! In fact administration of antibiotics can increase the plasma concentrations of LPS as circulating bacteria are lysed. Although the incidence of laminitis following colic surgery has been reduced by administration of flunixin (Templeton *et al.*, 1985) and heparin (Stashak, 1987), these therapeutic approaches are equivocally effective. This suggests that the condition is multifactorial and /or other factors are more pertinent.

One of the most plausible candidates for involvement in the pathogenesis is endothelin (ET). ET is activated by TNF, IL2, corticosteroids, other "stress" hormones and, most relevantly, by endotoxins themselves (Doherty, 1992). It is also activated by shear stress and thereby may then oppose vasodilatation brought about by inducible synthesis of nitric oxide and endotoxic shock (Rubanyi, 1992; Lüscher, 1995). ET may have a beneficial effect, similar to the treatment of septic shock by synthetic inhibitors of NOS (Doherty, 1992), or a detrimental effect if vasoconstriction is pathological. If ET is synthesised pathologically within the microcirculation of the equine digit, then the 2 - 3 hour duration of action would cause ischaemia and tissue damage. Reperfusion damage would be inevitable and the sequence of events would be those known to occur in equine

laminitis. Vasoconstriction precipitates opening of arteriovenous anastomoses (AVA) and redirection of blood away from the dermal and epidermal laminae. The same redirection of blood can be achieved by pathological opening of AVA by vasodilators such as VIP, calcitonin gene related peptide or neuropeptide Y (Molyneux *et al.*, 1994). These events are not mutually exclusive and can occur in combination. Obviously, there will be individual variations according to the exact initiating events.

If, for example, there is experimental induction of laminitis by carbohydrate overload, individual responses will vary according to basal physiological status alongside endocrine sensitivities. It is astonishing that no-one has asked the question - why, when laminitis is induced experimentally, that only 50 % develop laminitis, 25 % remain normal and 25 % die of shock? Most ponies develop diarrhoea and there are major variations in the responses to digestive disruption and to the endotoxic insult. Obviously, LPS will be absorbed from the digestive tract and have the same effect as endotoxins from other clinical conditions. Some ponies develop laminitis following vasoactive hormonal induction of ischaemia in the digital circulation. Other animals respond to the influx of endotoxins by the IL₂ activation of inducible nitric oxide synthase (iNOS) to counter the invasion of toxins with nitric oxide synthesised from macrophages; the production of nitric oxide is beneficial in that it is bactericidal but has the adverse effect of inappropriate vasodilatation and septic shock. It is again surprising that no attempts have as yet been made to prevent the death and morbidity of numerous ponies by the administration of inhibitors of NOS, like LNMMA, L-NAME or selective inhibitors of inducible NOS like aminoguanidines (Curzon *et al.*, 1993; Griffiths *et al.*, 1994).

The ponies that remain stoically normal despite repeated administration (up to six occasions!) of the carbohydrate gruel reported in the literature (e.g. Templeton *et al.* 1985) are of major interest. Do these ponies have specific protective mechanisms or are the ones that develop laminitis predisposed to the disease? Both aspects are probably true.

The ones that develop laminitis may have been 'primed' by dietary factors *vide supra* but those that will not develop laminitis may have some protection. The protection may be because: (i) they have resilient populations of enteric bacteria; (ii) robust l-arginine and constitutive nitric oxide pathways; (iii) no excess exposure to WSC; or (iv) because of an immune response. If the ponies have been exposed to LPS before the induction of laminitis, then they will have developed an immune response and have antibodies to the LPS molecule. Exposure to the fragments of any of the Gram negative bacteria will provide some protection; exposure to infections (infectious diarrhoea, septic wounds etc.) will prevent the effects of laminitis, or any of the damage of exposure to LPS since the ponies will rapidly lyse bacteria and clear the endotoxic fragments.

A new product, recently licensed for use in horses, may prove to be an effective approach to prevention and treatment of endotoxaemia. An anti-lipopolysaccharide antibody (Stegantox, Shering -Plough, Welwyn Garden City, Herts.) could be of great value in the treatment of endotoxaemia and therefore be a prophylactic agent in the prevention of equine laminitis. Of course, the efficacy of the antibody is very difficult to evaluate and although long term clinical studies are being undertaken, the results will be difficult to interpret because of individual variation in the administration of the drug, the severity^{of} the insult, the differences in clinical treatments and in the animals' individual responses. One way in which the efficacy of Stegantox could be assessed is by NIRS.

Since endotoxaemia causes a vascular response - vasoconstriction, vasodilatation (shock) of bleeding, it should be possible to undertake clinical trials with this methodology and the results, which with the latest version of the instrument are quantitative, would provide definitive data. More subjectively, thermography could be used in conjunction with NIRS to evaluate the role of various hormonal influences to gauge the effects or onset of laminitis clinically. Anything which shows compromised vascular integrity before the onset of lameness will improve prognosis.

Anecdotally, animals that have suffered one attack of laminitis are vulnerable to further attacks from any cause. The original insult will have damaged the blood vessels at the cellular level as well as altering the gross structure of the digital circulation. It is certainly possible that irreversible damage to the endothelium is a candidate for making laminitic horses vulnerable to successive attacks of laminitis. However, rather contrary to expectations, long term endocrine responses do not seem to be altered during chronic laminitis (Chapters 6 and 7). Subsequent attacks are because of the innate physiological responses of the animal which were the cause of the original attack.

Mares that have suffered *post partum* laminitis do not seem to be vulnerable to grass laminitis, nor do ponies that have had grass laminitis seem any more prone to endotoxic laminitis than normal animals. Morphological changes in the hoof and in the circulation may mean that there is physically less tissue maintaining the hoof and fewer afferent blood vessels to the distal limb. This may mean that any alteration of the blood flow / integrity of the tissue has an extreme effect. The fact that the tissue is compromised is borne out by the NIRS study which showed that chronic laminitics have slightly hypoxic laminal tissue and that cytochrome oxidase activity differs to normal horses (Chapter 4). Thermography also revealed circulatory differences in the coronary band (Chapter 4). Such observations are in line with *post mortem* angiographic studies of the hooves and digital circulation of chronic laminitics (Pollitt 1995). Naturally, compromised circulation in any microvascular bed will allow a local niche for the actions of paracrine systems such as the nitric oxide and endothelin regulators.

Knowledge of non-reproductive endocrinology of the horse is limited. This may in part be because of technical problems in processing equine plasma which for reasons unknown is often highly viscous, difficult to extract, and equine plasma proteins show great cross reactivity with antibodies used in RIAs. The only record of equine measurement of equine ANP (McKeever *et al* 1991) used an antiserum that did not serially dilute and was rejected in the present study; any differences seen in hormone

levels could reflect changes in plasma protein concentrations rather than changes in the measured hormone. Such changes were seen in the initial measurement of AII with various antisera and the specificity of the antiserum in binding site to the molecule is obviously crucial. Similarly extraction methods minimised protein concentrations in the final sample. These reasons explain why equine angiotensins have not been measured, although AI and AII have been extrapolated from renin activity. AII has been suggested as a key factor in the pathophysiology of equine laminitis (Hood, 1979; Stashak, 1987) but basal values have not been established.

The present study therefore establishes normal circulating values for AII in the horse and plasma concentrations differed between animals stabled and those at grass (Chapter 6). It is not suggested that there is a seasonal difference in AII but rather that seasonal changes reflect changes in diet particularly those related to sodium and potassium in grass and fodder (Chapter 6). The high potassium content of grass and alongside chronic sodium deficiency may explain these observations. Although sodium deficiency would increase the RAS activity and increase circulating levels of AII there is more 'leeway' in the range of plasma concentrations that can be tolerated than that of potassium. A potassium load will therefore account for the suppression of the RAS as there is kaliuresis at the expense of sodium. Concurrent seasonal differences in ANP, parallel to those of AII, were also observed (Figure 10.2); these too are likely to be the result of dietary electrolyte excess or deficiency (Chapter 7).

Sodium deficiency will result in lower plasma ANP thereby limiting sodium excretion. Dietary sodium deficiency is greatest in the summer months and plasma ANP concentrations are at their lowest. These interactions that occur with seasonal dietary changes are important as they may restrict the normal homeostatic mechanisms of the horse that prevent the onset of vasoconstriction associated with acute laminitis - for example, activation of ANP synthesis in early stages. It is also possible that chronic deficiency of dietary sodium may be exacerbated by digestive disturbance and further

intestinal electrolyte loss. Acute sodium loss could activate the RAS despite the ingestion of a potassium load in grass and cause AII vasoconstriction (Figure 10.1). This would be particularly acute if the AII receptors were upregulated to compensate for normally low plasma levels of AII. This study reveals that AII levels rose at the onset of acute laminitis but they did not remain elevated for more than 1 day (Chapter 6). ANP levels also increased when AII and BP increased (Chapters 3 and 7).

The activation of ANP under these circumstances is multifactorial. First, blood pressure increases cause atrial stretch and ANP mediated diuresis and natriuresis; secondly, increased heart rate activates ANP; thirdly, increased plasma sodium during acute laminitis will prompt ANP mediated natriuresis. Plasma sodium increased during the first days of acute laminitis but usually decreased soon afterwards. The *post partum* case retained plasma sodium for several weeks and displayed a sodium appetite. This case was refractory to treatment and remained hypertensive after the acute stage. Whether the hypertension was because of constant pain and secondary sodium retention because of corticosteroids, or because transient increases in AII caused upregulation of AII receptors and sodium retention is unknown.

Usually, as horses and ponies are treated, blood pressure declines, and there are certain to be many interactions between AII and ANP which are responsible, partly because of systemic effects and partly because of local feed back mechanisms with paracrine systems such as the endothelin / nitric oxide relationship. Attenuation of vascular responses by glycation, competitive inhibition of NO synthesis, or by vasoconstrictor agents (such as endothelin, angiotensin and catecholamines), may predispose horses to laminitis.

Lupton (1901) was the first to mention sub-acute or developmental laminitis and it is now thought that developmental, or mild, laminitis is not diagnosed as frequently as it should be (Ridgway, 1995). If developmental stages go largely unnoticed, then acute laminitis can also be misdiagnosed in mild or severe cases. Mild cases can be diagnosed as laminitis when, in fact, a stiff gait is the result of azoturia or pedal osteitis; mild cases of laminitis are often overlooked (Ridgway 1995). Severe cases of laminitis are often referred to veterinary hospitals as surgical colic and, on arrival, are diagnosed as laminitis (J.G.Lane, FRCVS; R.Fitzgerald MRCVS, personal communications). It may be of assistance to veterinary surgeons especially those newly qualified or inexperienced in equine matters to use a diagnostic chart (Figure 10.2) to assess the probability of the horse or pony having laminitis according to its clinical signs and recent history. If undecided on a diagnosis the history of the horse can be combined with the clinical presentation and the likelihood of laminitis assessed by noting the colour of the box that combines the history and symptoms. According to the symptoms and history, the number of green boxes can be added and compared with the number of red. This will provide a rough probability of the likelihood of the animals suffering from laminitis compared with other possible diseases. For example, a horse that was rolling and had abnormal abdominal sounds after ingesting grain would have two green squares and one red one for recumbency, suggesting that colic is the most urgent consideration although the horse may develop laminitis as well. Immobility following uterine prolapse would have a red square indicating a high risk of laminitis. This chart, once again but in a different way to Figure 10.1, emphasizes the complexity of the syndrome.

Figure 10.2 Diagnostic chart to assess probability of the horse or pony suffering acute laminitis compared with other diseases.

A CLINICAL GUIDE TO DIAGNOSING EQUINE LAMINITIS

SYMPTOMS

CLINICAL HISTORY

- Previous history of laminitis
- Depression of the coronary band
- Obesity
- On rich grazing
- High feed intake
- Retained placenta
- Uterine prolapse
- Septicaemia
- Assisted foaling
- Recent colic surgery
- Recent normal foaling
- Recent surgery
- Pituitary adenoma
- Foot infection
- Stress
- No known history

Symptom	Previous history of laminitis	Depression of the coronary band	Obesity	On rich grazing	High feed intake	Retained placenta	Uterine prolapse	Septicaemia	Assisted foaling	Recent colic surgery	Recent normal foaling	Recent surgery	Pituitary adenoma	Foot infection	Stress	No known history
Solar Prolapse	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High
Loss of hoof capsule	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High
Hypertension	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High
Digital pulse, 4 limbs	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High
Walks with wt on heels	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High
Laminitic stance	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High
Lameness, 4 feet	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High
Digital pulse, 2 F. Feet	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High
Lameness, 2 F. Feet	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High
Recumbency	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High
Immobility	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High
Increased heart rate	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High
Sweating	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High
Toxicity	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High
Lameness, 1 F. Foot	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High
Stiffness	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High
Rolling	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High
Abnormal intestinal sounds	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High
Convulsions	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High	High

KEY:

High probability of laminitis

Laminitis likely, particularly as a secondary disease.

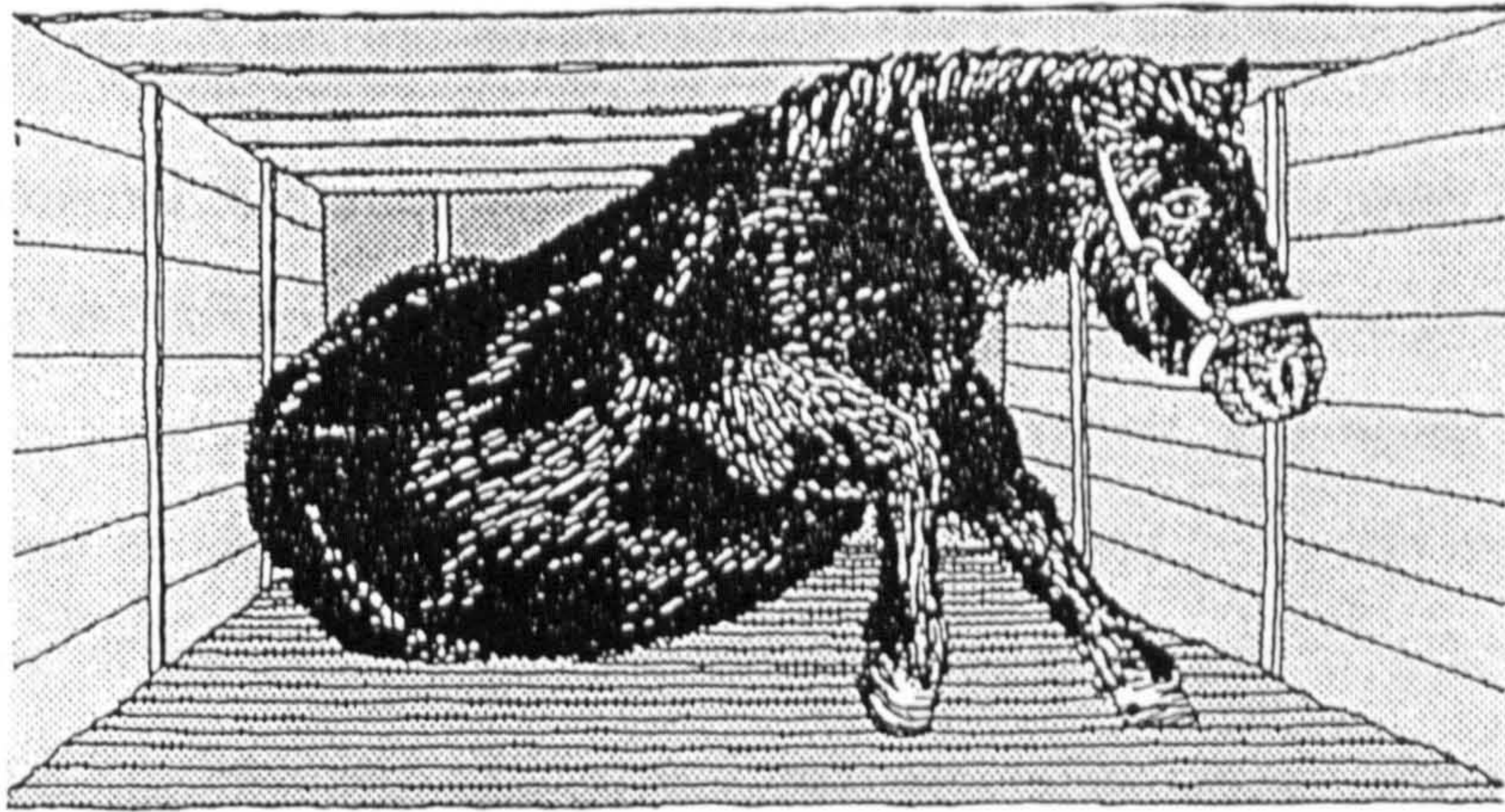
Consider differential diagnosis especially: COLIC; INFECTION; AZOTURIA

Laminitis unlikely, other conditions more likely

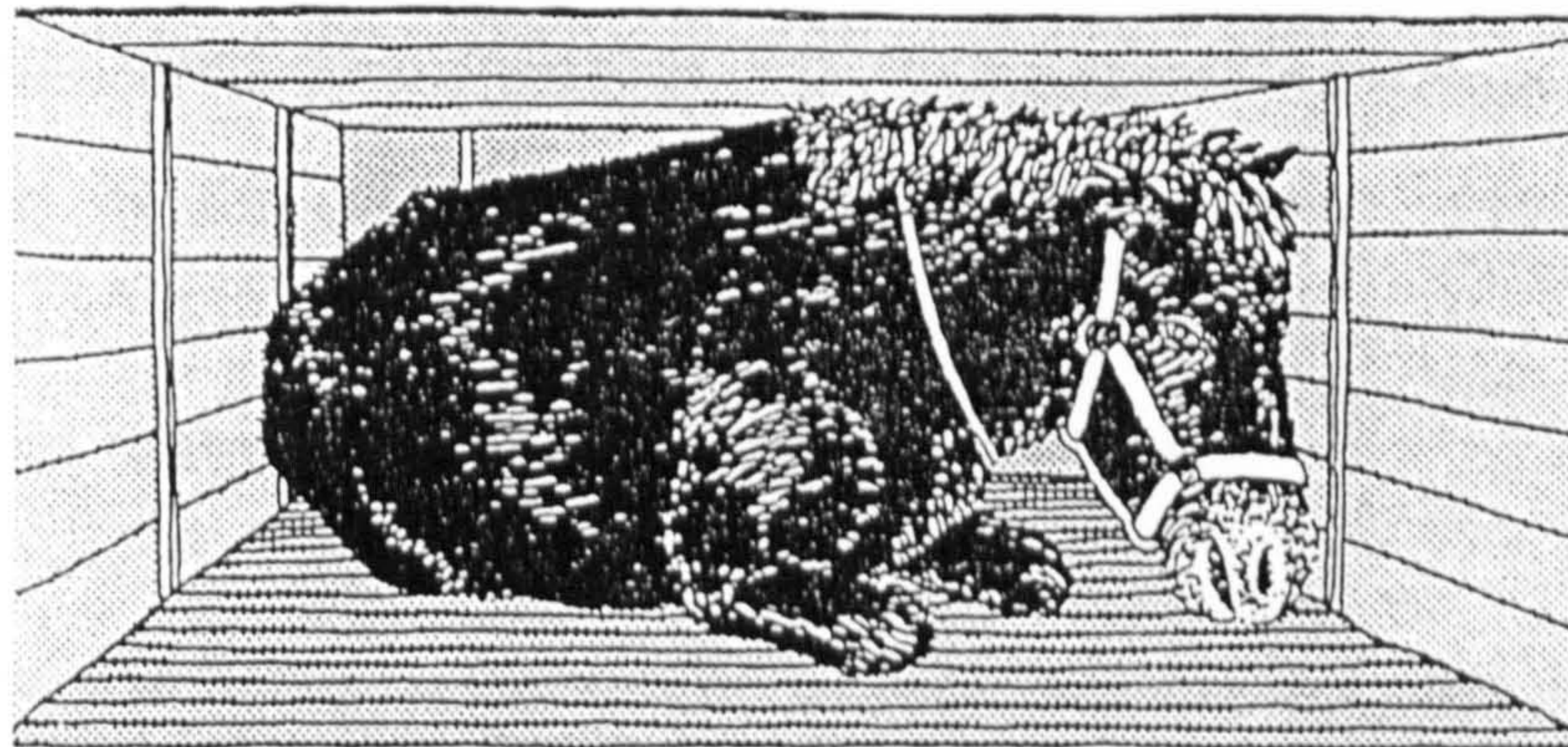
What are the most recent treatments that are available once acute stage is reached? There have been no major pharmacological advances in recent years and therapeutic measures have been surgical or physical nursing. It is quite remarkable that in the face of some understanding (if not definitive insight into) of laminitis, in recent years and months two "therapies" have been advocated which border upon the barbaric (Figures 10.3 and 10.4).



Figure 10.3 Amputation of the forelimb of a thoroughbred mare and the fitting with a prosthesis as treatment for acute laminitis. This mare lived for 6 months. (R.Redden)



When the reduction of the box height was moderate, the ponies succeeded in putting some load on the flexed front legs in trying to get up.



When the box was extremely low (about 125 % of the height of the thorax), the ponies lay calmly during the greater part of the experimental period.

Figure 10.4 Forced recumbency to prevent weightbearing during acute laminitis (Wattle *et al.*,1995)

Even in the 18th century, the leading equine veterinarian of the times, Professor James Clark, was frustrated by the lack of therapeutic measures and horrified by the dramatic interventions that were undertaken (Dunlop and Williams, 1995). He advised care, conservative treatment and rest in his publications (Clark, 1770; Clark, 1788) which are considered to be amongst the finest in veterinary literature. This advice was followed by J. Lupton in his version of Mayhew's Illustrated Horse Doctor (1901). His recommendations regarding nursing care and quiet, gentle, conservative care for the patient during the distress of the acute phase is as apt today as then.

Lupton (1901) recommended hot tubbing, slings, constant supervision and reassurance by a quiet attendant, hay and gruel diet but no dietary purgatives to upset the digestion and digitalis and belladonna as pharmaceutical remedies. Lupton recognised the importance of good nursing and that the patient should not be stressed, although he could have had no idea about the effect of stress hormones ! Lupton clearly had an understanding of horses on a personal basis and realised both as a horseman and physician that the best way to secure a recovery is to support the physiological mechanisms of the animal itself and do nothing that will cause the animal further deterioration. Lupton was undoubtedly one of the people of that era who with tremendous foresight, benefited the horse by creating understanding of their needs and so improving their conditions and welfare. Anna Sewell (1878) did the same thing in a different way by the publication of her book 'Black Beauty', which increased understanding of the needs of horses and reduced the hardship endured by a multitude of working horses, many of which suffered laminitis (Lupton 1901).

Unfortunately, such feeling for the horse do not seem to prevail in modern times and the literature on equine laminitis makes depressing reading to phillipic readers. Repeated induction of acute laminitis for experimental purposes with little apparent regard for the animals themselves is a regular finding. This particularly applies to the U.S.A. and the latest publication of treatments for laminitis uphold the view that there is little understanding or feeling for the horse itself - evidenced by Figures 10.3 and 10.4. Lupton (1901) would have been horrified. However, new understanding of physiological mechanisms especially endocrine control of vascular systems will undoubtedly bring new understanding of laminitis.

Preliminary evidence in this thesis (Chapter 5) indicates that nitric oxide assists the restoration of blood flow during acute laminitis. This can occur by providing the substrate l-arginine to enhance vasodilatation *in vivo* when the endothelium has reasonable integrity, or by administration of synthetic NO donors such as glyceryl

trinitrate which can be effective even when the endothelium is damaged. Other new compounds that release nitric oxide but do not induce tolerance -SPM - 5185 (Moncada and Higgs, 1995), and SP/W-5186 (Knuttel *et al.*, 1995) are also likely to be of benefit. Tolerance results from several mechanisms (Moncada and Higgs, 1995).

Increased exogenous nitric oxide may reduce basal production by direct inhibition of NOS at postranscriptional level; and desensitisation of cGMP occurs with high levels of NO administration. However, at lower doses there does not seem to be any desensitisation and long term inhalation of NO for treatment of adult respiratory distress syndrome (ARDS) is beneficial even after 53 days of treatment (Rossiant *et al.*, 1993; Moncada and Higgs, 1995). There are several mechanisms proposed for nitrate tolerance : depletion of tissue thiols ; deficiency of cGMP; or biological counterregulation, perhaps by enhanced superoxide/peroxynitrate formation (Mülsch *et al.*, 1995a; Scatchkov *et al.*, 1995) Nitrate induced tolerance is the subject of many studies (Mülsch *et al.*, 1995b; Bassenge, 1995) and may be related to dietary antioxidants (Laight and Änggård 1995; Bassenge, 1995) and therefore may be different in herbivores because of dietary influences. However, it is premature to suggest that nitric oxide donors could be used prophylactically.

In acute equine laminitis it is difficult to identify the site of action of NO. Is it within the microcirculation or is there also a more systemic effect ? Or both ? The variable responses of individuals to equine laminitis mean that one pony may have vasoconstriction in one area and another animal will be affected in a different area of the microcirculation; indeed, a case of an animal being affected in half of one foot is documented (Pollitt, 1995).

If nitric oxide is effective for all of the digital circulation then it is unimportant in clinical terms to know exactly what is happening; each case is unique. The principle of treatment however is the same in all cases - vasodilatation of vessels and restoration of blood flow through normal routes and / or by the action of glyceryl trinitrate which enhances collateral supply. The latter is outlined by Rang and Dale (1991) and shown in Figure 5.21. In a similar fashion, Pollitt (1995) shows the events that occur within the hoof in Figure 1.22 (Introduction). His assertion in the figure legend that the bounding pulses felt at the pasterns of affected horses is debatable. Pollitt (1995) states that reduced peripheral resistance causes the bounding arterial pulse - however, if there is less resistance through arterioles and capillaries, then a greater pulse would be present in the digital veins and it may be this that is felt at the pasterns. Pollitt (1995) bases this assertion on the inappropriate opening of AVA during acute stage laminitis but if vasoconstriction occurs in arterioles distal to the AVAs then the increased resistance in the arterioles would increase the pulse felt in the digital arteries.

In principle the events outlined in the equine hoof during acute laminitis are not disputed, as ischaemia occurs whether the cause is inappropriate opening of AVAs or vasoconstriction. A hypothesis for the action of NO in the equine digital circulation, which combines the mode of action of GTN (Rang and Dale, 1994) and the schematic events within the digital circulation (Pollitt 1995) is outlined in Figure 10.5 (i), (ii) (iii) below.

Figure 10.5 (i) shows the equine digital microcirculation in its normal state with circulation through the arterioles and capillaries supplying nutrients to the dermal and epidermal laminae. There are normal proportions of oxyhaemoglobin (O_2Hb) and deoxyhaemoglobin (HHb) which are in equilibrium and are represented by the proportions of O_2Hb (red) and HHb (purple) in the microcirculation. These quantities are in equilibrium and relate directly to the near infrared traces of O_2Hb and HHb seen in Chapter 5. The basal NIRS values can be visualised as these proportions.

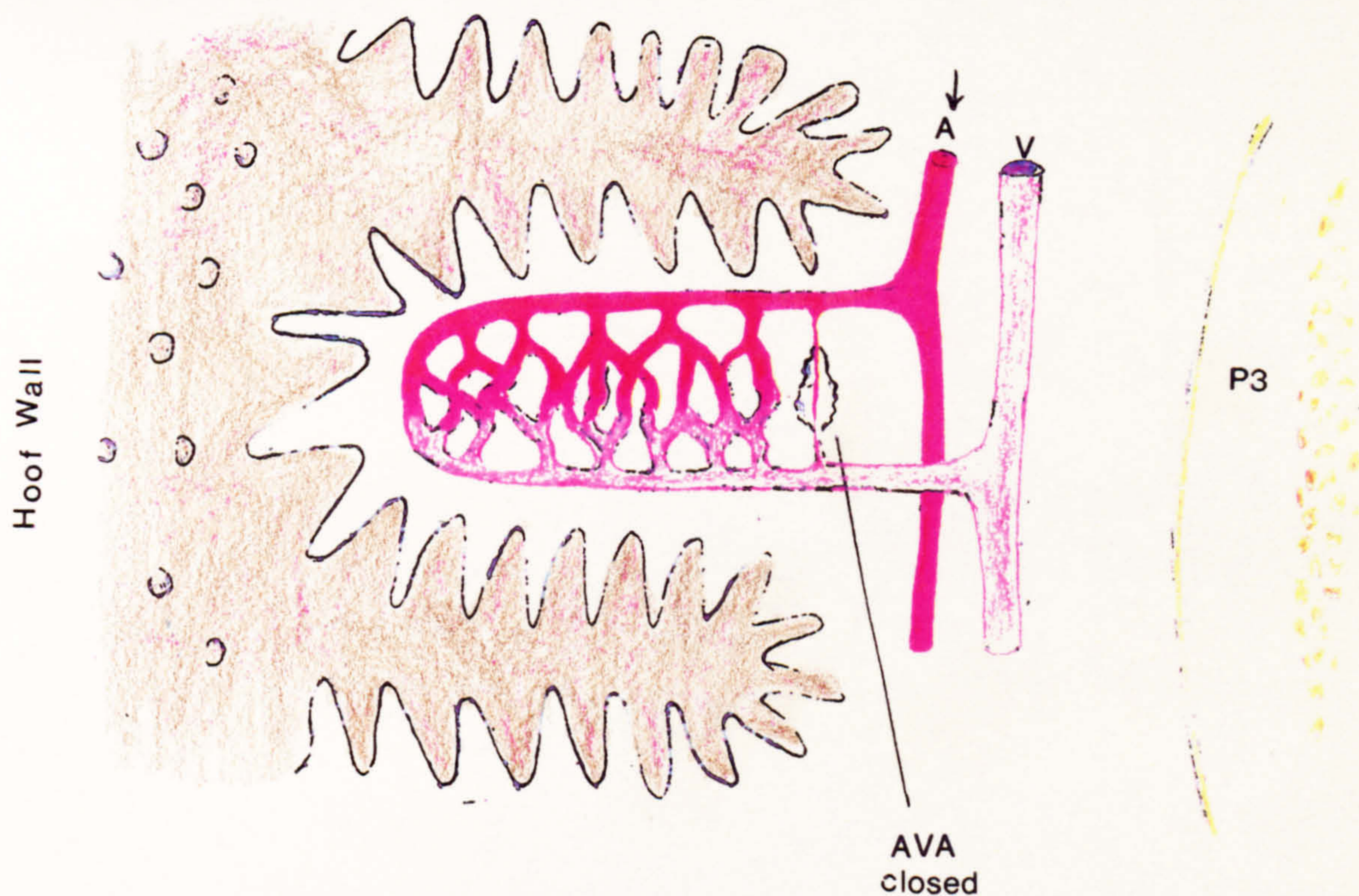


Figure 10.5 (i) - Normal equine digital microcirculation (after Pollitt 1995). Arteriovenous anastomoses (AVA) are closed to allow normal perfusion through arterioles and capillaries in the laminae.

Figure 10 5 (ii) shows the equine circulation during ischaemia. If vasoconstriction occurs distal to the AVA then blood will be redirected through the AVA which will open. The pathophysiology of vasoconstriction is indistinguishable from inappropriate dilation of AVA as both cause ischaemia within the dermal laminae. AVA are likely to respond to pressure changes within the hoof to prevent vascular damage during movement and weightbearing. This function is in addition to their role in thermoregulation. The afferent digital artery is slightly dilated because of increased heart rate and increased total flow to the hoof. The concentration of deoxyhaemoglobin in this figure is increased as the capillary bed is underperfused and O_2 is removed from blood during haemostasis. Venous pooling is shown. The picture is represented by the NIRS traces on deliberately induced ischaemia in normal horses and, in acute laminitis, by the 'resetting' of different baseline traces representing proportions of O_2Hb and HHb (see Chapters 4 and 5). The Critikon Model 2001 showed proportions of O_2Hb and HHb semi quantitatively and future models of the Critikon Near infrared spectroscope will provide quantitative data.

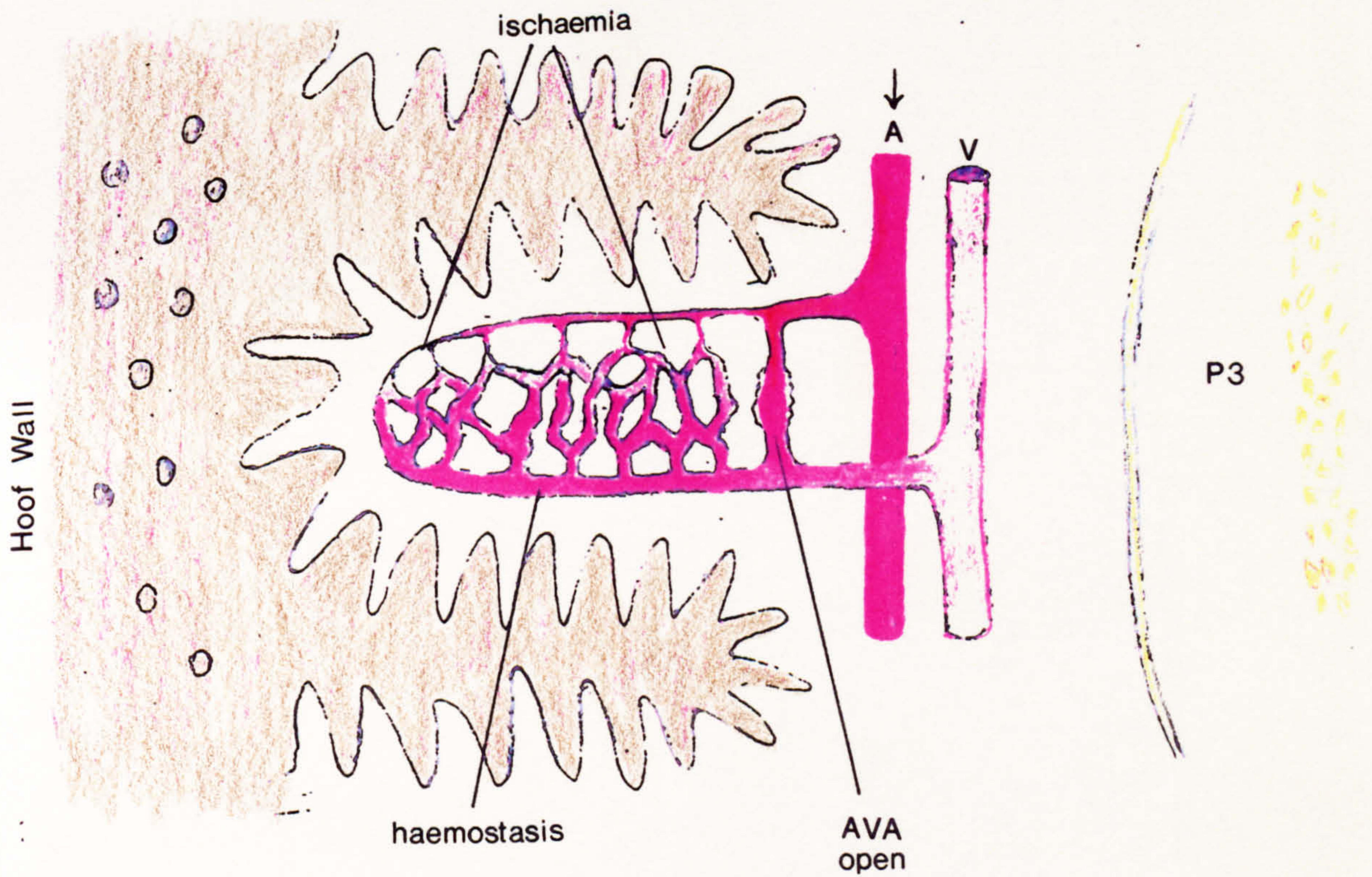
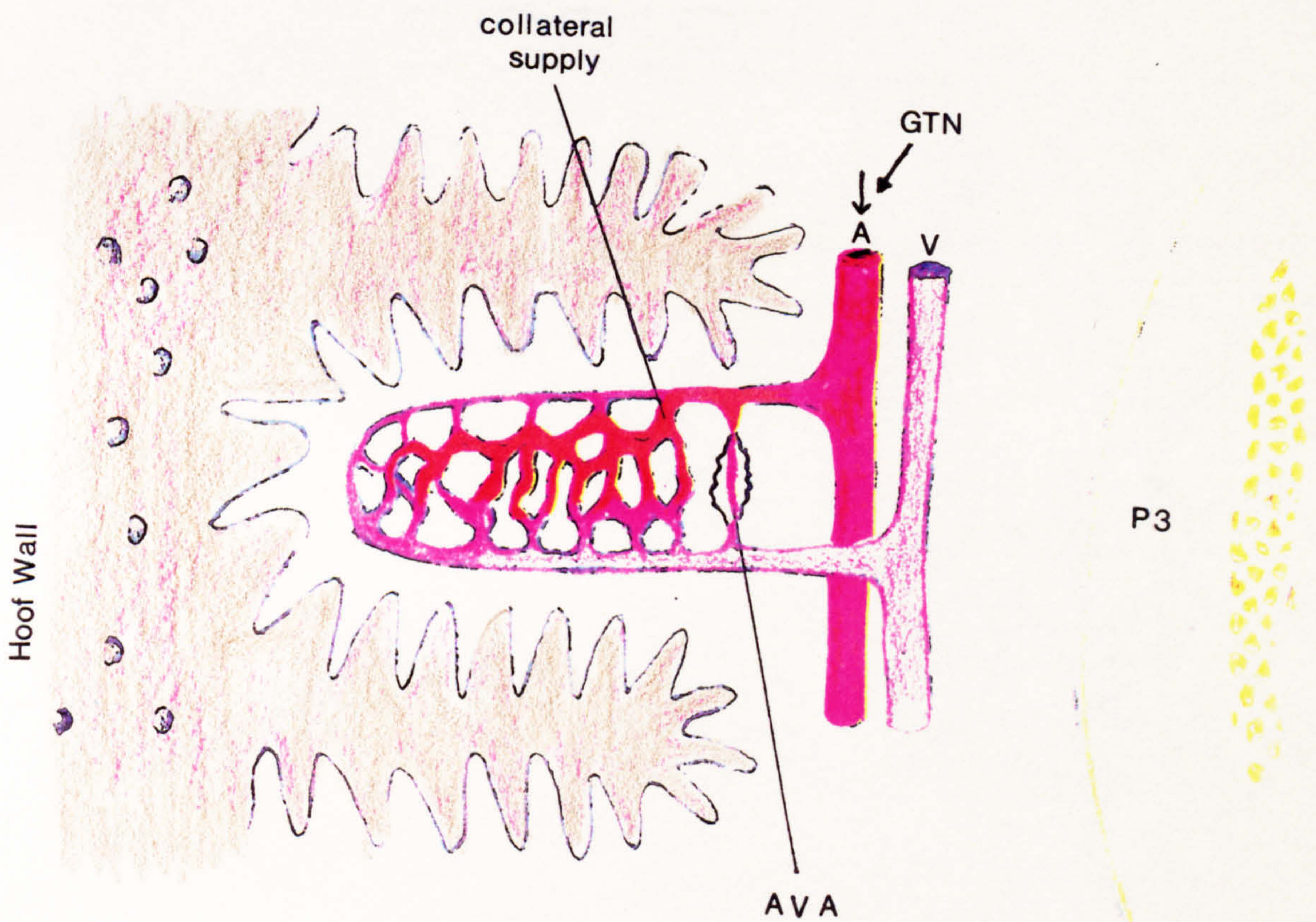


Figure 10.5 (ii) - Equine digital microcirculation showing opening of arteriovenous anastomoses (AVA) and resultant haemostasis and ischaemia in lamellar tissue.

Figure 10.5 (iii) - Equine digital microcirculation after administration of l-arginine and/or glyceryl trinitrate (GTN) to the afferent blood supply showing the proposed mechanism for restoration of blood flow to ischaemic areas by vasodilatation and enhancement collateral blood supply and 'washout' of capillary venous pooling.



The possible events that occur within the hoof are illustrated in Figure 10.5 (iii). If l-arginine or glyceryl trinitrate (GTN) is administered proximal to the digital microcirculation then nitric oxide will vasodilate the arterioles close to the AVA. The mode of action of nitric oxide within the digital vasculature is unknown but a plausible theory is that collateral supply in the arterioles is enhanced in a manner outlined by Rang and Dale (1994) although AVAs are not shown in their scheme (Figure 5.21). Nitric oxide will diffuse freely through tissue and vasodilate vessels in the vicinity of collateral supplies. Increased NO may dilate AVAs further and possibly initiate counter regulation and closing of AVA by novel homeostatic adjustments. Figure 10.5 (iii) also shows a greater proportion than both previous figures of O₂Hb indicating a 'washout' and reperfusion of vessels. There is gradual reperfusion of the dermal laminae. Those vessels that are last to be reperfused still have a high concentration of HHb but this will be transient. This picture is equivalent to the analysis shown on the traces of the NIRS where HHb is rapidly decreased and O₂Hb greatly increased during arginine infusion in normal and acutely laminitic horses. In acute laminitis, the washout process returns baseline values towards normal proportions/concentrations and, after a while, normal values are fully restored. When arginine is given to normal horses blood flow to the area is increased.

Nitric oxide is likely to be an important therapeutic agent for equine laminitis in the future. The benefits are not restricted to vasodilatation but include beneficial influences : anti platelet aggregation, anti-proliferative effects and inhibition of pathological vasoconstriction by a variety of agents. A therapy that alleviates suffering in the 3% of the equine population that is afflicted by laminitis will have considerable welfare implications.

Near infrared spectroscopy has been invaluable in this study as a novel method for assessing vascular function within the hoof without introducing artefacts that have previously been detrimental (Robinson, 1990). Its usefulness will not be limited to

investigations of the hoof and the application of NIRS to exercise physiology and assessment of normal and diseased states in all tissues will be of interest in years to come. The method is particularly suited to the equine studies as horses are so susceptible to the stress of invasive methods of investigation.

As perhaps with most investigations the present research has raised more questions than it has answered; in answering one question several others seem to arise. If horses have low renin activity (Gutherie *et al.*, 1980; Purohit *et al.*, 1979.) and low plasma AII, do horses have increased sensitivity to AII because of upregulation of receptor density? If this is the case, do increased levels of AII observed during acute laminitis cause acute vasoconstriction even though they are within the normal human range? What causes sodium hypertension in refractory laminitis, and why do these cases have the poorest prognosis? Do horses have seasonal alterations in aldosterone in response to ingestion of potassium loaded grass? Why do cases that have blood stained exudate at the coronary band, observed by Lupton (1901), Colles (1991b) and in this study, have invariably to be destroyed? Does endothelin play a role in acute laminitis within the microcirculation of the equine digit? What are the interactions of local mechanisms regulating pedal haemodynamics in the equine foot? To what extent do ADMA regulate NOS and vasodilatation in the hoof? What are the features of diet that cause chronic laminitic animals to have increased ADMA in summer months? Where is the principle action of nitric oxide within the hoof? Can a prophylactic be developed? Can blood or urine tests be developed that will predict the onset of, or predisposition to, acute laminitis? All these questions require answers at present.

In conclusion, this thesis has attempted to throw light on normal equine endocrinology and normal pedal haemodynamics by developing, validating and using novel methods of assessment whether this be near infrared spectroscopy or novel radioimmunoassay. The work began with the aim of increasing understanding of normal equine physiology and its relationship to the modern environment, and to investigate the environmental factors that have changed during evolutionary history and to determine how these have affected the horse during its evolution over thousands of years. By combining history with current knowledge, and by combining rigorous scientific investigation with veterinary medicine, the rifts between the different disciplines have been bridged. Specialised disciplines rarely communicate and much understanding of physiological processes *in vivo* are lost because of this omission. It is hoped that the work and ideas within this thesis will increase understanding of equine physiology in health and disease and that generations of horses will benefit.

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APPENDIX I

Individual equine blood pressure measurements

Individual BP values and heart rates of normal ponies taken during winter months

	Blood pressure (mmHg)			Heart rate (bts/min)
	Systolic	Diastolic	Mean	
Cracker II	99	52	68	41
	102	53	71	42
	97	56	72	39
Mean	99	54	70	41
SE	1	1	1	1
Marroa	101	61	80	38
	91	55	67	38
	76	50	60	44
	92	53	68	39
Mean	90	55	69	40
SE	5	2	4	1
Despina	51	33	45	39
	122	74	85	40
	85	67	76	39
Mean	86	58	69	39
SE	21	13	12	0
Snowflake	136	75	103	61
	125	61	72	47
	136	67	91	39
Mean	132	68	89	49
SE	4	4	9	6
Selluci	105	53	71	42
	101	57	74	43
	104	54	70	44
Mean	103	55	72	43
SE	1	1	1	1
Jasper	105	60	72	39
	115	56	74	39
	126	67	88	40
Mean	115	61	78	39
SE	6	3	5	0

Individual values of BP of normal ponies taken during summer months

	Blood pressure (mmHg)		Heart rate (bts/min)
	Systolic	Diastolic	
Normal			
Cracker II	105	68	47
Tess	111	63	54
Beckford	101	61	45
Marroa	96	70	43
	99	63	40
	104	64	39
Mean	100	66	41
SE	2	2	1
Snowflake	116	73	38

		Summer BP measurements			May-95
		Blood Pressure (mmHg)			
		Systolic	Diastolic	Mean	Ht rate
Normal Ponies					
Cracker II		108	57	81	43
		113	62	78	43
		108	59	72	45
	Mean	110	59	77	44
	SE	2	1	3	1
Despina		102	55	66	38
		92	60	73	40
		91	59	75	40
	Mean	95	58	71	39
	SE	4	2	3	1
Marroa		101	59	74	38
		110	55	71	40
		110	59	76	43
	Mean	107	58	74	40
	SE	3	1	1	1
Sellaci		108	49	72	52
		132	57	77	47
		135	62	91	46
	Mean	125	56	80	48
	SE	9	4	6	2

		Summer BP measurements			May-95
		Blood Pressure (mmHg)			
		Systolic	Diastolic	Mean	Ht rate
Laminitic Ponies (chronic)					
Cobweb		127	54	80	56
		133	68	86	57
		115	60	78	53
	Mean	125	61	81	55
	SE	5	4	2	1
Domino		112	57	78	49
		107	55	75	50
		103	46	66	49
		99	57	68	50
	Mean	105	54	72	50
	SE	3	3	3	0
Misty		137	69	99	68
		109	63	88	67
		120	72	98	57
	Mean	122	68	95	64
	SE	8	3	4	4
Tess		96	53	64	48
		118	33	43	47
	Mean	107	43	54	48
	SE	11	10	11	1

Summary of Summer BP measurements (ponies)					
		Blood Pressure (mmHg)			Ht rate
		Systolic	Diastolic	Mean	
Normal Ponies					
Cracker II	Mean	110	59	77	44
Despina	Mean	95	58	71	39
Marron	Mean	107	58	74	40
Selluci	Mean	125	56	80	48
Group Mean		109	58	76	43
Standard Error		6	1	2	2
Minimum		95	56	71	39
Maximum		125	59	80	48
Laminitic Ponies					
Cobweb	Mean	125	61	81	55
Domino	Mean	105	54	72	50
Misty	Mean	122	68	95	64
Tess	Mean	107	43	54	48
Group Mean		115	57	76	54
Standard Error		5	5	9	4
Minimum		105	43	54	48
Maximum		125	68	95	64

Difference in heart rate between laminitic and normal groups (summer)		
t-Test: Two-Sample Assuming Equal Variances		
	Variable 1	Variable 2
Mean	54.25	42.75
Variance	50.91667	16.91667
Observations	4	4
Pooled Variance	33.91667	
Hypothesized Mean difference	0	
df	6	
t	2.792584	
P(T<=t) one-tail	0.015735	
t Critical one-tail	1.943181	
P(T<=t) two-tail	0.03147	
t Critical two-tail	2.446914	
There is a significant difference		
p < 0.05		

Mean BP values and heart rates of normal ponies taken during spring months

	Blood pressure (mmHg)		Mean	Heart rate (bts/min)
	Systolic	Diastolic		
Cracker II	105	68	89	47
Despina	-	-	-	-
Jasper	-	-	-	-
Marron	100	66	77	41
Selluci	-	-	-	-
Snowflake	116	73	87	38
Tess	111	63	80	54
Group Mean	108	68	83	45
Standard Error	3	2	3	4
Range	(100 - 116)	(63 - 73)	(77 - 89)	(38 - 54)

n = 4
Number of tests = 11

Mean BP values and heart rates of normal ponies taken during winter months :

	Blood pressure (mm Hg)		Mean	Heart rate (bts/min)
	Systolic	Diastolic		
Cracker II	99	54	70	41
Despina	86	58	69	39
Jasper	115	61	78	39
Marron	90	55	69	40
Selluci	103	55	72	43
Snowflake	132	68	89	49
Group Mean	104	59	73	43
Standard Error	7	2	3	1
Range	(86 - 132)	(54 - 68)	(69 - 89)	(39 - 49)

n = 6
Number of tests = 18

Mean BP values and heart rates of normal ponies taken during summer months

	Blood pressure (mmHg)			Heart rate (bts/min)
	Systolic	Diastolic	Mean	
Cracker II	110	59	77	44
Despina	95	58	71	39
Marron	107	58	74	40
Selluci	125	56	80	48
Group Mean	109	58	76	43
Standard Error	6	1	2	2
Range	(95 - 125)	(56 - 59)	(71 - 80)	(39 - 48)

n = 4

Number of tests = 21

Mean BP values and heart rates of normal horses taken during winter months :

	Blood pressure (mmHg)			Heart rate (bts/min)
	Systolic	Diastolic	Mean	
Albert	87	41	57	65
Amanda	99	51	65	43
Annabella	88	44	65	43
Beckford	101	61	74	45
Biscuit	100	55	68	48
Blossom	93	57	70	57
Dolly	89	68	66	52
Emily	108	62	81	50
Maree Gray	80	39	54	58
Melody	118	50	63	46
Poppy	79	41	54	52
Tango	104	58	73	59
Group Mean	95	51	65	52
Standard Error	4	3	2	2
Range	(79 - 118)	(39 - 68)	(54 - 81)	(43 - 65)

n = 12

Number of tests = 36

Comparison of BP of pregnant and non-pregnant mares.

Mean values for BP of pregnant mares (in the last trimester) are compared with non-pregnant mares.

	Blood pressure (mm Hg)			Heart rate (bts/min)
	Systolic	Diastolic	Mean	
Bryn	120	64	81	46
Dolly	96	61	72	51
Maree Gray	88	42	57	40
Rose	90	51	70	41
Tango	100	56	75	48
Group Mean	99	55	71	45
Standard Error	6	4	4	2
Range	(88 - 120)	(42 - 64)	(57 - 81)	(40 - 51)
No of tests = 15				
n=5				
Amanda	90	53	67	48
Annabella	82	48	63	57
Biscuit	94	50	62	45
Blossom	84	44	55	53
Emily	102	58	77	43
Melody	94	43	57	45
Poppy	86	49	57	45
Group Mean	90	49	63	48
Standard Error	3	2	3	2
Range	(82 - 102)	(43 - 58)	(55 - 77)	(43 - 57)
No of tests = 21				
n=7				

BP measurements of normal horses during winter months

		BP measurements of normal horses			Winter 95
		Blood Pressure (mmHg)			
		Systolic	Diastolic	Mean	Ht rate
Albert		93	35	47	67
		86	45	63	67
		81	44	60	62
	Mean	87	41	57	65
	SE	3	3	5	2
Amanda		97	54	70	44
		101	51	62	42
		99	49	63	42
	Mean	99	51	65	43
	SE	1	1	3	1
Annabella		100	51	72	55
		90	44	57	56
		74	36	50	66
	Mean	88	44	60	59
	SE	8	4	6	4
Biscuit		93	56	67	49
		106	55	68	47
		100	53	68	48
	Mean	100	55	68	48
	SE	4	1	0	1
Beckford		101	61	74	45
	SE				

		BP measurements of normal horses			Winter 95
		Blood Pressure (mmHg)			
		Systolic	Diastolic	Mean	Ht rate
Blossom		99	60	73	54
		84	53	71	57
		96	58	67	59
	Mean	93	57	70	57
	SE	5	2	2	1
Bryn					
	Mean				
	SE				
Dolly		99	94	68	48
		86	50	64	57
		82	60	65	52
	Mean	89	68	66	52
	SE	5	13	1	3
Emily		104	58	83	50
		107	57	77	49
		113	70	83	51
	Mean	108	62	81	50
	SE	3	4	2	1

Mean BP measurements of normal horses during winter months

	Mean BP measurements of normal horses				Winter 95
	Blood Pressure (mmHg)				
	Systolic	Diastolic	Mean	Ht rate	
Albert	Mean	87	41	57	65
Amanda	Mean	99	51	65	43
Annabella	Mean	88	44	65	43
Beckford	Mean	101	61	74	45
Biscuit	Mean	100	55	68	48
Blossom	Mean	93	57	70	57
Dolly	Mean	89	68	66	52
Emily	Mean	108	62	81	50
Maree G	Mean	80	39	54	58
Melody	Mean	118	50	63	46
Poppy	Mean	79	41	54	52
Tango	Mean	104	58	73	59
Group mean		95	52	65	52
SE		4	3	2	2
Range		79	39	54	43
n = 11		118	68	81	65

BP measurements of normal horses during winter months (contd)

	BP measurements of normal horses (Contd)				Winter 95
	Blood Pressure (mmHg)				
	Systolic	Diastolic	Mean	Ht rate	
Maree G	84	40	53	66	
	83	40	57	54	
	72	37	51	54	
Mean	80	39	54	58	
SE	4	1	2	4	
Melody	160	49	65	45	
	96	48	64	47	
	97	52	61	46	
Mean	118	50	63	46	
SE	21	1	1	1	
Poppy	80	43	57	52	
	80	43	53	52	
	77	38	53	53	
Mean	79	41	54	52	
SE	1	2	1	0	
Tango	91	64	75	62	
	112	53	75	61	
	109	57	68	53	
Mean	104	58	73	59	
SE	7	3	2	3	

Mean BP measurements of chronic laminitic horses during winter months

	Mean BP Measurements of chronic laminitic horses		Winter 95		
	Blood Pressure (mmHg)				
	Systolic	Diastolic		Mean	Ht rate
Isadora	Mean	96	48	65	39
Rose M	Mean	102	60	72	40
Group mean					
		99	54	68	39
SE		3	6	3	1
Range		96 - 102	48 - 60	65 - 72	39 - 40
n = 2					

BP measurements of chronic laminitic horses during winter months

	BP measurements of chronic laminitic horses			Winter 95
	Blood Pressure (mmHg)			
	Systolic	Diastolic	Mean	
Isadora	102	50	62	39
	90	46	68	38
Mean	96	48	65	39
SE	6	2	3	1
Rose M	98	62	72	42
	107	56	68	38
	100	61	75	39
Mean	102	60	72	40
SE	3	2	2	1

BP measurements of normal horses during summer months

		BP measurements of normal horses			Summer 95
		Blood Pressure (mmHg)			
		Systolic	Diastolic	Mean	Ht rate
Albert					
	Mean				
	SE				
Amanda		102	51	66	48
		97	55	67	47
		94	53	67	49
	Mean	98	53	67	48
	SE	2	1	0	1
Annabella		86	47	65	51
		78	47	63	61
		81	51	60	58
	Mean	82	48	63	57
	SE	2	1	1	3
Biscuit		92	54	68	45
		97	51	62	45
		94	44	56	46
	Mean	94	50	62	45
	SE	1	3	3	0
Blossom		84	44	57	53
		84	41	54	54
		83	46	55	51
	Mean	84	44	55	53
	SE	0	1	1	1

		BP measurements of normal horses			Summer 95
		Blood Pressure (mmHg)			
		Systolic	Diastolic	Mean	Ht rate
Bryn		108	59	74	45
		117	54	67	46
		136	78	103	47
	Mean	120	64	81	46
	SE	8	7	11	1
Dolly		99	61	66	48
		94	60	76	52
		94	62	75	52
	Mean	96	61	72	51
	SE	2	1	3	1
Emily		103	55	73	43
		101	59	81	43
		102	59	78	42
	Mean	102	58	77	43
	SE	1	1	2	0
Maree G		83	40	55	40
		84	39	55	41
		98	47	61	39
	Mean	88	42	57	40
	SE	5	3	2	1
Melody		100	44	61	45
		95	43	57	45
		87	41	53	46
	Mean	94	43	57	45
	SE	4	1	2	0

Mean BP measurements of normal horses during summer months

		Mean BP measurements of normal horses				
		Blood pressure (mm Hg)			Mean	Ht rate
	Mean	Systolic	Diastolic	Mean		
Amanda	Mean	98	53	67		48
Annabella	Mean	82	48	63		57
Biscuit	Mean	94	50	62		45
Blossom	Mean	84	44	55		53
Bryn	Mean	120	64	81		46
Dolly	Mean	96	61	72		51
Emily	Mean	102	58	77		43
Maree G	Mean	88	42	57		40
Melody	Mean	94	43	57		45
Poppy	Mean	86	49	57		45
Rose	Mean	90	51	70		41
Tango	Mean	100	56	75		48
Group mean		94	52	66		47
SE		3	2	3		1
Range		82 - 120	42 - 64	55 - 81		40 - 57
n = 12						

BP measurements of normal horses during summer months (Contd)

		BP measurements of normal horses (Contd)			Summer 95
		Blood Pressure (mm Hg)			Ht rate
	Mean	Systolic	Diastolic	Mean	
Poppy		87	53	68	43
		85	43	54	47
		87	50	56	42
	Mean	86	49	59	44
	SE	1	3	4	2
Rose		100	58	83	42
		79	50	65	42
		92	45	62	39
	Mean	90	51	70	41
	SE	6	4	7	1
Tango		110	59	82	51
		115	53	80	42
		74	57	64	51
	Mean	100	56	75	48
	SE	13	2	6	3

Comparison of BP measurements of pregnant and non-pregnant mares

		Comparison of pregnant and non-pregnant mares			Summer 95
		Blood Pressure (mmHg)			
		Systolic	Diastolic	Mean	Ht rate
Normal					
Bryn	Mean	120	64	81	46
Dolly	Mean	96	61	72	51
Maree G	Mean	88	42	57	40
Rose	Mean	90	51	70	41
Tango	Mean	100	56	75	48
Group mean					
		99	55	71	45
SE		6	4	4	2
Range		88	42	57	40
		120	64	81	51
n=5					
Amanda					
	Mean	90	53	67	48
Annabella	Mean	82	48	63	57
Biscuit	Mean	94	50	62	45
Blossom	Mean	84	44	55	53
Emily	Mean	102	58	77	43
Melody	Mean	94	43	57	45
Poppy	Mean	86	49	57	45
Group mean					
		90	49	63	48
SE		3	2	3	2
Range		82 - 102	43 - 58	55 - 77	43 - 57
n=7					

CHRONIC LAMINITIS

Individual BP values of chronic laminitis cases taken during winter months :

	BP measurements of chronic cases				Ht rate
	Systolic	Diastolic	Mean		
Rose Marie	94	56	70		39
	100	57	66		39
	90	61	73		41
	Mean	58	70		40
	SE	3	2		1
Bean	110	57	74		42
	121	54	73		39
	106	59	84		40
	100	54	78		40
	Mean	109	56	77	40
	SE	4	1	2	1
Domino			55		
	98	53	64		39
	81	46	57		40
	85	56	66		38
	Mean	88	52	62	39
	SE	5	3	3	1
Tess	113	71	93		40
	106	54	74		39
	108	56	77		39
	Mean	109	60	81	39
	SE	2	5	6	0
Misty	102	59	76		43
	100	50	66		45
	87	52	67		49
	100	57	80		49
	Mean	97	55	72	47
	SE	3	2	3	2
Cobweb	101	53	71		44
	79	57	67		46
	106	59	76		43
	104	57	74		40
	Mean	98	57	72	44
	SE	6	1	2	1

NORMAL

Individual BP values of normal cases taken during winter months :

	BP measurements of normal cases				Ht rate
	Systolic	Diastolic	Mean		
9.12.94					
Normal					
Cracker II	99	54	68		41
Marron	90	55	69		40
Despina	86	58	69		40
Snowflake	132	68	89		49
Selluci	103	55	72		43
Jasper	115	61	72		43
	Mean	104	59	73	43
	SE	7	2	3	1
	p	0.545751	0.395696	0.845974	

T-test assuming unequal variance two tailed probability

Individual values of BP of chronic laminitis cases taken during summer months

	Blood Pressure (mmHg)			Summer 94
	Systolic	Diastolic	Mean	
Domino	68	39	49	Ht rate 40
	55	38	44	96
	121	95	103	55
	88	66	75	75
	117	48	71	40
	100	65	77	50
	83	42	56	52
Mean	90	56	68	58
SE	9	8	8	8
Beau	103	58	73	54
	105	57	64	45
Mean	104	58	69	50
SE	1	1	5	5
Rose Marie	112	73	92	51
	123	75	102	56
	117	74	92	54
Mean	117	74	95	54
SE	3	1	3	1

Difference in heart rate between laminitic and normal groups (summet)
t-Test: Two-Sample Assuming Equal Variances

	Variable 1	Variable 2
Mean	54.25	42.75
Variance	50.91667	16.91667
Observations	4	4
Pooled Variance	33.91667	
Hypothesized Mean Difference	0	
df	6	
t	2.792584	
P(T<=t) one-tail	0.015735	
t Critical one-tail	1.943181	
P(T<=t) two-tail	0.03147	
t Critical two-tail	2.446914	
There is a significant difference		
	P < 0.05	

ACUTE LAMINITIS

Individual cases of grass induced acute laminitis

Blood pressure readings of acute laminitis cases during the summer months

		BP measurements of acute cases			Summer 94
		Blood Pressure (mmHg)			
		Systolic	Diastolic	Mean	Ht rate
Beau1		172	93	131	75
		209	118	175	74
		186	90	122	75
		177	92	133	76
		179	86	123	71
		176	103	137	70
		181	94	134	54
		181	99	145	61
	Mean	183	97	138	70
	SE	4	4	6	3
Count	8				
Beau2		187	85	121	61
		188	86	124	50
	Mean	188	86	123	56
	SE	1	1	2	6
Count	2				
Beau3		163	80	113	54
		167	70	99	53
	Mean	165	75	106	54
	SE	2	5	7	1
Count	2				

		BP measurements of acute cases (Contd)			Summer 94
		Blood Pressure (mmHg)			
		Systolic	Diastolic	Mean	Ht rate
Misty1		171	88	129	61
Misty2		165	90	118	71
Misty3		151	97	115	72
		159	91	127	78
	Mean	155	94	121	75
	SE	4	3	6	3
Count	2				
Cobweb1		155	84	117	62
Cobweb2		168	80	118	57
		185	91	132	57
	Mean	177	86	125	57
	SE	9	6	7	0
Count	2				
Domino		131	81	98	51
Tess		174	77	116	51
		155	77	111	52
		162	84	117	54
	Mean	164	79	115	52
	SE	6	2	2	1
Count	3				

Mean BP values for animals with grass induced acute laminitis 1994

Blood Pressure of acute laminitis cases 1994						
Animal code	Systolic mmHg	Diastolic mmHg	Mean mmHg	Ht rate	Obel Grade	
B1	183	97	138	70	4	
B2	188	86	123	56	3.5	
B3	165	75	106	54	3	
M1	171	88	129	61	3.5	
M2	165	90	118	71	3	
M3	155	94	121	75	3.5	
C1	155	84	117	62	2	
C2	177	86	125	57	4	
D	131	81	98	51	3	
T	164	79	115	52	2	
Mean	165	86	119	61		
Standard Error	5	2	4	3		
No. Cases	10					

Key: B1 - Case 1
 B2 - Case 2
 B3 - Case 3
 M1 - Case 4
 M2 - Case 5
 M3 - Case 6
 C1 - Case 7
 C2 - Case 8
 D - Case 9
 T - Case 10

Mean BP and heart rates during grass induced acute laminitis 1994 and the relationship to Obel Grade of lameness

Animal Code	Systolic mmHg	Diastolic mmHg	Mean mmHg	Ht rate	Obel Grade
Case 1	183	97	138	70	4
Case 2	188	86	123	56	3.5
Case 3	165	75	106	54	3
Case 4	171	88	129	61	3.5
Case 5	155	94	121	75	3.5
Case 6	177	86	125	57	4
Case 7	131	81	98	51	3
Case 8	164	79	115	52	2
Case 9	165	90	118	71	3
Case 10	155	84	117	62	2
Mean	165	86	119	61	3
Standard Error	5	2	4	3	
Range	(131-188)	(75-97)	(98-138)	(51-75)	(2-4)
Number of Cases	10				
Number of tests	23				

APPENDIX II

Clinical and Experimental Blood Pressures

Blood pressure measurements of a normal horse during l-arginine infusion					
(Indirect, tail cuff method, Dinamap Vital Signs Monitor, Critikon, U.K.)					
L-arginine iv					
Beckford					
Basal readings					
		systolic	diastolic	heart rate	
		mm Hg	mmHg	b/min	
11.12.93		120	69	43	
		112	61	39	
		122	65	41	
		97	62	43	
		83	53	48	
		76	53	62	
		106	58	53	
		Mean	102	60	47
		Minimum	76	53	39
		Maximum	122	69	62
14.12.93		122	78	51	
		96	58	41	
		111	55	41	
		91	47	45	
		82	60	39	
		104	54	45	
		98	72	51	
		112	70	40	
		Mean	102	62	44
		Minimum	82	47	39
Infusion		Maximum	122	78	51

Beckford (continued)

10%+ arginine			systolic	diastolic	heart rate	
First 15 mins			mm Hg	mmHg	b/min	
			101	59	49	
			108	59	47	
		Mean	107	58	45	
		Minimum	101	56	39	
		Maximum	112	59	49	
15mins -30mins			116	63	53	
			148	88	40	
			134	79	40	
		Mean	133	77	44	
		Minimum	116	63	40	
		Maximum	148	88	53	
30 -45 mins			137	89	69	
			127	75	59	
			132	84	42	
		Mean	132	83	57	
		Minimum		75	42	
		Maximum		89	69	
45-60 mins			112	70	43	
			101	70	40	
			100	71	40	
			123	85	35	
		Mean	109	74	40	
		Minimum	100	70	35	
		Maximum	123	85	43	

BEAU	JUN 1994										
Statistics before, during and after l-arginine infusion											
Before infusion	Sys	Dias	Mean BP	Heart rt							
Mean	184	97	143	72							
Standard Error	3.94	3.54	7.16	1.72							
Median	181.00	93.50	135.50	72.50							
Standard Deviation	11.14	10.01	20.24	4.87							
Range	37.00	32.00	53.00	15.00							
Minimum	172.00	86.00	122.00	61.00							
Maximum	209.00	118.00	175.00	76.00							
Count	8.00	8.00	8.00	8.00							
During Infusion											
Mean	171	95	131	68							
Standard Error	2.09	3.80	3.60	1.41							
Median	172.00	95.00	131.00	68.00							
Standard Deviation	5.54	10.06	9.52	3.73							
Range	16.00	29.00	27.00	10.00							
Minimum	161.00	80.00	119.00	64.00							
Maximum	177.00	109.00	146.00	74.00							
Count	7.00	7.00	7.00	7.00							
After Infusion											
Mean	184	101	136	79							
Standard Error	3.26	3.45	3.06	2.84							
Median	184.00	100.00	131.00	74.00							
Standard Deviation	9.79	10.36	9.17	8.51							
Range	29.00	33.00	26.00	26.00							
Minimum	172.00	84.00	128.00	72.00							
Maximum	201.00	117.00	154.00	98.00							
Count	9.00	9.00	9.00	9.00							

BEAU	JUN 94									
BP and heart rate during an acute attack of laminitis and l-arginine infusion i.v.										
Date	Time	Systolic mm Hg	Diastolic mm Hg	Mean	Ht-rate b/min	Obel Grd	Comment			
2.6.94	12.25	172	93	171	75	4				
	12.37	209	118	175	74					
	15.00	186	90	122	75		Resp 31 /min			
	15.45	177	92	133	76		sedated 0.15ml detomidine i.v.			
	15.50	179	86	123	71		and 0.3 ml butorphanol i.v.			
	16.00	186	103	137	70		0.3 ml butorphanol i.v.			
	16.08	181	94	134	70					
	16.15	181	99	145	61		iv catheter inserted			
	16.22	177	101	136	64		Infusion started at 16.21			
	16.25	174	95	126	64					
	16.30	161	85	119	68					
	16.40	172	91	131	68					
	16.45	168	80	121	68					
	16.49	176	101	146	72					
	16.51	169	109	136	74		Infusion stopped			
	16.56	191	100	136	73					
	17.00	184	84	129	72					
	17.02	184	102	129	74					
	17.05	201	92	129	74					
	17.10	194	110	144	84					
	17.13	172	96	131	84					
	17.17	176	96	128	74					
	17.21	184	117	154	76					
	17.25	173	111	143	98					

Case Report 1 BEAU I									
Changes in vital signs during application of glyceryl trinitrate patches topically (half an inch of percutol paste on each branch of digital artery ie two on each pastern, on RF LF and LH)									
Date	Time	Systolic mm Hg	Diastolic mm Hg	Mean	Ht.rate b/min	Obel Grd	Pulse (digital)	Comment	
3.6.94	13.05	205	108	139	62	4	yes	recumbent	
	13.32	176	81	118	61				
	Apply patches								
	19.45	169	89	125	57	3+			
4.6.94	16.00	174	80	106	49				
	Apply patches								
5.6.94	17.05	158	81	118	42	3			
	Apply patches								
6.6.94	19.15	152	69	105	40	3-			
	Apply patches								
7.6.94	13.15	132	69	84	40	2			
8.6.94	20.15	108	59	76	39	2-			
Mean		149	75	102	45				
Standard Error		#N/A	#N/A	#N/A	#N/A				
Median		155.00	74.50	105.50	41.00				
Standard Deviation		24.84	10.80	19.02	7.12				
Range		66.00	30.00	49.00	18.00				
Minimum		108.00	59.00	76.00	39.00				
Maximum		174.00	89.00	125.00	57.00				
Count		6	6	6	6	6			

Case Report 1 (contd.) BEAU I (contd.)									
Changes in vital signs during application of glyceryl trinitrate patches topically (quarter of an inch of percutol paste on each branch of the digital artery ie two on each pastern, on RF LF and LH)									
Date	Time	Systolic mm Hg	Diastolic mm Hg	Mean	Ht.rate b/min	Obel Grd	Pulse (digital)	Comment	
9.6.94	19.35	126	54	74	39				
10.6.94	19.45	121	59	78	38				
11.6.94	19.45	100	60	60	39				
12.6.94	19.45	100	54	65	41				
13.6.94	19.45	97	53	78	38				
Mean		108.8	56	71	39				
Standard Error		#N/A	#N/A	#N/A	#N/A				
Median		100	54	74	39				
Standard Deviation		13.59044	3.24037	8.1240	1.22474				
Variance		184.7	10.5	66	1.5				
Range		29	7	18	3				
Minimum		97	53	60	38				
Maximum		126	60	78	41				
Count		5	5	5	5				

Infusion of l-arginine and application of glyceryl trinitrate patches as a treatment for equine laminitis									
Vital signs before, during and after infusion of l-arginine									
Date	Time	Systolic mm Hg	Diastolic mm Hg	Mean mm Hg	Ht. rate b/min	Obel Grd	Pulse (digital)	Comment	
Before infusion									
Mean		184	97	143	72	33%			
During infusion									
Mean		171	95	131	68	34%			
After infusion									
Mean		184	101	136	79	42%			
After application of patches (initial dose)									
Mean		149	75	102	45				
After application of patches (half dose)									
Mean		109	56	71	39				

Case Report 2									
Beau 2									
	Glyceryl trinitrate	Glyceryl trinitrate total	Systolic mmHg	Diastolic mmHg	Mean mmHg	Heart rate			
Basal Values (Clinically Normal)			105	69	86	54			
26.7.94	0	0	187	85	121	61			3.5
27.8.94	0	0	188	86	124	50			3.5
28.7.94	64.8	64.8	173	87	129	43			3
29.7.94	86.4	141.2	171	73	104	43			3
30.7.94	86.4	237.6	120	59	78	53			2
31.7.94	0	237.6	123	61	78	30			1
1.8.94	53.76	291.36	112	61	81	40			1

Case Report 3
Beau 3

	Glyceryl trinitrate	Glyceryl trinitrate total	Blood Pressure			Heart rate	Obel Grade
			Systolic mmHg	Diastolic mmHg	Mean mmHg		
Basal Values (Clinically Normal)			105	69	86	54	
13/8/94	40	40	163	80	107	54	2.5
13/8/94	43	83	167	70	99	53	2.5
14/8/94	40		158	80	96	48	2
15/8/94			165	82	109	49	
16/8/94			135	55	81	42	2
17/8/94			106	44	57	86	1.5
18/8/94			127	63	81	39	1
19/8/94			127	58	86	41	

Case Report 4
Misty 1

Changes in vital signs during mild, untreated laminitis

Jul-Aug 94	Blood Pressure			Heart rate	Obel Grade
	Systolic mmHg	Diastolic mmHg	Mean mmHg		
Acute laminitis					
24.7.94	165	90	118	71	3
25.7.94	109	51	70	48	3
26.7.94	109	58	78	42	3
27.8.94	98	54	78	48	2
28.7.94	88	49	63	45	2
29.7.94	99	51	68	41	2
30.7.94	95	60	66	45	1

Case Report 5									
Misty 2									
Changes in vital signs during mild, untreated laminitis									
Jul-Aug 94									
	Glyceryl trinitrate	Glyceryl trinitrate	Blood Pressure	Diastolic	Mean	Heart rate	Obel Grade		
Date	mg	total mg	mmHg	mmHg	mmHg	beats/min			
7.8.94			151	97	115	72			
			159	91	127	78	3.75		
8.8.94	86.4	86.4	128	70	70	52	4		
9.9.94	86.4	172.8	92	47	64	42	2.75		
10.8.94	26.8	259.2	97	45	64	41	1.5		
12.8.94	0	259.2	96	54	71	42	1		

Case Report 6									
Misty 3									
Vital signs during topical treatment of laminitis with glyceryl trinitrate patches									
Jul-Aug 94									
	Glyceryl trinitrate	Glyceryl trinitrate	Blood Pressure	Diastolic	Mean	Heart rate			
Date	mg	total mg	mmHg	mmHg	mmHg	beats/min			
Basal values	0	0	105	69	86	54			
<i>(clinically normal)</i>									
<i>Acute laminitis</i>									
	0	0	171	88	129	61			
<i>Treatment</i>									
Day									
1	64.8	64.8	143	84	105	52			
2	32.4	97.2	116	66	92	49			
3	40.32	137.52	139	74	97	50			
4	40.32	178.04	105	61	81	47			
5	20.16	198.2	98	56	72	42			
6	0	198.2	90	47	62	40			
7	0	198.2	91	33	48	41			
			81	50	67	2			

Case Report 7						
Cobweb 1						
Changes in vital signs during mild, untreated laminitis						
	Blood Pressure			Heart rate	Obel Grade	
	Systolic mmHg	Diastolic mmHg	Mean mmHg	beats/min		
Mild laminitis						
24.7.94	155	84	117	62	2	
25.7.94	152	73	103	52	2	
26.7.94	129	64	91	41	2	
27.8.94	114	46	91	49	1	
28.7.94	104	61	82	61	1	
29.7.94	123	55	82	39	0	
30.7.94	117	59	82	43	0	

Case Report 8						
Cobweb 2						
	Glyceryl trinitrate		Blood Pressure		Heart rate	Obel Grade
	mg	total mg	Systolic mmHg	Diastolic mmHg	Mean mmHg	
12.8.94	0	0	177	86	125	57
13.8.94	86.4	86.4	147	75	114	51
14.8.94	86.4	172.8	93	49	65	66
15.8.94	0	172.8	135	71	99	46
16.8.94	13.44	186.24	142	71	97	48

Case Report 9
Domino

	Glyceryl trinitrate		Blood Pressure		Heart rate	Obel Grade
	mg	total mg	Systolic mmHg	Diastolic mmHg		
7/8/94	53.76	53.76	131	81	51	3
8/8/94	53.76	107.52	111	48	40	3
9/8/94	0	107.52				2
10/8/94	0	107.52				1
12/8/94	0	107.52	80	44	40	

Case Report 10
Tess

Vital signs during topical treatment of intermediate severity acute laminitis with glyceryl trinitrate patches

Date	Glyceryl trinitrate		Blood Pressure		Heart rate	Obel Grade
	mg	total mg	Systolic mmHg	Diastolic mmHg		
Basal values	0	0				
(clinically normal)						
Acute laminitis	0	0	174	77	116	2
			155	77	111	2
			162	84	117	2
	Mean		164	79	115	2
	Standard Error		6	2	2	1
	Standard Deviation		10	4	3	2
Treatment with GTN		43.2				

Domino and Beau - Acute laminitis 1995

	Systolic	Diastolic	Mean	Ht rate
<i>Domino</i>	100	60	71	48
	120	88	76	48
	115	59	77	47
	105	54	78	47
	98	56	71	46
Mean	108	63	75	47
SE	4	6	2	0
No of tests	5			
<i>Beau</i>	194	125	167	77
	189	97	141	80
	195	96	134	81
	219	137	193	75
	187	109	139	80
Mean	197	113	155	79
SE	6	8	11	1
No of tests	5			

Biscuit - Acute post partum laminitis 1995

Day/time	Blood pressure (mm Hg)			Heart rt	No. Tests	Obel Gd
	Systolic	Diastolic	Mean			
<i>Day 1</i>						
12.30pm	133	78	103	77		
	136	84	105	76		
	127	70	82	85		
Mean	132	77	97	79	3	3
SE	3	4	7	3		
7.30am	133	96	124	64		
	126	73	106	67		
	108	71	85	67		
Mean	127	79	101	73	6	4
SE	4	4	6	3		
<i>Day 2</i>						
7.15am	179	96	138	87		
	182	101	127	87		
	186	88	116	90	3	4
Mean	182	95	127	88		
SE	2	4	6	1	3	4
11am	155	87	87	100		
	154	90	131	101		
	148	83	95	109		
	151	81	103	103		
	145	88	106	101	5	4

Biscuit - Acute post partum laminitis 1995 (contd)

Day/time	Blood pressure (mmHg)			Heart rt	No. Tests	Obel Gd
	Systolic	Diastolic	Mean			
	Mean	151	86	104	103	
	SE	2	2	7	2	
7.45pm		135	93	107	82	
		133	91	107	80	
		133	78	108	87	4
	Mean	134	87	107	83	
	SE	1	5	0	2	
11pm		148	96	109	86	
		135	91	107	86	
		138	93	111	85	
		127	92	103	89	4
	Mean	137	93	108	87	
	SE	4	1	2	1	
Day 3						
7.15 am		152	93	118	69	
		147	91	111	72	
		123	81	97	72	
		143	83	111	76	4
	Mean	141	87	109	72	
	SE	6	3	4	1	

Biscuit - Acute post partum laminitis 1995 (contd)

Day/time	Blood pressure (mmHg)			Heart rt	No. Tests	Obel Gd
	Systolic	Diastolic	Mean			
11am		139	92	106	76	
		137	78	98	76	
		134	90	108	74	4
	Mean	137	87	104	75	
	SE	1	4	3	1	
8pm		132	91	106	76	
		134	106	116	76	
		133	80	106	75	4
	Mean	133	92	109	76	
	SE	1	8	3	0	
Day 4						
7.15am		154	77	108	67	
		135	92	115	74	
		122	84	109	73	
		144	79	102	72	4
	Mean	139	83	109	72	
	SE	7	3	3	2	

Biscuit - Acute post partum laminitis 1995 (contd)

Day/time	Blood pressure (mm Hg)			Heart rt	No. Tests	Obel Gd
	Systolic	Diastolic	Mean			
Day 6						
10.30am	151	109	133	70		
	163	108	129	70		oestrus
	173	116	138	70		
	152	95	128	67	4	3
	Mean	160	107	132		
	SE	5	4	2	1	
7.30pm	136	99	116	67		
	132	97	106	67		
	152	99	118	63	*	
	158	104	126	61	*	
	142	103	116	61		
	143	91	113	61	6	3
	Mean	144	99	116		
	SE	4	2	3	1	

Biscuit - Acute post partum laminitis 1995 (contd)

Day/time	Blood pressure (mm Hg)			Heart rt	No. Tests	Obel Gd
	Systolic	Diastolic	Mean			
8pm						
	151	81	112	67		
	143	78	102	64		
	133	71	95	66		
	135	80	99	68		
	136	81	106	71	5	3
	Mean	140	78	103		
	SE	3	2	3	1	
Day 5						
10.30am	161	91	122	67		
	161	103	126	61		
	176	111	138	65		
	176	117	143	61	4	3
	Mean	169	106	132		
	SE	4	6	5	2	
7.30pm	154	90	113	67		
	126	78	90	65		
	133	88	107	65		
	144	101	117	61		
	145	94	116	64	5	3
	Mean	140	90	109		
	SE	5	4	5	1	

Biscuit - Acute post partum laminitis 1995 (contd)

Day/time	Blood pressure (mm Hg)			Heart rt	No. Tests	Obel Gd
	Systolic	Diastolic	Mean			
Day 7						
9.40am	157	88	118	65		
	163	94	118	67		
	162	85	109	64		
	163	99	123	65	*foal suckling	
	159	97	114	63		
	158	91	124	64	6	3
	Mean	92	118	65		
	SE	2	2	1		
7.0pm	144	87	116	66		
	153	87	109	64		
	159	81	109	64		
	147	77	102	65		
	138	81	107	67	5	3
	Mean	83	109	65		
	SE	4	2	1		

Biscuit - Acute post partum laminitis 1995 (Contd)

Day/time	Blood pressure (mm Hg)			Heart rt	No. Tests	Obel Gd
	Systolic	Diastolic	Mean			
Day 8						
7.15am	153	107	123	69		
	151	96	115	60		
	158	91	106	60		
	159	95	117	59	4	3
	Mean	97	115	62		
	SE	3	4	2		
8.0pm	146	96	116	57		
	143	93	109	57		
	143	93	113	57	3	3
	Mean	94	113	57		
	SE	1	2	0		
Day 9						
7.30am	153	101	119	66		
	164	100	121	64		
	158	94	121	61	3	3
	Mean	98	120	64		
	SE	3	1	1		

Biscuit - Acute post partum laminitis 1995

Day/time	Blood pressure (mmHg)			Heart rt	No. Tests	Obel Gd
	Systolic	Diastolic	Mean			
7.30 pm	155	88	106	58		
	148	88	106	55		
	145	83	116	60		
	146	81	106	56	4	3
	Mean	149	109	57		
	SE	2	3	1		
Day 10						
7.45pm	148	90	112	51		
	147	92	114	55		
	117	93	106	56		
	134	84	102	57		
	136	89	107	55	5	3
	Mean	136	108	55		
	SE	6	2	1		
Day 11						
8.0pm	152	82	93	53		
	145	87	108	51		
	128	72	86	53		
	144	88	105	55	4	3
	Mean	142	98	53		
	SE	5	5	1		

Biscuit - Acute post partum laminitis 1995 (Contd)

Day/time	Blood pressure (mmHg)			Heart rt	No. Tests	Obel Gd
	Systolic	Diastolic	Mean			
Day 12						
7.45pm	144	87	105	55		
	158	89	111	55		
	159	83	107	56	3	3
	Mean	154	108	55		
	SE	5	2	0		
Day 13						
7.45pm	156	101	125	51	foal sucking	
	155	94	119	49	*	
	154	102	121	50	*	
	161	103	125	48	*	
	Mean	157	123	50	4	3
	SE	2	2	1		
Day 14						
7.45pm	161	86	113	45		
	142	73	91	44		
	128	75	92	45		
	115	75	90	45		
	135	74	99	44	5	2+
	Mean	136	97	45		
	SE	8	4	0		

Biscuit - Acute post partum laminitis 1995 (Contd)

Day/time	Blood pressure (mm Hg)			Heart rt	No. Tests	Obel Gd
	Systolic	Diastolic	Mean			
Day 15	192	120	165	50		
7.45pm	188	120	144	54		
	181	116	147	52		
	178	106	137	55	4	2+
	Mean	116	148	53		
	SE	3	6	1		
Day 16	127	73	93	43		
7.45pm	134	85	103	41		
	135	92	115	42		
	133	82	98	41		
	128	82	99	39	5	2+
	Mean	83	102	41		
	SE	2	4	1		

Biscuit - Acute post partum laminitis 1995 (Contd)

Day/time	Blood pressure (mm Hg)			Heart rt	No. Tests	Obel Gd
	Systolic	Diastolic	Mean			
Day 17						
7.0pm	148	91	113	43		
	146	85	104	43		
	143	84	109	44		
	136	88	105	44		
	131	86	104	47	5	2+
	Mean	87	107	44		
	SE	3	2	1		
Day 18	145	79	103	51		
7.0pm	153	84	107	50		
	142	91	107	52		
	145	74	101	51	4	2+
	Mean	82	105	51		
	SE	2	2	0		
Day 19	138	82	99	42		
6.0pm	138	88	108	42		
	137	86	101	42	3	2+
	Mean	85	103	42		
	SE	0	2	3		

Biscuit - Acute post partum laminitis 1995 (Contd)

Day/time	Blood pressure (mmHg)			Heart rt	No. Tests	Obel Gd
	Systolic	Diastolic	Mean			
Day 20						
5.0pm	152	87	116	42	*foal	
	138	86	104	55		
	152	99	124	55		
	142	80	106	51		
	139	81	106	50	7	2+
	164	97	122	51	*	
	157	98	119	50	*	
	Mean	149	90	114		
	SE	4	3	3	2	
Day 21						
8.0pm	126	76	93	44		
	117	79	92	44		
	115	74	90	45	3	2+
	Mean	119	76	92		
	SE	3	1	1	0	

Biscuit - Acute post partum laminitis 1995 (Contd)

Day/time	Blood pressure (mmHg)			Heart rt	No. Tests	Obel Gd
	Systolic	Diastolic	Mean			
Day 22						
7.30 pm	143	92	113	43		
	129	88	103	43		
	146	77	102	44		
	138	78	103	44		
	128	89	105	44	5	3
	Mean	137	85	105		
	SE	4	3	2	0	
Day 23						
10.15 pm	189	103	145	45		
	185	108	141	43		
	191	103	128	44		
	194	102	129	45	4	3
	Mean	190	136	44		
	SE	2	4	0		

Biscuit - Acute post partum laminitis 1995 (Contd)

Day/time	Blood pressure (mm Hg)			Heart rt	No. Tests	Obel Gd
	Systolic	Diastolic	Mean			
Day 24						
10.0pm	144	92	107	51		
	146	91	115	54		
	152	89	114	50		
	145	89	101	51		
	147	82	102	52		
	135	83	102	54		
	143	85	107	52	7	3
	Mean	87	107	52		
	SE	2	2	1		
Day 25						
10.0pm	144	92	107	51		
	146	91	115	54		
	152	89	114	50		
	145	89	107	51		
	147	82	102	53		
	135	83	102	54		
	Mean	87	107	52		
	SE	2	2	1		

Biscuit - Acute post partum laminitis 1995 (Contd)

Day/time	Blood pressure (mm Hg)			Heart rt	No. Tests	Obel Gd
	Systolic	Diastolic	Mean			
Day 26						
6.0pm	120	71	87	48		
	128	81	96	49		
	133	84	99	48		
	Mean	79	94	48		
	SE	4	4	0	3	2+
Day 27						
7.30pm	152	89	111	50		
	138	81	106	50		
	171	125	151	106	*foal suckling	
	143	80	103	72	*foal suckling	
	131	84	98	88	5	2+
	Mean	92	114	73		
	SE	7	10	11		

Biscuit - Acute post partum laminitis 1995 (Contd)										
Day/time	Systolic	Diastolic	Blood pressure (mmHg)			Heart rt	No. Tests	Obel Gd		
			Mean							
Day 28										
7.30pm	161	101	112		61		*foal annoys			
	135	83	102		56					
	136	79	99		50					
	129	71	91		53					
	122	64	78		54					
	136	91	106		59					
	144	93	115		53	7				2+
	Mean	138	83	100	55					
	SE	5	5	5	1					
Day 35										
7.30pm	154	103	133		62					
	181	120	143		60					
	177	108	139		65					
	166	98	125		63					
	175	113	131		65					
	166	101	129		64	6				3
	Mean	170	107	133	63					
	SE	4	3	3	1					

L-Name Infusions						
Cracker II	Systolic mmHg	Diastolic mmHg	Mean BP mmHg	Heart rate B/min	Comments	
Before infusion	71	57	64	47		
	120	90	108	45		
	126	74	104	40		
	116	74	95	39		
	103	65	88	41		
	113	67	95	42		
	99	67	88	40		
	95	48	66	82		
Mean	105	68	89	47		
Standard Error	6	4	6	5		
Standard Deviation	18	12	16	14		
n=8						
1-2pm						
Infusion (I)4mls	103	66	80	44		
	100	73	92	41		
	124	66	91	45		
	101	54	71	42		
	74	47	60	49		

(ii)15mls	122	58	76	48	
	113	64	85	43	
	103	58	75	41	
	115	64	92	44	
	115	56	78	42	
	111	61	73	43	
	103	65	78	40	
	80	57	70	41	
	106	58	74	40	
	98	55	65	42	
	82	55	70	41	
	115	56	85	43	
	75	46	62	41	v.quiet
	94	55	64	39	swaying
	83	53	64	39	
	94	63	76	39	
Mean	101	59	75	42	
Standard Error	3	1	2	1	
	15	6	10	3	
n=21					

Hypotensive episode	67	51	56	62 reared
2 hours later	137	52	72	42
	144	57	122	43
Mean	141	55	97	43
Standard Error	4	3	25	1
Standard Deviation	5	4	35	1
n=2				

L-Name Infusions(Contd)					
Tess	Systolic mmHg	Diastolic mmHg	Mean BP mmHg	Heart rate B/min	
Basal	131	60	82	56	
	97	67	88	52	
	92	53	68	50	
	112	63	57	57	
	129	59	74	63	
	122	69	107	57	
	95	68	86	44	
Mean	111	63	80	54	
Standard Error	6	2	6	2	
Standard Deviation	17	6	16	6	
n=7					
Infusion	127	85	106	48	
	136	76	102	40	
	142	93	115	43	
	178	96	123	39	
	132	87	114	39	
	134	79	98	41 unsteady	
	130	100	123	40 swaying	
	142	86	103	41	
	152	67	95	41	
Mean	141	85	109	41	
Standard Error	6	3	3	1	
Standard Deviation	16	10	10	3	
n=9					

Appendix III

Serum Electrolytes

Serum electrolytes of normal horses and ponies								
Date of Collection:	17/04/94							
Normal	Sodium	Potassium	Na : K Ratio	Chloride	Inorganic P	Calcium	Magnesium	PCV
	mmol/l	mmol/l		mmol/l	mmol/l	mmol/l	mmol/l	%
Despina	134	4.20	31.90	99.00	1.06	3.15	0.76	47
Jasper	135	4.30	31.40	100.00	1.15	3.16	0.82	46
Marron	136	4.60	29.57	101.00	0.91	3.21	0.73	45
Selluci	134	4.00	33.50	99.00	1.29	2.97	0.79	38
Snowflake	137	4.80	28.54	102.00	1.20	3.26	0.93	48
Tess*	138	4.80	28.75	102.00	1.09	3.25	0.83	48
Group Mean	136	4.45	30.61	100.50	1.12	3.17	0.81	45
Standard Error	0.67	0.14	0.81	0.56	0.05	0.04	0.03	2
Range	(134 - 138)	(4.00 - 4.80)	(28.54 - 33.50)	(99.00 - 102.00)	(0.91 - 1.29)	(2.97 - 3.26)	(0.73 - 0.93)	(38 - 48)
Normal Range	(132 - 146)	(3.30 - 5.40)	(28.00 - 40.00)	(89.00 - 108.00)	(0.90 - 1.80)	(2.50 - 3.60)	(0.60 - 1.00)	(32 - 53)
No. of Tests	6	6	6	6	6	6	6	6
(* - Tess before the first attack of laminitis)								
Serum electrolytes for chronically laminitic horses and ponies								
Date of Collection:	17/04/94							
Chronic Laminitic	Sodium	Potassium	Na : K Ratio	Chloride	Inorganic P	Calcium	Magnesium	PCV
	mmol/l	mmol/l		mmol/l	mmol/l	mmol/l	mmol/l	%
Beau	135	3.90	34.62	103.00	1.04	3.15	0.90	45
Cobweb	134	4.00	33.50	100.00	1.10	2.91	0.68	40
Domino	135	4.00	33.75	101.00	0.92	3.21	0.88	33
Misty	135	3.70	36.49	99.00	1.43	2.98	0.61	40
Rose Marie	135	3.60	37.50	102.00	0.97	2.97	0.69	37
Group Mean	135	3.84	35.17	101.00	1.09	3.04	0.75	39
Standard Error	0	0.08	0.78	0.71	0.09	0.06	0.06	2
Range	(134 - 135)	(3.60 - 4.00)	(33.50 - 37.50)	(99.00 - 103.00)	(0.92 - 1.43)	(2.91 - 3.21)	(0.61 - 0.90)	(33 - 45)
Normal Range	(132 - 146)	(3.30 - 5.40)	(28.00 - 40.00)	(89.00 - 108.00)	(0.90 - 1.80)	(2.50 - 3.60)	(0.60 - 1.00)	(32 - 53)
No. of Tests	5	5	5	5	5	5	5	5

Serum electrolytes of normal horses and ponies								
Date of Collection:	30/04/94							
Normal	Sodium	Potassium	Na : K Ratio	Chloride	Inorganic P	Calcium	Magnesium	PCV
	mmol/l	mmol/l		mmol/l	mmol/l	mmol/l	mmol/l	%
<i>Cracker II</i>	133.00	4.60	28.91	96.00	1.08	3.00	0.70	50
<i>Despina</i>	147.00	5.40	27.22	111.00	1.13	3.83	0.79	47
<i>Marron</i>	146.00	4.30	33.95	108.00	0.99	3.57	0.79	52
<i>Selluci</i>	167.00	6.70	27.54	116.00	1.67	3.87	0.89	40
<i>Snowflake</i>	163.00	6.30	25.87	123.00	1.13	4.06	1.03	50
<i>Tess*</i>	158.00	4.70	33.62	120.00	1.04	3.85	0.90	50
Group Mean	150.87	5.17	29.52	112.50	1.17	3.68	0.85	48.17
Standard Error	4.45	0.31	1.41	3.94	0.10	0.15	0.05	1.76
Range	(133 - 163)	(4.30 - 6.30)	(25.87 - 33.95)	(96.00 - 123.00)	(0.99 - 1.67)	(3.00 - 4.06)	(0.70 - 1.03)	(40 - 50)
Normal Range	(132 - 148)	(3.30 - 5.40)	(28.00 - 40.00)	(89.00 - 108.00)	(0.90 - 1.80)	(2.50 - 3.80)	(0.60 - 1.00)	(32 - 53)
No. of Tests	6	6	6	6	6	6	6	6
(* - Tess before the first attack of laminitis)								
Serum electrolytes for chronically laminitic horses and ponies								
Date of Collection:	30/04/94							
Chronic laminitic	Sodium	Potassium	Na : K Ratio	Chloride	Inorganic P	Calcium	Magnesium	PCV
	mmol/l	mmol/l		mmol/l	mmol/l	mmol/l	mmol/l	%
<i>Beau</i>	181.00	6.80	31.21	142.00	1.27	4.66	1.07	45
<i>Cobweb</i>	212.00	6.90	30.72	166.00	1.57	5.18	1.48	
<i>Domino</i>	149.00	4.40	33.88	113.00	1.13	3.45	0.85	
<i>Misty</i>	178.00	6.70	31.40	135.00	1.18	4.35	0.94	46
Group Mean	180.25	5.70	31.80	139.00	1.29	4.41	1.09	45
Standard Error	12.87	0.51	0.70	10.92	0.10	0.36	0.14	0
Range	(149 - 212)	(4.40 - 6.90)	(30.72 - 33.88)	(113.00 - 166.00)	(1.13 - 1.57)	(3.45 - 5.18)	(0.85 - 1.48)	(45 - 46)
Normal Range	(132 - 148)	(3.30 - 5.40)	(28.00 - 40.00)	(89.00 - 108.00)	(0.90 - 1.80)	(2.50 - 3.80)	(0.60 - 1.00)	(32 - 53)
No. of Tests	4	4	4	4	4	4	4	2

Serum electrolytes of normal horses and ponies								
Date of Collection:			12/06/94					
Normal	Sodium	Potassium	Na : K Ratio	Chloride	Inorganic P	Calcium	Magnesium	PCV
	mmol/l	mmol/l		mmol/l	mmol/l	mmol/l	mmol/l	%
<i>Annabella</i>	131.00	4.90	26.73	99.00	1.07	3.26	0.80	41
<i>Cracker II</i>	128.00	3.80	33.68	96.00	0.69	2.86	0.54	38
<i>Despina</i>	133.00	4.20	31.67	101.00	1.07	3.13	0.82	41
<i>Jasper</i>	131.00	4.10	31.95	100.00	1.14	3.22	0.84	36
<i>Marron</i>	132.00	4.10	32.20	100.00	0.87	3.28	0.74	44
<i>Melody</i>	131.00	4.80	27.29	100.00	1.00	2.95	0.72	47
<i>Selluci</i>	133.00	4.00	33.25	95.00	1.27	2.90	0.78	44
<i>Snowflake</i>	131.00	4.50	29.11	96.00	1.21	3.18	0.77	51
<i>Tess*</i>	131.00	4.50	29.11	100.00	1.20	3.19	0.78	46
Group Mean	131.22	4.32	30.55	98.56	1.06	3.11	0.75	43
Standard Error	0.49	0.12	0.85	0.75	0.06	0.05	0.03	2
Range	(128 - 133)	(3.80 - 4.90)	(26.73 - 33.68)	(95.00 - 101.00)	(0.69 - 1.27)	(2.86 - 3.28)	(0.54 - 0.84)	(36 - 51)
Normal Range	(132 - 146)	(3.30 - 5.40)	(28.00 - 40.00)	(89.00 - 108.00)	(0.90 - 1.80)	(2.50 - 3.60)	(0.60 - 1.00)	(32 - 53)
No. of Tests	9	9	9	9	9	9	9	9
(* - Tess before the first attack of laminitis)								
Serum electrolytes for chronically laminitic horses and ponies								
Date of Collection:			12/06/94					
Chronic Laminitic	Sodium	Potassium	Na : K Ratio	Chloride	Inorganic P	Calcium	Magnesium	PCV
	mmol/l	mmol/l		mmol/l	mmol/l	mmol/l	mmol/l	%
<i>Beau</i>	133.00	4.10	32.44	99.00	0.75	3.25	0.75	44
<i>Cobweb</i>	129.00	3.20	40.31	99.00	0.50	2.91	0.61	36
<i>Domino</i>	133.00	4.10	32.44	101.00	1.27	3.29	0.75	38
<i>Misty</i>	132.00	4.10	32.20	100.00	1.00	3.08	0.63	37
<i>Rose Mare</i>	133.00	3.30	40.30	102.00	1.04	3.08	0.70	39
Group Mean	132.00	3.76	35.54	100.20	0.91	3.12	0.69	39
Standard Error	0.77	0.21	1.95	0.58	0.13	0.07	0.03	1
Range	(129 - 133)	(3.20 - 4.10)	(32.20 - 40.31)	(99.00 - 102.00)	(0.50 - 1.27)	(2.91 - 3.29)	(0.61 - 0.75)	(36 - 44)
Normal Range	(132 - 146)	(3.30 - 5.40)	(28.00 - 40.00)	(89.00 - 108.00)	(0.90 - 1.80)	(2.50 - 3.60)	(0.60 - 1.00)	(32 - 53)
No. of Tests	5	5	5	5	5	5	5	5

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Serum electrolytes of normal horses and ponies								
Date of Collection:	17/07/94							
Normal	Sodium mmol/l	Potassium mmol/l	Na : K Ratio	Chloride mmol/l	Inorganic P mmol/l	Calcium mmol/l	Magnesium mmol/l	PCV %
<i>Afnabella</i>	162.00	5.00	30.40	116.00	1.33	3.89	0.99	30
<i>Cracker II</i>	162.00	6.00	25.33	113.00	1.37	3.79	0.83	44
<i>Despina</i>	176.00	6.20	28.23	133.00	1.54	4.20	1.02	35
<i>Jasper</i>	146.00	4.70	31.06	109.00	1.38	3.48	0.78	36
<i>Marron</i>	178.00	6.70	31.23	133.00	1.57	4.28	0.93	42
<i>Melody</i>	187.00	6.30	29.68	141.00	1.77	4.63	1.20	36
<i>Selluci</i>	182.00	6.30	28.89	138.00	1.87	4.12	1.07	36
<i>Snowflake</i>	174.00	6.10	28.52	131.00	1.68	4.37	1.11	50
<i>Tess*</i>	180.00	6.00	30.00	136.00	1.88	4.46	1.04	41
Group Mean	169.56	5.81	29.26	127.56	1.62	4.14	1.00	38.89
Standard Error	5.08	0.19	0.60	3.96	0.08	0.12	0.04	1.98
Range	(146 - 187)	(4.70 - 6.30)	(25.33 - 31.23)	(109.00 - 141.00)	(1.33 - 1.88)	(3.48 - 4.63)	(0.78 - 1.20)	(30 - 50)
Normal Range	(132 - 146)	(3.30 - 5.40)	(28.00 - 40.00)	(89.00 - 108.00)	(0.80 - 1.80)	(2.50 - 3.60)	(0.60 - 1.00)	(32 - 53)
No. of Tests	9	9	9	9	9	9	9	9
(* - Tess before the first attack of laminitis)								
Serum electrolytes for chronically laminitic horses and ponies								
Date of Collection:	17/07/94							
Chronic Laminitic	Sodium mmol/l	Potassium mmol/l	Na : K Ratio	Chloride mmol/l	Inorganic P mmol/l	Calcium mmol/l	Magnesium mmol/l	PCV %
<i>Beau</i>	164.00	3.50	44.00	118.00	0.84	3.57	0.96	38
<i>Cobweb</i>	163.00	4.50	36.22	123.00	2.02	3.95	1.04	42
<i>Domino</i>	187.00	6.80	27.50	141.00	1.52	4.86	1.11	36
<i>Misty</i>	136.00	4.30	31.63	104.00	0.89	3.12	0.64	37
<i>Roxmead</i>	178.00	5.00	35.60	136.00	1.30	4.07	0.96	40
Group Mean	163.60	4.82	34.99	124.20	1.31	3.91	0.94	38.60
Standard Error	8.97	0.55	2.74	6.51	0.22	0.29	0.08	1.08
Range	(136 - 187)	(3.50 - 6.80)	(27.50 - 44.00)	(104.00 - 141.00)	(0.84 - 2.02)	(3.12 - 4.86)	(0.64 - 1.11)	(36 - 42)
Normal Range	(132 - 146)	(3.30 - 5.40)	(28.00 - 40.00)	(89.00 - 108.00)	(0.80 - 1.80)	(2.50 - 3.60)	(0.60 - 1.00)	(32 - 53)
No. of Tests	5	5	5	5	5	5	5	5

Appendix III

Serum electrolytes of normal horses and ponies								
Date of Collection:	14/08/94							
Normal	Sodium	Potassium	Na : K Ratio	Chloride	Inorganic P	Calcium	Magnesium	PCV
	mmol/l	mmol/l		mmol/l	mmol/l	mmol/l	mmol/l	%
Albert	139.00	4.30	32.33	103.00	0.99	3.28	0.85	45
Annabella	167.00	5.70	29.30	128.00	1.58	4.07	0.97	37
Cracker II	132.00	4.00	33.00	95.00	1.38	3.08	0.68	35
Despina	205.00	6.50	31.54	158.00	2.30	4.74	1.27	35
Jasper	177.00	5.50	32.18	137.00	1.59	4.53	1.22	37
Marron	117.00	3.70	31.62	86.00	0.92	2.82	0.65	41
Melody	154.00	5.30	29.06	116.00	1.30	3.57	0.82	35
Selluci	175.00	5.00	35.00	135.00	1.98	3.79	0.85	38
Snowflake	160.00	6.70	23.88	118.00	1.71	3.88	0.92	47
Tess*	134.00	4.20	31.90	100.00	1.16	3.31	0.74	47
Group Mean	156.00	5.09	30.98	117.60	1.49	3.71	0.90	40
Standard Error	8.31	0.33	0.95	7.04	0.14	0.20	0.07	2
Range	(117 - 205)	(3.70 - 6.70)	(23.88 - 35.00)	(86.00 - 158.00)	(0.92 - 2.30)	(2.82 - 4.74)	(0.65 - 1.27)	(35 - 47)
Normal Range	(132 - 146)	(3.30 - 5.40)	(28.00 - 40.00)	(89.00 - 108.00)	(0.90 - 1.80)	(2.50 - 3.60)	(0.60 - 1.00)	(32 - 53)
No. of Tests	10	10	10	10	10	10	10	10
(* - Tess before the first attack of laminitis)								
Serum electrolytes for chronically laminitic horses and ponies								
Date of Collection:	14/08/94							
Chronic Laminitic	Sodium	Potassium	Na : K Ratio	Chloride	Inorganic P	Calcium	Magnesium	PCV
	mmol/l	mmol/l		mmol/l	mmol/l	mmol/l	mmol/l	%
Beau	162.00	5.20	31.15	123.00	1.46	3.97	0.95	44
Cobweb	211.00	6.80	31.03	163.00	1.46	5.28	1.27	48
Domino	162.00	4.60	35.22	126.00	1.17	3.92	0.78	44
Misty	151.00	5.20	29.04	116.00	0.97	3.73	0.76	46
Rose Marie	177.00	5.30	33.40	138.00	1.35	3.98	0.90	44
Group Mean	172.50	5.42	31.97	133.20	1.28	4.18	0.93	45
Standard Error	10.45	0.37	1.07	8.25	0.09	0.28	0.09	1
Range	(151 - 211)	(4.60 - 6.80)	(29.04 - 35.22)	(116.00 - 163.00)	(0.97 - 1.46)	(3.73 - 5.28)	(0.76 - 1.27)	(44 - 48)
Normal Range	(132 - 146)	(3.30 - 5.40)	(28.00 - 40.00)	(89.00 - 108.00)	(0.90 - 1.80)	(2.50 - 3.60)	(0.60 - 1.00)	(32 - 53)
No. of Tests	5	5	5	5	5	5	5	5

Serum electrolytes of normal horses and ponies								
Date of Collection:	21/08/94							
Normal	Sodium	Potassium	Na : K Ratio	Chloride	Inorganic P	Calcium	Magnesium	PCV
	mmol/l	mmol/l		mmol/l	mmol/l	mmol/l	mmol/l	%
<i>Albert</i>	143.00	4.50	31.78	108.00	0.97	3.64	0.83	47
<i>Annabella</i>	145.00	4.60	31.52	110.00	1.07	3.52	0.87	36
<i>Cracker II</i>	164.00	5.30	29.08	116.00	1.48	3.54	0.90	37
<i>Despina</i>	164.00	6.60	29.29	127.00	1.18	3.64	1.02	36
<i>Jasper</i>	201.00	5.30	37.92	166.00	1.39	6.11	1.37	36
<i>Marron</i>	126.00	4.00	31.25	94.00	1.18	2.89	0.71	38
<i>Melody</i>	144.00	6.60	26.71	107.00	1.34	3.10	0.76	35
<i>Snowflake</i>	166.00	6.60	23.85	116.00	1.55	3.66	1.04	47
<i>Tess*</i>	168.00	6.80	28.97	129.00	1.58	3.97	0.99	38
Group Mean	155.44	5.24	29.93	117.89	1.37	3.67	0.94	38.89
Standard Error	7.10	0.25	1.34	5.83	0.11	0.21	0.07	1.57
Range	(125 - 201)	(4.00 - 6.50)	(23.85 - 37.92)	(94.00 - 155.00)	(0.97 - 1.99)	(2.99 - 5.11)	(0.71 - 1.37)	(35 - 47)
Normal Range	(132 - 146)	(3.30 - 5.40)	(28.00 - 40.00)	(89.00 - 108.00)	(0.90 - 1.80)	(2.50 - 3.60)	(0.60 - 1.00)	(32 - 53)
No. of Tests	9	9	9	9	9	9	9	9
(* - Tess before the first attack of laminitis)								
Serum electrolytes for chronically laminitic horses and ponies								
Date of Collection:	21/08/94							
Chronic Laminitic	Sodium	Potassium	Na : K Ratio	Chloride	Inorganic P	Calcium	Magnesium	PCV
	mmol/l	mmol/l		mmol/l	mmol/l	mmol/l	mmol/l	%
<i>Beau</i>	171.00	6.30	27.14	132.00	1.39	3.98	0.84	44
<i>Cobweb</i>	169.00	6.90	26.96	123.00	0.93	3.76	0.95	48
<i>Domino</i>	176.00	7.10	24.79	133.00	1.01	4.46	1.13	36
<i>Misty</i>	144.00	6.60	26.71	112.00	0.88	3.38	0.72	42
<i>Rose Mare</i>	161.00	5.20	29.04	117.00	1.31	3.44	0.83	40
Group Mean	160.20	6.02	26.73	123.40	1.10	3.80	0.89	42.00
Standard Error	5.98	0.32	0.72	4.11	0.10	0.20	0.07	2.00
Range	(144 - 176)	(5.20 - 7.10)	(24.79 - 29.04)	(112.00 - 133.00)	(0.88 - 1.39)	(3.38 - 4.46)	(0.72 - 1.13)	(36 - 48)
Normal Range	(132 - 146)	(3.30 - 5.40)	(28.00 - 40.00)	(89.00 - 108.00)	(0.90 - 1.80)	(2.50 - 3.60)	(0.60 - 1.00)	(32 - 53)
No. of Tests	5	5	5	5	5	5	5	5

Serum electrolytes of normal horses and ponies								
Date of Collection:	28/08/94							
Normal	Sodium	Potassium	Na : K Ratio	Chloride	Inorganic P	Calcium	Magnesium	PCV
	mmol/l	mmol/l		mmol/l	mmol/l	mmol/l	mmol/l	%
Albert	166.00	6.60	27.86	120.00	1.19	3.77	0.87	43
Annabella	163.00	5.40	30.19	127.00	1.21	4.12	0.97	39
Cracker II	168.00	6.20	27.10	127.00	1.60	4.12	0.92	50
Despina	134.00	4.60	29.13	102.00	0.95	3.00	0.70	39
Jasper	128.00	4.40	29.09	95.00	1.11	3.02	0.64	36
Marron	132.00	4.00	33.00	99.00	0.86	3.15	0.65	38
Melody	120.00	5.00	24.00	89.00	0.92	2.86	0.69	39
Sailuci	166.00	6.40	26.78	126.00	1.78	3.36	0.85	44
Snowflake	192.00	7.30	24.30	141.00	1.90	4.90	1.19	48
Tess*	198.00	8.30	23.86	148.00	2.41	4.55	1.01	43
Group Mean	155.60	5.78	27.43	117.40	1.39	3.74	0.85	41.90
Standard Error	8.48	0.48	0.95	6.38	0.16	0.22	0.06	1.43
Range	(120 - 198)	(4.00 - 8.30)	(23.86 - 33.00)	(89.00 - 148.00)	(0.86 - 2.41)	(2.86 - 4.90)	(0.64 - 1.19)	(38 - 50)
Normal Range	(132 - 146)	(3.30 - 5.40)	(28.00 - 40.00)	(89.00 - 108.00)	(0.90 - 1.80)	(2.50 - 3.60)	(0.60 - 1.00)	(32 - 53)
No. of Tests	10	10	10	10	10	10	10	10
(* - Tess before the first attack of laminitis)								
Serum electrolytes for chronically laminitic horses and ponies								
Date of Collection:	28/08/94							
Chronic Laminitic	Sodium	Potassium	Na : K Ratio	Chloride	Inorganic P	Calcium	Magnesium	PCV
	mmol/l	mmol/l		mmol/l	mmol/l	mmol/l	mmol/l	%
Beau	190.00	6.20	30.65	149.00	0.98	4.91	1.16	36
Cobweb	229.00	7.80	29.38	186.00	1.68	6.64	1.44	43
Domino	141.00	4.80	29.37	108.00	1.18	3.43	0.83	34
Misty	177.00	6.90	30.00	139.00	1.18	4.28	0.91	40
Rose Mare	148.00	6.50	26.31	117.00	0.72	3.48	0.78	48
Group Mean	177.00	6.04	29.28	139.60	1.15	4.34	1.02	40
Standard Error	15.83	0.50	0.63	13.53	0.16	0.43	0.12	2
Range	(141 - 229)	(4.80 - 7.80)	(26.91 - 30.65)	(108.00 - 186.00)	(0.72 - 1.68)	(3.43 - 6.64)	(0.78 - 1.44)	(34 - 48)
Normal Range	(132 - 146)	(3.30 - 5.40)	(28.00 - 40.00)	(89.00 - 108.00)	(0.90 - 1.80)	(2.50 - 3.60)	(0.60 - 1.00)	(32 - 53)
No. of Tests	5	5	5	5	5	5	5	5

Serum electrolytes of normal horses and ponies								
Date of Collection:	04/09/94							
Normal	Sodium	Potassium	Na : K Ratio	Chloride	Inorganic P	Calcium	Magnesium	PCV
	mmol/l	mmol/l		mmol/l	mmol/l	mmol/l	mmol/l	%
Albert	134.00	2.40	56.83	100.00	0.83	3.26	0.81	50
Annabella	133.00	4.30	30.93	101.00	1.07	3.10	0.69	38
Cracker II	132.00	4.20	31.43	99.00	1.14	3.27	0.75	40
Despina	130.00	4.70	27.66	98.00	0.98	3.16	0.79	38
Jasper	133.00	4.40	30.23	100.00	1.05	3.25	0.74	37
Marron	133.00	4.20	31.67	101.00	0.99	3.30	0.67	40
Melody	132.00	5.00	26.40	100.00	1.27	3.22	0.74	38
Selluci	132.00	6.60	23.57	99.00	1.14	3.05	0.67	44
Snowflake	131.00	4.90	26.73	98.00	1.10	3.14	0.82	48
Tess*	132.00	4.50	29.33	99.00	1.28	3.31	0.75	44
Group Mean	132.20	4.42	31.38	99.50	1.09	3.21	0.74	42
Standard Error	0.36	0.26	2.84	0.34	0.04	0.03	0.02	1
Range	(130 - 134)	(2.40 - 5.60)	(23.57 - 55.83)	(98.00 - 101.00)	(0.83 - 1.28)	(3.05 - 3.31)	(0.67 - 0.82)	(37 - 50)
Normal Range	(132 - 146)	(3.30 - 5.40)	(28.00 - 40.00)	(89.00 - 108.00)	(0.90 - 1.80)	(2.50 - 3.60)	(0.60 - 1.00)	(32 - 53)
No. of Tests	10	10	10	10	10	10	10	10
(* - Tess before the first attack of laminitis)								
Serum electrolytes for chronically laminitic horses and ponies								
Date of Collection:	04/09/94							
Chronic Laminitic	Sodium	Potassium	Na : K Ratio	Chloride	Inorganic P	Calcium	Magnesium	PCV
	mmol/l	mmol/l		mmol/l	mmol/l	mmol/l	mmol/l	%
Beau	132.00	4.50	29.33	101.00	0.82	3.18	0.82	38
Cobweb	131.00	3.80	34.47	101.00	0.81	3.11	0.76	44
Domino	130.00	3.90	33.33	99.00	1.15	3.19	0.72	36
Misty	131.00	4.30	30.47	100.00	0.86	3.14	0.74	40
Rose Mare	133.00	3.80	35.00	103.00	0.86	3.17	0.71	42
Group Mean	131.40	4.06	35.00	100.80	0.90	3.16	0.75	40
Standard Error	0.51	0.14	1.12	0.66	0.06	0.01	0.02	1
Range	(130 - 133)	(3.80 - 4.50)	(29.33 - 35.00)	(99.00 - 103.00)	(0.81 - 1.15)	(3.11 - 3.19)	(0.71 - 0.82)	(36 - 44)
Normal Range	(132 - 146)	(3.30 - 5.40)	(28.00 - 40.00)	(89.00 - 108.00)	(0.90 - 1.80)	(2.50 - 3.60)	(0.60 - 1.00)	(32 - 53)
No. of Tests	5	5	5	5	5	5	5	5

Serum electrolytes of normal horses and ponies								
Date of Collection:	11/09/94							
Normal	Sodium	Potassium	Na : K Ratio	Chloride	Inorganic P	Calcium	Magnesium	PCV
	mmol/l	mmol/l		mmol/l	mmol/l	mmol/l	mmol/l	%
Albert	138.00	3.90	35.38	103.00	0.82	2.97	0.53	45
Annabella	138.00	4.10	33.17	103.00	1.09	3.25	0.72	38
Cracker II	137.00	4.10	33.41	100.00	1.25	3.00	0.67	44
Desana	133.00	4.50	30.67	102.00	1.13	3.02	0.72	41
Jasper	138.00	4.10	33.17	100.00	1.15	3.08	0.72	39
Merron	139.00	4.20	33.10	104.00	0.98	2.98	0.64	41
Melody	137.00	3.90	35.13	102.00	1.39	3.21	0.75	34
Selluci	133.00	4.40	30.23	101.00	0.99	2.91	0.71	43
Snowflake	133.00	4.50	29.58	98.00	1.12	3.08	0.74	44
Tess*	135.00	4.40	30.68	101.00	1.18	3.13	0.83	42
Group Mean	138.20	4.21	32.45	101.40	1.11	3.08	0.73	41
Standard Error	0.65	0.07	0.65	0.58	0.05	0.03	0.02	1
Range	(133 - 139)	(3.90 - 4.50)	(29.58 - 35.38)	(98.00 - 104.00)	(0.82 - 1.39)	(2.91 - 3.25)	(0.64 - 0.83)	(34 - 45)
Normal Range	(132 - 148)	(3.30 - 5.40)	(28.00 - 40.00)	(89.00 - 108.00)	(0.90 - 1.80)	(2.50 - 3.60)	(0.60 - 1.00)	(32 - 53)
No. of Tests	10	10	10	10	10	10	10	10
(* - Tess before the first attack of laminitis)								
Serum electrolytes for chronically laminitic horses and ponies								
Date of Collection:	11/09/94							
Chronic Laminitic	Sodium	Potassium	Na : K Ratio	Chloride	Inorganic P	Calcium	Magnesium	PCV
	mmol/l	mmol/l		mmol/l	mmol/l	mmol/l	mmol/l	%
Beau	137.00	4.00	34.25	107.00	0.80	3.22	0.78	44
Cobweb	138.00	3.80	36.32	102.00	1.25	3.05	0.73	44
Domino	137.00	3.90	35.13	102.00	1.14	3.18	0.73	38
Misty	138.00	4.70	28.94	100.00	1.00	3.17	0.71	43
Rose Mane	134.00	4.30	31.18	105.00	0.60	2.69	0.67	43
Tess								
Group Mean	138.40	4.14	33.18	103.20	0.98	3.08	0.72	42
Standard Error	0.68	0.18	1.38	1.24	0.12	0.10	0.02	1
Range	(134 - 138)	(3.80 - 4.70)	(28.94 - 36.32)	(100.00 - 107.00)	(0.60 - 1.25)	(2.69 - 3.22)	(0.67 - 0.78)	(38 - 44)
Normal Range	(132 - 148)	(3.30 - 5.40)	(28.00 - 40.00)	(89.00 - 108.00)	(0.90 - 1.80)	(2.50 - 3.60)	(0.60 - 1.00)	(32 - 53)
No. of Tests	5	5	5	5	5	5	5	5

Serum electrolytes of normal horses and ponies								
Date of Collection:	16/10/94							
Normal	Sodium	Potassium	Na : K Ratio	Chloride	Inorganic P	Calcium	Magnesium	PCV
	mmol/l	mmol/l		mmol/l	mmol/l	mmol/l	mmol/l	%
Albert	137.00	5.00	27.40	102.00	0.86	3.26	0.72	43
Annapela	138.00	4.90	28.16	105.00	1.23	3.25	0.75	39
Cracker II	140.00	4.40	31.82	102.00	1.29	3.22	0.61	47
Despina	134.00	4.50	29.78	99.00	1.39	2.98	0.64	41
Jasper	134.00	4.60	29.13	100.00	1.26	3.15	0.73	42
Marron	138.00	4.20	32.86	103.00	0.99	3.08	0.62	42
Melody	135.00	4.90	27.55	101.00	1.25	3.26	0.77	45
Seluc	135.00	4.30	31.40	98.00	1.37	2.95	0.65	42
Snowflake	135.00	5.70	23.88	98.00	1.24	3.11	0.66	43
Tess*	134.00	5.00	26.80	99.00	1.25	3.20	0.65	50
Group Mean	136.00	4.75	28.86	100.70	1.21	3.14	0.68	43
Standard Error	0.67	0.14	0.87	0.73	0.05	0.04	0.02	1
Range	(134 - 140)	(4.20 - 5.70)	(23.68 - 32.86)	(98.00 - 105.00)	(0.86 - 1.39)	(2.85 - 3.25)	(0.61 - 0.77)	(39 - 50)
Normal Range	(132 - 146)	(3.30 - 5.40)	(28.00 - 40.00)	(89.00 - 108.00)	(0.90 - 1.80)	(2.50 - 3.60)	(0.60 - 1.00)	(32 - 53)
No. of Tests	10	10	10	10	10	10	10	10
(* - Tess before the first attack of laminitis)								
Serum electrolytes for chronically laminitic horses and ponies								
Date of Collection:	16/10/94							
Chronic Laminitic	Sodium	Potassium	Na : K Ratio	Chloride	Inorganic P	Calcium	Magnesium	PCV
	mmol/l	mmol/l		mmol/l	mmol/l	mmol/l	mmol/l	%
Beau	135.00	4.00	33.75	103.00	1.22	2.96	0.73	40
Cobweb	135.00	4.90	27.55	104.00	0.82	3.20	0.80	46
Domino	136.00	4.20	32.38	105.00	0.68	3.10	0.73	32
Misty	135.00	5.10	26.47	101.00	1.58	3.16	0.67	44
Rose Mare	134.00	4.50	29.78	104.00	0.73	3.14	0.65	43
Group Mean	135.00	4.54	29.99	103.40	1.01	3.11	0.72	41
Standard Error	0.32	0.21	1.38	0.68	0.17	0.04	0.03	2
Range	(134 - 136)	(4.00 - 5.10)	(26.47 - 33.75)	(101.00 - 105.00)	(0.68 - 1.58)	(2.96 - 3.20)	(0.65 - 0.80)	(32 - 46)
Normal Range	(132 - 146)	(3.30 - 5.40)	(28.00 - 40.00)	(89.00 - 108.00)	(0.90 - 1.80)	(2.50 - 3.60)	(0.60 - 1.00)	(32 - 53)
No. of Tests	5	5	5	5	5	5	5	5

Serum electrolytes of normal horses and ponies									
Date of Collection:	19/11/94								
Normal	Sodium	Potassium	Na : K Ratio	Chloride	Inorganic P	Calcium	Magnesium	PCV	
	mmol/l	mmol/l		mmol/l	mmol/l	mmol/l	mmol/l	%	
Albert	140.00	4.80	29.17	106.00	0.78	3.08	0.72	48	
Annabelle	135.00	6.90	19.57	104.00	0.96	3.13	0.67	42	
Cracker	136.00	5.40	25.19	103.00	1.01	3.22	0.62	46	
Casone	136.00	5.10	26.67	104.00	1.03	3.11	0.68	39	
Jasoor	137.00	5.20	26.35	102.00	1.04	3.18	0.69	42	
Marron	137.00	4.60	29.78	102.00	0.81	3.04	0.57	46	
Meady	136.00	6.40	21.25	103.00	0.97	3.10	0.71	46	
Seluc	134.00	6.80	19.71	100.00	1.23	3.02	0.67	38	
Snowflake	132.00	7.10	18.59	100.00	1.18	3.19	0.63	51	
Tess*	133.00	6.80	19.56	101.00	0.94	3.16	0.68	47	
Group Mean	135.60	5.91	23.58	102.50	1.00	3.12	0.66	45	
Standard Error	0.72	0.31	1.36	0.60	0.04	0.02	0.01	1	
Range	(132 - 140)	(4.60 - 7.10)	(18.59 - 29.78)	(100.00 - 106.00)	(0.78 - 1.23)	(3.02 - 3.22)	(0.57 - 0.72)	(38 - 51)	
Normal Range	(132 - 146)	(3.30 - 5.40)	(28.00 - 40.00)	(89.00 - 108.00)	(0.90 - 1.80)	(2.50 - 3.60)	(0.60 - 1.00)	(32 - 53)	
No. of Tests	10	10	10	10	10	10	10	10	
(* - Tess before the first attack of laminitis)									
Serum electrolytes for chronically laminitic horses and ponies									
Date of Collection:	19/11/94								
Chronic Laminitic	Sodium	Potassium	Na : K Ratio	Chloride	Inorganic P	Calcium	Magnesium	PCV	
	mmol/l	mmol/l		mmol/l	mmol/l	mmol/l	mmol/l	%	
Bees	136.00	3.70	36.76	102.00	0.85	2.97	0.74	37	
Cowboy	134.00	5.10	26.27	102.00	0.87	3.21	0.76	40	
Domino	134.00	4.90	27.35	103.00	1.04	2.95	0.68	34	
Misty	136.00	3.80	35.79	104.00	0.89	3.17	0.71	40	
Rose Mare	132.00	6.20	21.29	106.00	0.87	3.09	0.63	38	
Group Mean	134.40	4.74	29.49	103.40	0.86	3.08	0.70	37.80	
Standard Error	0.75	0.46	2.96	0.75	0.06	0.05	0.02	1.11	
Range	(132 - 136)	(3.70 - 6.20)	(21.29 - 36.76)	(102.00 - 106.00)	(0.67 - 1.04)	(2.95 - 3.21)	(0.63 - 0.76)	(34 - 40)	
Normal Range	(132 - 146)	(3.30 - 5.40)	(28.00 - 40.00)	(89.00 - 108.00)	(0.90 - 1.80)	(2.50 - 3.60)	(0.60 - 1.00)	(32 - 53)	
No. of Tests	5	5	5	5	5	5	5	5	

Serum electrolytes of normal horses and ponies								
Date of Collection:	21/01/95							
Normal	Sodium	Potassium	Na : K Ratio	Chloride	Inorganic P	Calcium	Magnesium	PCV
	mmol/l	mmol/l		mmol/l	mmol/l	mmol/l	mmol/l	%
Albert	135.00	4.80	28.12	105.00	0.81	3.31	0.72	46
Amanda	134.00	4.90	27.35	106.00	0.62	2.93	0.50	43
Annabella	137.00	5.50	24.91	108.00	0.87	3.21	0.64	39
Biscuit	134.00	5.30	25.28	105.00	0.85	3.08	0.78	42
Blossom	134.00	5.40	24.81	104.00	0.95	3.20	0.65	42
Cracker II	135.00	5.80	23.28	102.00	0.93	3.18	0.74	37
Despina	134.00	5.10	26.27	102.00	1.01	3.23	0.72	43
Dolly	134.00	4.40	30.45	104.00	0.77	3.26	0.75	45
Emity	134.00	5.40	24.81	104.00	0.99	3.09	0.68	45
Jasper	135.00	6.10	22.13	103.00	1.16	3.23	0.80	43
Marron	134.00	4.50	29.78	98.00	0.58	3.22	0.63	43
Maree Gray	134.00	5.00	26.80	105.00	0.82	3.11	0.63	40
Melody	136.00	5.40	25.19	103.00	0.91	2.89	0.63	40
Poppy	128.00	5.30	23.77	100.00	0.72	3.18	0.67	42
Seluci	134.00	5.20	25.77	102.00	1.41	2.70	0.70	36
Tango	132.00	4.20	31.43	100.00	1.02	3.18	0.62	38
Group Mean	133.88	5.14	26.26	103.19	0.90	3.13	0.68	42
Standard Error	0.59	0.13	0.65	0.63	0.05	0.04	0.02	1
Range	(128 - 137)	(4.20 - 6.10)	(22.13 - 31.43)	(98.00 - 108.00)	(0.58 - 1.41)	(2.70 - 3.31)	(0.50 - 0.80)	(38 - 48)
Normal Range	(132 - 146)	(3.30 - 5.40)	(28.00 - 40.00)	(89.00 - 108.00)	(0.90 - 1.80)	(2.50 - 3.60)	(0.60 - 1.00)	(32 - 53)
No. of Tests	18	18	18	18	18	18	18	18
Serum electrolytes for chronically laminitic horses and ponies								
Date of Collection:	21/01/95							
Chronic Laminitic	Sodium	Potassium	Na : K Ratio	Chloride	Inorganic P	Calcium	Magnesium	PCV
	mmol/l	mmol/l		mmol/l	mmol/l	mmol/l	mmol/l	%
Beau	138.00	3.00	46.00	105.00	0.71	3.16	0.87	37
Cobweb	133.00	4.60	28.91	102.00	0.64	3.11	0.88	48
Domino	134.00	4.50	29.78	102.00	0.73	3.09	0.69	38
Misty	137.00	4.90	27.96	102.00	0.81	3.14	0.65	43
Tess	135.00	6.00	22.50	104.00	0.83	3.23	0.74	46
Group Mean	135.40	4.60	31.03	103.00	0.74	3.15	0.77	42
Standard Error	0.93	0.48	3.95	0.63	0.03	0.02	0.05	2
Range	(133 - 138)	(3.00 - 6.00)	(22.50 - 46.00)	(102.00 - 105.00)	(0.64 - 0.83)	(3.09 - 3.23)	(0.65 - 0.88)	(38 - 48)
Normal Range	(132 - 146)	(3.30 - 5.40)	(28.00 - 40.00)	(89.00 - 108.00)	(0.90 - 1.80)	(2.50 - 3.60)	(0.60 - 1.00)	(32 - 53)
No. of Tests	5	5	5	5	5	5	5	5

Appendix III

Serum electrolytes of normal horses and ponies								
Date of Collection:	08/03/95							
Normal	Sodium	Potassium	Na : K Ratio	Chloride	Inorganic P	Calcium	Magnesium	PCV
	mmol/l	mmol/l		mmol/l	mmol/l	mmol/l	mmol/l	%
Albert	138.00	4.00	34.00	103.00	0.66	3.35	0.74	48
Amanda Gien	137.00	4.60	29.78	108.00	0.84	3.14	0.68	41
Annabella Gien	135.00	4.70	28.72	104.00	0.63	3.13	0.72	42
Biscuit	138.00	4.40	30.91	105.00	0.74	3.10	0.75	43
Blossom	135.00	4.60	29.35	105.00	0.66	3.12	0.71	39
Dolly	133.00	4.80	27.71	105.00	0.86	3.34	0.69	36
Emily	129.00	3.90	33.08	98.00	0.66	3.15	0.76	41
Isadora	134.00	4.80	27.92	104.00	0.77	3.09	0.72	39
Maree Gray	135.00	4.30	31.40	105.00	0.77	3.17	0.75	47
Melody	140.00	4.40	31.82	109.00	0.69	3.09	0.84	39
Parnell	135.00	3.90	34.62	105.00	0.74	3.19	0.64	42
Poppy	135.00	4.10	32.93	106.00	0.67	3.13	0.68	40
Tango	140.00	4.40	31.82	109.00	0.88	3.18	0.74	40
Group Mean	135.33	4.41	30.84	105.08	0.73	3.15	0.72	41
Standard Error	0.85	0.09	0.63	0.80	0.03	0.02	0.01	1
Range	(129 - 140)	(3.90 - 4.80)	(27.71 - 34.62)	(98.00 - 109.00)	(0.55 - 0.88)	(3.09 - 3.35)	(0.64 - 0.84)	(36 - 48)
Normal Range	(132 - 146)	(3.30 - 5.40)	(28.00 - 40.00)	(89.00 - 108.00)	(0.90 - 1.80)	(2.50 - 3.60)	(0.60 - 1.00)	(32 - 53)
No. of Tests	12	12	12	12	12	12	12	12

Beau	Sodium mmol/l	Potassium mmol/l	Na : K Ratio	Chloride mmol/l	Inorganic P mmol/l	Calcium mmol/l	Magnesium mmol/l	PCV %	Comments
Normal range	(132 - 146)	(3.30 - 5.40)	(28.00 - 40.00)	(89.00 - 108.00)	(0.90 - 1.80)	(2.50 - 3.60)	(0.60 - 1.00)		
10/10/93	107.00	2.80	38.21	82.00	0.65	2.22	0.50		
17/10/93	100.00	3.30	30.30	75.00	0.57	2.19	0.53		
31/10/93	125.00	3.80	32.89	92.00	1.20	2.72	0.67		
05/12/93	125.00	3.10	40.32	95.00	1.09	2.96	0.73		
30/05/95	162.00	3.90	41.54	124.00	1.59	3.83	0.91		
31/05/95	160.00	6.20	25.81	125.00	1.50	3.87	0.98		
01/06/95	163.00	5.40	30.19	129.00	1.15	4.00	0.97		
02/06/95	130.00	6.00	21.67	101.00	0.83	3.07	0.77		
03/06/95	152.00	6.80	22.35	118.00	1.27	3.62	0.94		
04/06/95	188.00	9.60	19.58	156.00	1.61	4.22	0.93		
Group Mean	141.20	5.09	30.29	109.70	1.15	3.27	0.79		
Standard Error	8.87	0.67	2.51	7.89	0.12	0.23	0.06		
Range	(100 - 188)	(2.80 - 9.60)	(19.58 - 41.54)	(75.00 - 156.00)	(0.57 - 1.61)	(2.19 - 4.22)	(0.50 - 0.98)	32 - 55	
No. of Tests	10	10	10	10	10	10	10	0	

Changes in serum electrolytes and packed cell volume during grass induced acute laminitis (Beau - Case Report 1).

Beau	Sodium mmol/l	Potassium mmol/l	Na : K Ratio	Chloride mmol/l	Inorganic P mmol/l	Calcium mmol/l	Magnesium mmol/l	PCV %	Comments
Date of collection									
16/03/94	158.00	4.50	35.11	116.00	1.36	3.67	0.96	42	
17/04/94	135	3.90	34.62	103.00	1.04	3.15	0.90	45	
30/04/94	181.00	5.80	31.21	142.00	1.27	4.66	1.07	45	
15/05/94	130.00	4.50	28.89	94.00	1.80	3.02	0.85	43	
20/05/94	132.00	3.90	33.85	96.00	1.10	3.14	0.78	ND	
22/05/94	134.00	4.10	32.68	98.00	1.04	2.99	0.77	45	
03/06/94	103.00	4.70	21.91	76.00	0.81	2.35	0.65	34	
04/06/94	92.00	4.70	19.57	72.00	0.71	1.94	0.41	42	
05/06/94	134.00	3.90	34.36	103.00	1.06	3.03	0.75	42	
12/06/94	133.00	4.10	32.44	99.00	0.75	3.25	0.75	44	
19/06/94	152.00	4.80	31.67	113.00	0.88	3.54	1.06	46	
24/06/94	180.00	5.10	35.29	136.00	0.96	4.04	1.09	46	
03/07/94	160.00	4.40	36.36	119.00	1.31	3.65	1.13	ND	
09/07/94	126.00	3.60	35.00	91.00	1.06	2.89	0.75	40	
17/07/94	154.00	3.50	44.00	118.00	0.84	3.57	0.96	38	
24/07/94	148.00	4.20	35.24	109.00	1.31	3.49	0.96	45	
26/07/94	145.00	3.60	40.28	107.00	1.49	3.32	0.91	38	
29/07/94	144.00	4.70	30.64	108.00	1.14	3.29	0.82	36	
31/07/94	127.00	3.80	33.42	94.00	0.71	2.84	0.76	40	
07/08/94	159.00	5.00	31.80	117.00	1.43	3.81	1.05	42	
14/08/94	162.00	5.20	31.15	123.00	1.46	3.97	0.95	44	
21/08/94	171.00	6.30	27.14	132.00	1.39	3.98	0.84	44	
28/08/94	190.00	6.20	30.65	149.00	0.98	4.91	1.16	36	
04/09/94	132.00	4.50	29.33	101.00	0.82	3.18	0.82	38	
11/09/94	137.00	4.00	34.25	107.00	0.80	3.22	0.78	44	
16/10/94	135.00	4.00	33.75	103.00	1.22	2.96	0.73	40	
19/11/94	136.00	3.70	36.76	102.00	0.85	2.97	0.74	37	
14/03/95	139.00	4.10	33.90	105.00	0.91	3.06	0.81	ND	
Mean	147.81	4.43	33.98	111.56	1.11	3.44	0.89	41	
Standard Error	4.34	0.22	1.04	3.64	0.07	0.13	0.03	0.7	
Range	(92 - 190)	(3.50 - 6.30)	(19.57 - 44.00)	(72.00 - 149.00)	(0.71 - 1.80)	(1.94 - 4.91)	(0.41 - 1.16)	(34 - 46)	
Normal Range	(132 -	(3.30 - 5.40)	(28.00 - 40.00)	(89.00 - 108.00)	(0.90 - 1.80)	(2.50 - 3.60)	(0.60 - 1.00)	(32 - 53)	
No. of Tests	28	28	28	28	28	28	28	25	

Biscuit	Sodium mmol/l	Potassium mmol/l	Na : K Ratio	Chloride mmol/l	Inorganic P mmol/l	Calcium mmol/l	Magnesium mmol/l	PCV %	Comments
Date/Time of collection									
23/05/95	130.00	4.60	28.26	104.00	0.64	2.74	0.43		
24/05/95 7.00 am	152.00	6.60	23.03	120.00	1.28	3.53	0.56	37	
24/05/95 11.00 am	137.00	4.90	27.96	107.00	1.05	3.10	0.47	37	
24/05/95 7.00 pm	158.00	5.60	28.21	125.00	1.01	3.49	0.57	33	
24/05/95 11.00 pm	158.00	5.60	28.21	126.00	1.08	3.68	0.60	38	
25/05/95	164.00	4.60	35.65	128.00	1.17	3.75	0.68		
26/05/95	153.00	5.50	27.82	120.00	1.46	3.56	0.74	33	Salt appetite
27/05/95	144.00	5.10	28.24	114.00	1.25	3.28	0.66	35	
28/05/95	155.00	6.60	23.48	123.00	1.14	3.54	0.76	36	
29/05/95	132.00	5.00	26.40	103.00	1.26	3.02	0.61	36	
30/05/95	135.00	4.30	31.40	107.00	1.13	2.86	0.55	36	
31/05/95	138.00	5.90	23.39	110.00	1.05	3.00	0.84	36	
01/06/95	146.00	5.10	28.63	115.00	1.06	3.27	0.65	36	
02/06/95	177.00	5.70	31.05	141.00	1.39	4.01	0.83	36	
03/06/95	157.00	7.50	20.93	125.00	1.33	3.52	0.79	39	
04/06/95	187.00	10.30	18.16	146.00	1.53	4.66	1.24	35	
12/06/95	186.00	5.80	32.07	151.00	1.30	3.73	1.03	41	Ventral oedema
14/06/95	134.00	7.00	19.14	97.00	1.35	2.70	0.67	45	Ventral oedema
Group Mean	152.39	5.87	26.78	120.11	1.19	3.41	0.70		
Standard Error	4.15	0.33	1.09	3.53	0.05	0.11	0.05		
Range	(130 - 187)	(4.30 - 10.30)	(18.16 - 35.65)	(97.00 - 151.00)	(0.64 - 1.53)	(2.70 - 4.66)	(0.43 - 1.24)	(33 - 45)	
Normal Range	(132 - 146)	(3.30 - 5.40)	(28.00 - 40.00)	(89.00 - 108.00)	(0.90 - 1.80)	(2.50 - 3.60)	(0.60 - 1.00)		
No. of Tests	18	18	18	18	18	18	18	16	

APPENDIX IV

Treatment of *post partum* laminitis

Post partum Acute Laminitis

A Registered Irish Draught mare (600 kg b.wt.) developed acute laminitis after dystocia, retained placental membranes and endotoxaemia. The case exhibited Obel Grade 4 lameness.

Treatment of acute laminitis. Acute laminitis was treated with transdermal glyceryl trinitrate (GTN) (Pericutol Paste, Cusi, Welwyn Garden City, UK) applied to the palmar surfaces of the pasterns once daily. This case received adjunct therapy of analgesics/ cyclooxygenase inhibitors - i.v. flunixin meglumine (FX) (Finadyne, Schering -Plough Animal Health, Suffolk, UK); i.v. non steroidal anti inflammatory agents - Phenylbutazone/ Ramifenazone (PBZ) (Intervet UK Ltd., Cambridge, UK) twice daily i.v.; and α -adrenergic blockade- acepromazine maleate (ACP) (ACP, C-Vet, Bury St. Edmonds, UK) four times daily. Systemic antibiotics of 25 mls penicillin /streptomycin (AB1) (Streptopen, Pitman Moore, Cheshire, UK) and later 25 mls of gentamycin (Gentaject 10%, Franklin Pharmaceuticals, Trim, Co Meath, Eire) (AB2) were given i.m. twice daily. Uterine irrigation (UI) of 5L 0.9% sterile saline (Baxter, Thetford, UK), followed by twice daily treatment of 6mls each of penicillin (Penillin, Univet, Oxford, UK) and framomycin (15% Framomycin, C-Vet, Bury St. Edmonds, UK) solutions respectively. 25 i.u. oxytocin (OX) i.v. (Leo Animal Health, Princes Risborough, Bucks) was given four times daily. Oral PBZ (Equipalazone, Arnolds Veterinary Products, Shrewsbury, UK) was given throughout the treatment at a dose of 4g/day. A summary of treatment is detailed in the Table below.

Table IV Treatment of *post partum* acute laminitis.

	GTN (mg/day)	PBZ (mls/day)	FX (mls/day)	ACP (mls/day)	Other
Day 1	100	-	12	-	AB 1
Day 2 (7.00 hr)	-	-	10	4	AB 1
Day 2 (11.00 hr)	56	20	10	7	AB 2, UI, OX
Day 2 (19.00 hr)	-	-	10	7	AB 2, OX
Day 2 (23.00 hr)	-	20	10	7	OX
Day 3	56	20	40	28	AB 2, OX
Day 4	56	20	40	28	AB2, UI, OX
Day 5	56	-	40	28	AB2, OX
Day 6	56	-	40	28	AB2, OX
Day 7	56	-	40	28	AB2, OX
Day 8	56	-	40	28	AB2, OX
Day 9	56	-	40	28	-
Day 10	56	-	40	28	-
Day 11	56	-	40	28	-
Day 12	24	-	40	28	-
Day 13	24	-	40	28	-
Day 14	24	-	40	28	-
Day 15	24	-	40	28	-
Day 16	24	-	40	28	-
Day 17	24	-	40	28	-
Day 18	24	-	40	28	-
Day 19	24	-	40	28	-

Clinical notes

The mare, Biscuit, and her foal were kept in a large loose box on a semi-deep litter bed of shavings and sand, which was covered with straw. The mare refused to lie down at any stage. Hay was fed *ad libitum* and a small feed provided as a vehicle for medicines. The mare's body weight was reduced in an attempt to minimise the weightbearing load on the hooves. The foal was allowed to remain with its dam to suckle, although supplementary milk was given to the foal.

Ventral and digital oedema was marked on Day 2 of the acute stage and ventral oedema remained a feature of the disease for several weeks. Salt appetite was avid in the first two weeks and the mare would gnaw salt blocks, on occasions consuming a whole block overnight.

There was little evidence of orthopaedic changes in the early stages of the disease and only slight depressions could be felt at the dorsal coronary band. A bloody exudate appeared at the coronary band on Day 21.

The reproductive cycle was quickly established after parturition and an interesting feature, on ultrasound scanning of the reproductive tract, was that the mature ovarian follicle became haemorrhagic during transdermal application of glyceryl trinitrate paste (R.Gunstone, MRCVS).

Unfortunately, the mare escaped from her stable on Day 22 and was outside at liberty throughout the night. She had, by morning, done a considerable amount of walking of her own volition. Although there were no apparent ill effects it was impossible to determine changes within the hoof. Under these circumstances, and because of the unusually hot weather and the fact that the foal needed to exercise for proper development, the mare was transported to a very small paddock and allowed to stay outside. She did not walk around the paddock or exercise; indeed water was carried to her, but morale was considerably improved.

After several weeks at grass (7 weeks after the onset of laminitis), lameness worsened and depressions around the coronary bands developed, suggesting 'sinking' of the bony column through the hoof capsule. Pain was severe and could not be controlled despite increased analgesia. Radiography confirmed gross displacement of P3 which quickly descended and prolapsed through the soles of both front feet. The mare was destroyed and the foal hand reared.

Publications and Presentations arising from this Thesis

Publications:

Hinckley K.A., Fearn S., Howard B.R. and Henderson I.W. (1996) Nitric oxide donors as treatment for acute grass induced laminitis in ponies. *Equine Veterinary Journal.* 28 : 17 - 28

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Hinckley K. A. and Henderson I.W. (1995) Plasma endothelins in normal and acutely laminitic horses. *Journal of Endocrinology.* 147 Supplement : P 81

Hinckley K. A. and Henderson I.W. (1995) Laminitis : Hypertension in acute and chronic stages ? Proceedings of the 34th Annual British Equine Veterinary Association Congress, Harrogate, September 1995. : 20

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Treatment of Equine Laminitis (October 1995) presented to Intervet, at the University of Sheffield.

Treatment of Equine Laminitis (May 1995) presented to Intervet, Cambridge.

Vascular Influences in Equine Laminitis (March 1995) Post Graduate Symposium, University of Sheffield.

Endocrine Aspects of Equine Laminitis. (November 1994) Institute of Endocrinology Workshop, University of Sheffield.

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Treatment of Equine Laminitis (December 1993). presented to Pfizer UK Ltd., Sandwich, Kent.

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POSTER 11

LAMINITIS - HYPERTENSION IN ACUTE AND CHRONIC STAGES?

K A Hinckley and I W Henderson

Institute of Endocrinology, University of Sheffield, S10 2TN

Laminitic equids may be hypertensive but not unequivocally so. Few data are available for blood pressures (BP) taken over the course of the disease. Normal equine BP are difficult to acquire because of handling and/or invasive measurement stresses. Basal values for normal ponies and for acute and chronic laminitic ponies were determined.

A herd of experimental ponies, kept under controlled management, were accustomed to being regularly handled by the investigators. BP and heart rates (HR) were measured non-invasively by tail cuff plethysmography (Dinamap 8711, Critikon).

**BP and HR (\pm SE) of normal,
chronic and acute laminitic ponies**

	Blood pressure (mmHg)			Hear rate (bts./min)
	Systolic	Diastolic	Mean	
Normal (n=8;63 tests)	107 \pm 4	62 \pm 2	78 \pm 2	44 \pm 1
Chronic laminitic (n=6;42 tests)	101 \pm 3	58 \pm 2	74 \pm 3	46 \pm 2
Acute laminitic (n=10;23 tests)	165 \pm 5	86 \pm 2	119 \pm 4	61 \pm 3

The hypertension of acute laminitis may reflect a response to pain and an activated renin - angiotensin - aldosterone system. BP were related to Obel Grade of lameness.

103 THE ROLE OF NITRIC OXIDE PRECURSORS AND INHIBITORS IN ACUTE EQUINE LAMINITIS.

K. A. HINCKLEY, P.R. KEEN, B.R. HOWARD * AND I. W. HENDERSON. *Institute of Endocrinology, Department of Animal and Plant Sciences, and *Field Laboratories, University of Sheffield, Sheffield S10 2TN UK.*

The present study assesses the potential role of nitric oxide in acute equine laminitis, a disease characterised by compromised circulation within the hoof. Endothelial nitric oxide (NO) produced by the action of NO synthase (NOS) on its substrate L-arginine, relaxes vascular smooth muscle to cause vasodilatation. Reduced substrate levels, or the presence of competitive analogues of L-arginine, potentially reduce normal vasodilatory tone and be involved in the pathophysiology of acute equine laminitis. An intravenous infusion of 10% L-arginine in 0.9% saline had little effect on blood pressure of normal horses. Haemostasis in the hoof of an acute laminitic pony was detected non-invasively using near infrared spectroscopy. An intravenous infusion of 10% L-arginine in 0.9% saline dramatically induced reperfusion of laminal tissues within 4 minutes. Infusions of a competitive analogue of L-arginine, N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME) an inhibitor of NOS synthesis, were given iv to normal horses. A dose of 0.31mg/kg had no observable effect, while a dose of 1.5mg/kg L-NAME increased blood pressure (mmHg) from basal values of $111 \pm 6 / 63 \pm 2$ (mean 80 ± 6) to $141 \pm 6 / 105 \pm 3$ (mean 109 ± 3), although laminitis was not induced. Plasma levels of an endogenous inhibitor of NOS, asymmetric N^G, N^G-dimethylarginine (ADMA), were determined in normal, chronic or acute laminitic horses kept under controlled husbandry. Laminitic horses had lower mean levels of plasma ADMA $\mu\text{mol/l}$ 0.62 ± 0.29 than controls, 1.01 ± 0.16 . Plasma levels of L-arginine were similar in all groups although one acute laminitic pony had lower values for one month before the attack. The aetiology of laminitis is clearly multifactorial but endothelial factors, in particular NO and endothelin, together with glycation, are likely to be key elements in the disease.

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: P81 (1995)

P81 PLASMA ENDOTHELINS IN NORMAL AND ACUTELY LAMINITIC HORSES

K A Hinckley and I W Henderson; Institute of Endocrinology, Centre for Equine Research, Department of Animal and Plant Sciences, University of Sheffield, Western Bank, Sheffield S10 2TN

Endothelins (ET) are the most potent vasoconstrictor peptides known. Secreted by the vascular endothelium, they are held to regulate vascular tone and are their plasma concentrations may be elevated in a number of vascular diseases, especially vasospastic conditions and sepsis. Equine laminitis has a multifactorial aetiology and pathogenesis including those associated with digestive disturbance and endotoxaemia. The disease is characterised by a compromised microcirculation within the hoof; accordingly ET alongside other local and systemic vasoconstrictors are likely to participate in the pathogenesis of the disease.

The aims of the present investigation were to assess the potential involvement of ET in laminitis. Total ET were determined by radioimmunoassay (Nichols Institute Diagnostics BV, The Netherlands) in normal horses, in horses with acute grass-induced laminitis and in a case of *post partum* laminitis in which there was endometritis and endotoxaemia. Normal (n = 16) horses gave plasma ET concentrations of $1.78 \pm 0.2 \text{ pg ml}^{-1}$ (range 0.45 - 3.11). In acute grass-induced laminitis, plasma ET levels were within the normal range (0.64 - 0.93). A mare's *prepartum* values, prior to endotoxaemia determined for previous months, were within the normal range (normal: 0.8, 1.1). At the onset of laminitis plasma ET concentrations were reduced (0.75, 0.2) and during the most severe phase of the attack, they fell to below the level of detection of the assay (3 separate specimens in one day < 0.1); normal concentrations (>0.42) returned after three days and were sustained for several weeks thereafter. There were no obvious associations between plasma ET concentrations and caudal arterial blood pressure. The seemingly, and perhaps surprisingly, reduced plasma levels of ET prior to the onset of endotoxaemia-induced laminitis could have resulted from endotoxin-induced ET receptor characteristics including increased affinity alongside reduced ET synthesis, perhaps compounded by concurrent administration of analgesics, nitrovasodilators and adrenergic receptor antagonists. Moreover, measurement of ET in peripheral jugular venous blood may not be a reliable index of ET paracrine activity in the microcirculation of the hoof.

Nitric oxide donors as treatment for grass induced acute laminitis in ponies

K. A. HINCKLEY, S. FEARN*, B. R. HOWARD† and I. W. HENDERSON

Institute of Endocrinology, Department of Animal and Plant Sciences and †Field Laboratories, University of Sheffield, Sheffield S10 2TN, UK.

Keywords: horse; laminitis; nitric oxide; arginine; glyceryl trinitrate

Summary

The potential for participation of the arginine-nitric oxide system in the aetiology of acute equine laminitis has been assessed. Nitric oxide (NO), produced by the action of NO synthase (NOS) on its substrate *l*-arginine, relaxes vascular smooth muscle to cause vasodilatation. An attenuated normal vasodilatory tone may characterise the pathogenesis of acute equine laminitis. An intravenous infusion of 10% *l*-arginine in 0.9% saline caused vasodilatation in the hoof of a normal pony and immediate reperfusion of laminal tissues in an acutely laminitic pony, detected noninvasively by near infrared spectroscopy (NIRS), but the amino acid had little effect on systemic blood pressure. Treatment of acute laminitis with glyceryl trinitrate applied topically to the pasterns reduced the typical 'bounding pulses' in treated limbs, reduced lameness and lowered systemic blood pressure. Nitric oxide is likely to participate in the multifactorial pathogenesis of equine laminitis.

Introduction

Equine laminitis features as a commonplace disease in the earliest books of veterinary medicine and farriery (Hodson *et al.* 1672). The syndrome remains incompletely understood and the many potential aetiological factors include carbohydrate overload, endometritis and surgical complications (Hood *et al.* 1993). The initiation of acute laminitis is certainly multifactorial and attempted induction gives inconsistent and equivocal results. For example, injected endotoxins fail to induce laminitis (Fessler *et al.* 1982) and an oral carbohydrate overload to induce the disease experimentally results in some animals developing the disease others die of shock and others are apparently unaffected.

Fundamentally, laminitis is a vascular disease associated with areas of ischaemia or haemostasis within the hoof (Coffman *et al.* 1970; Hood *et al.* 1978). In the overt stages, total blood flow to the hoof may increase (Robinson 1990), but arteriovenous shunts open (Hood *et al.* 1978; Pollitt 1990), to decrease perfusion of dermal laminal tissues (Galey *et al.* 1990) and cause local ischaemia distally. However, the exact mechanisms that compromise pedal circulation within the hoof are arguable (Hunt 1991; Hood *et al.* 1993). There are a number of hypotheses

reviewed by Hood *et al.* (1993). Laminitis may be a sequel to disturbed keratin metabolism within the hoof. Endotoxaemia may instigate the acute phase when circulating endotoxins precipitate a cascade of events that releases inflammatory mediators, which in turn compromise normal circulation. The mechanisms responsible for pedal haemostasis are unclear but the more plausible features include: 1) pooling as a result of venoconstriction, increased post capillary resistance and secondary physical occlusion of capillaries by microthrombi (Weiss *et al.* 1994); 2) inappropriate function of arteriovenous anastomoses as a result of the actions of vasoactive mediators such as vasoactive intestinal peptide (VIP), calcitonin gene related peptide (CGRP), Substance P or CNS activated hormones (Molyneux *et al.* 1994) to modify the distribution of blood within the hoof; 3) and vasoconstriction as a result of primary vascular events (Hunt 1991; Hood *et al.* 1993).

Whatever the exact pathogenesis of laminitis, it is clear that endocrine and pedal paracrine factors regulating vascular tone are key. It is now recognised that the endothelium of the vasculature represents a major source of materials that regulate, primarily or secondarily, blood pressure and the distribution of flow within tissues and organs (Änggård 1990; Vane *et al.* 1990; Griffith 1994). Endothelial-derived relaxing factor (EDRF) is a potent vasodilator, discovered by Furchgott and Zawadzki (1980) and later identified as nitric oxide (Palmer *et al.* 1987). Nitric oxide (NO), produced by vascular endothelial cells, maintains circulatory tone in a paracrine fashion (Rees *et al.* 1989; Moncada *et al.* 1991). Moncada *et al.* (1991) notes that nitric oxide 'maintains the vascular system in a state of constant vasodilatation'. Nitric oxide is probably an important factor in the regulation of pedal microcirculatory homeostasis, is synthesised from the guanidino group of *l*-arginine (Palmer *et al.* 1988) and is responsible for cGMP mediated relaxation of vascular smooth muscle. *l*-arginine is, therefore, the substrate from which NO originates. The synthesis of NO from *l*-arginine is endothelium dependent while NO derived from synthetic donors, such as glyceryl trinitrate, is endothelium independent. The responses of equine digital vessels to vasoconstrictive agents have been studied *in vitro* (Baxter *et al.* 1989). Vessels from horses given endotoxins *i.v.* react differently to endothelium dependent and endothelial independent donors of NO (Baxter 1994a,b). The role of NO in modulating responses to vasoconstrictive and vasorelaxant agents in equine digital veins has also been studied *in vitro* (Elliott *et al.* 1994; Schneider *et al.* 1994). Since laminitis is a disease of a compromised microcirculation, endothelial derived hormones are potentially of vital significance. The present study addresses, *in vivo*, the potential role of NO in regulating pedal blood flow under normal and laminitic circumstances. Some of the present

*Present address: c/o Johnson and Johnson Medical Ltd., Coronation Road, Ascot, Berkshire SL5 9EY, UK.



Fig 1: Near infrared spectroscopy emitter/sensor in place on the dorsal wall of the equine hoof.

data have appeared in abstract form (Hinckley *et al.* 1994).

Materials and methods

Investigations

1) Blood pressure and heart rates were measured noninvasively in normal ponies and during the treatment of acute laminitis with nitric oxide donors, 2) Pedal haemodynamics of a pony with acute laminitis were assessed noninvasively using NIRS, 3) the effects of i.v. administration of *l*-arginine during acute laminitis were examined, 4) a 10% *l*-arginine solution was infused i.v. into normal horses and compared with an infusion of 0.9% saline and 5) glyceryl trinitrate was administered to ponies with acute laminitis of mild and moderate severity. Responses were compared with those of untreated cases of mild laminitis.

Animals

Fifteen animals were used as described in Table 1. They were kept as a group for at least 6 months prior to study and were subject to careful management regimes of feeding, anthelmintic prophylaxis and farriery. The animals were turned out to grass on unfertilised old pasture. Some ponies had a history of laminitis when at grass in previous years, but were not lame or in need of special farriery. Acute cases described (repeated attacks in 5 animals) were lame in four limbs and the lameness described by Obel (1948). *Grade 1*: lifts feet incessantly, no lameness evident in walk but trot is a shortened or stilted gait; *Grade 2*: horse is willing to move but with characteristic gait; *Grade 3*: horse reluctant to move and will not lift forefeet; *Grade 4*: horse moves only when forced (Obel 1948). In this study, the categories were subdivided into half grades (e.g. 2.5) to describe the slight changes in lameness existing between the grades. Animals were restrained in a standard crush unless sedated. All procedures were carried out under the Animals (Scientific Procedures) Act 1986. Intravenous infusions were administered through indwelling catheters (Ohmeda, Hatfield, Herts, UK) placed in the left jugular vein, secured with superglue (Vetbond, 3M, UK) and tape (Treatplast, Animalcare Ltd., York, UK).

Measurements

Blood pressure and heart rate: Systolic, diastolic and mean blood pressures and heart rates were measured noninvasively using a tail cuff attached to a Dinamap Vital Signs Monitor Model 8711 (Critikon, Johnson and Johnson, Ascot, UK). Animals had been accustomed to the routine over the previous 3 months during

TABLE 1: Description of animals used in the study and procedures

Name of animal	Breed	Procedures*	Normal/clinical	Case No.
Albert	Hunter	NIRS, sedated, BP	normal	-
Beckford	Hunter	<i>l</i> -arginine i.v, BP	normal	-
Beau	Welsh cross	NIRS, <i>l</i> -arginine i.v, GTN, BP	laminitis	1, 2, 3
Cobweb	Welsh cross	GTN, BP	normal/laminitis	7, 8, 11
Cracker II	Welsh cross	BP	normal	-
Despina	Welsh cross	NIRS, <i>l</i> -arginine i.v, BP	normal	-
Domino	Appaloosa	BP	laminitis	9
Jasper	Welsh cross	BP	normal	-
Marron	Welsh cross	NIRS, sedated, saline i.v, BP	normal	-
Maree Gray	Hunter	NIRS, sedated, BP	normal	-
Misty	Welsh cross	GTN, BP	laminitis	4, 5, 6, 12
Poppy	Reg Irish Draught	NIRS, unsedated	normal	-
Selluci	Welsh cross	BP	normal	-
Snowflake	Welsh cross	BP	normal	-
Tess	Welsh cross	BP	normal/laminitis	10

Key: BP - blood pressure measured noninvasively; NIRS - near infrared spectroscopy; GTN - glyceryl trinitrate; *see Table 5.

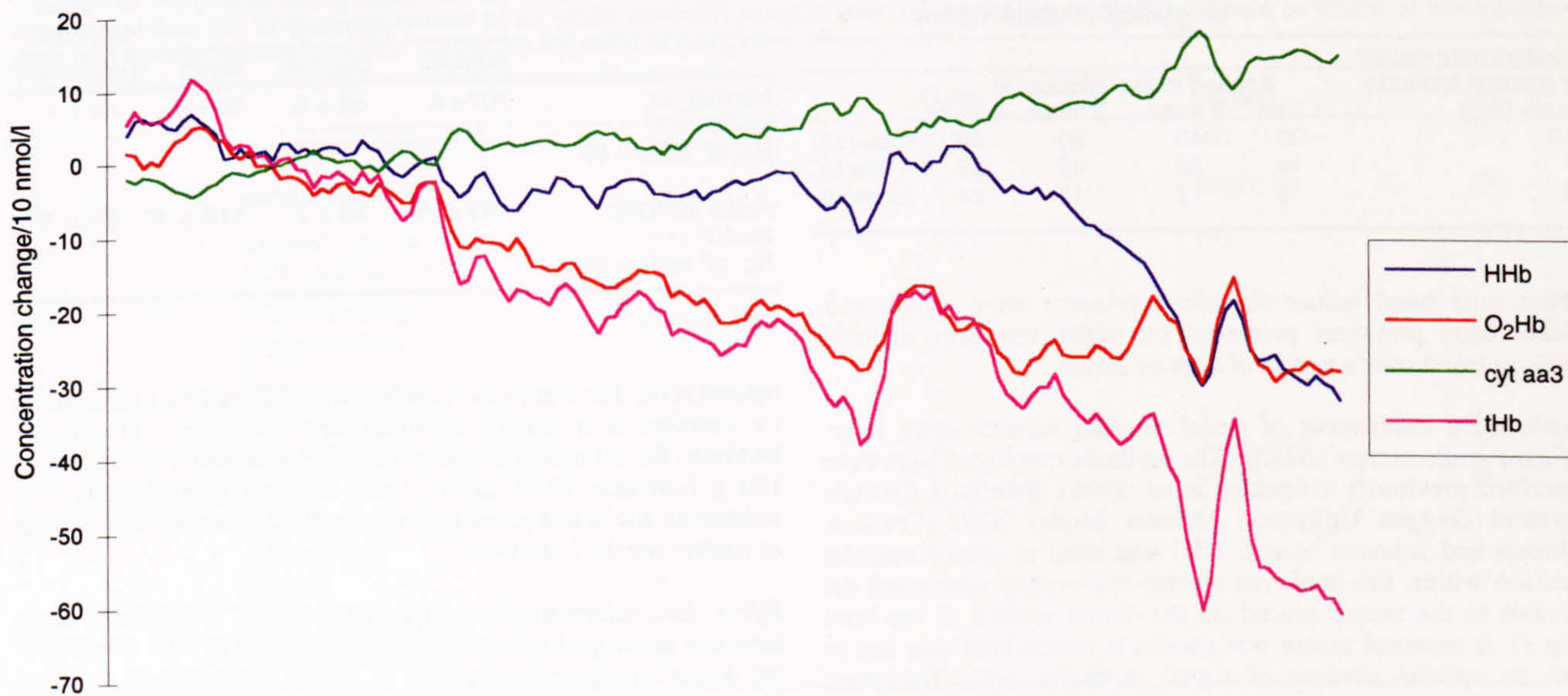


Fig 2: NIRS assessment of pedal haemodynamics of an unsedated normal horse; traces were recorded over a period of 30 min. Key: HHb - deoxyhaemoglobin; cyt aa3 - cytochrome oxidase; O₂Hb - oxyhaemoglobin; tHb - total haemoglobin.

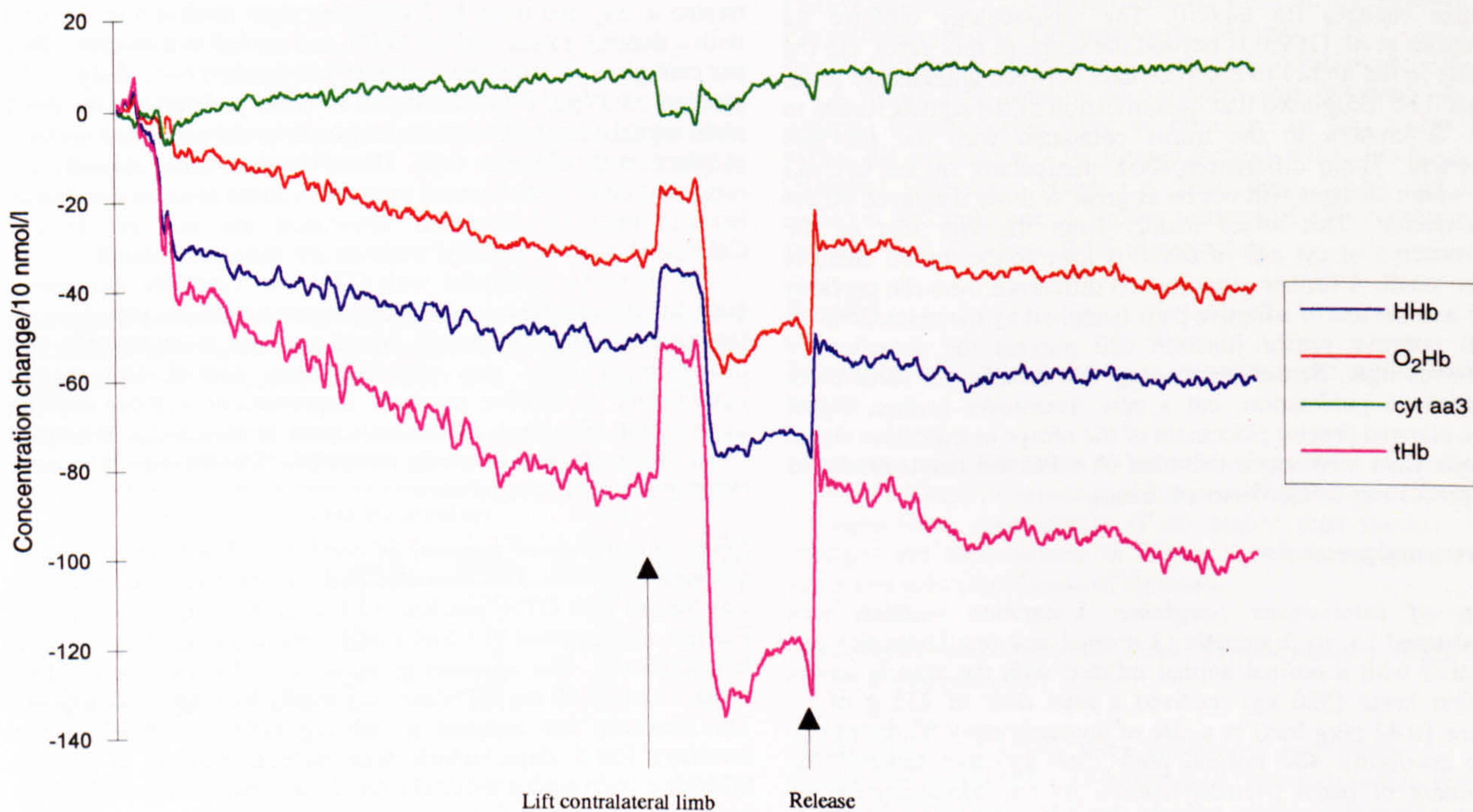


Fig 3: NIRS assessment of pedal haemodynamics of a sedated normal horse; traces were recorded over a period of 30 min. Key: HHb - deoxyhaemoglobin; cyt aa3 - cytochrome oxidase; O₂Hb - oxyhaemoglobin; tHb - total haemoglobin.

TABLE 2: Doses of glyceryl trinitrate given to acutely laminitic ponies

Approximate weight of glyceryl trinitrate paste (mg)	Total approximate amounts of glyceryl trinitrate mg/day				
	Applied in two aliquots to:				
	1 limb	2 limbs	3 limbs	4 limbs	
10	20	40	60	80	(dose a)
7	14	28	42	56	(dose b)
3	6	12	18	24	(dose c)

which time basal values for blood pressure were established. Mean blood pressures presented on tables represent multiple readings taken over a period of days or months.

Noninvasive assessment of pedal haemodynamics using near infrared spectroscopy (NIRS): The methods employed have been described previously (Hinckley *et al.* 1995). Briefly, a Critikon Cerebral Oxygen Utilisation Monitor Model 2000 (Critikon, Johnson and Johnson, Ascot, UK) was used to assess vascular function within the hoof. An electro-optic cable connected the monitor to the sensor placed on the dorsal surface of the hoof (Fig 1). A neonatal sensor was chosen to match hoof size and to give an optimal strength of signal. Adhesive tape (Treatplast, Animalcare Ltd, York) or polythene adhesive tape (Gaffatape) ensured exclusion of ambient light and constant sensor to hoof coupling. Real time qualitative changes in deoxygenated haemoglobin (HHb), oxygenated haemoglobin (O₂Hb), cytochrome aa3 (cyt aa3) and total haemoglobin (tHb) were displayed on the monitor cathode ray screen and recorded on a portable computer (Thinkpad, IBM, Southampton, UK). In later procedures, an improved version of the NIRS instrumentation, the Critikon Cerebral Redox Monitor Model 2001, was used to quantify changes (in $\mu\text{mol/l}$). The methodology outlined by Essenspries *et al.* (1993) is beyond the scope of this paper, but the benefits in the ability to compare data between animals are clear. It should be recognised that quantification of the signals results in some differences in the traces compared with the previous instrument. These differences show particularly on the cyt aa3 data, where changes will not be as great as those displayed on the trend device. This arises mainly from the fact that as the concentration of cyt aa3 in tissue is low, concentration changes will be small. A further experimental difference from the previous study was the use of adhesive pads (supplied by the manufacturer) which improve sensor function and prevent the detection of extraneous light. Sensor positioning was broadly the same as in the previous publication, but a new instrument feature, signal status, allowed precise placement of the sensor to maximise signal strength. Data were again recorded on a PC and charts produced in a spreadsheet using Microsoft Excel.

Experimental protocols

Effects of intravenous L-arginine: L-arginine solution was administered i.v. to 3 animals (2 normal and one laminitic) and compared with a normal animal infused with the vehicle alone. The first horse (550 kg) received a total dose of 231 g of L-arginine (0.42 g/kg bwt) at a rate of approximately 50 ml/min (1 mg/kg bwt/min). The normal pony (360 kg) was sedated, for assessment of pedal haemodynamics, by i.v. administration of 0.006 mg/kg bwt detomidine hydrochloride and 0.012 mg/kg bwt butorphanol. This animal received a total dose of 40 g L-arginine (0.12 g/kg bwt) at a rate of approximately 27 ml/min (0.7 mg/kg bwt/min). The pony suffering acute laminitis was sedated, for assessment of pedal haemodynamics, by i.v. administration of 0.006 mg/kg bwt detomidine hydrochloride and 0.012 mg/kg bwt

TABLE 3: Blood pressure and heart rates of normal and laminitic ponies

	Blood pressure (mmHg)			Heart rate (beats/min)
	Systolic	Diastolic	Mean	
Normal (n=8) No. of tests = 63	107 ± 4	62 ± 2	78 ± 2	44 ± 1
Acute laminitic (n=10) No. of tests = 23	165 ± 5*	86 ± 2*	119 ± 4*	61 ± 3*

* (P<0.001).

butorphanol. The L-arginine solution was administered through an i.v. catheter at a rate of approximately 40 ml/min (16 mg/kg bwt/min) for 30 min. This pony (250 kg) received a total dose of 120 g L-arginine (0.48 mg/kg bwt). Another normal pony was sedated as above and given the vehicle of 0.9% saline alone to act as control for the L-arginine.

Effect of transdermal glyceryl trinitrate (GTN): Ten cases of acute laminitis developed spontaneously whilst at grass. The severity of the disease varied between mild (2 cases) characterised by Obel *Grade 2* (Obel 1948) and moderate (10 cases) Obel *Grades 3 or 4*. On diagnosis, ponies were stabled, fed only hay and no other treatment or medication was applied except for one pony mare which received 1 g oral Phenylbutazone daily because of minor accidental trauma. The caudal surface of the pasterns was clipped and shaved over the area of the digital vessels extending medially and laterally, then rinsed and dried. The GTN patches were secured on the pasterns with stretch adhesive tape (Treatplast) to position them over digital vessels. Two, 3 or all 4 limbs were treated at any one time. In 2 cases the right hindlimb was bound with a dummy patch, without GTN, and served as a control. Two per cent glyceryl trinitrate ointment was applied once daily to the pasterns as a 'patch'; each aliquot of paste positioned over the 2 main digital vessels, covered with grease proof paper and secured in place with adhesive tape. Doses for individual animals are estimated under experimental protocols - these are approximations because rates of cutaneous absorption are not yet known. Calculated doses of glyceryl trinitrate are shown in Table 2.

In all cases, treatment with GTN was carefully monitored, both by blood pressure and any improvement in lameness. If lameness markedly improved, blood pressure invariably fell and the dose of GTN was reduced. Each animal was treated individually to achieve maximal improvement without causing hypotension. Ten cases of laminitis were treated and 2 very mild cases were stabled but left untreated. Treated animals were compared with untreated ones.

Effect of intravenous arginine in combination with transdermal glyceryl trinitrate: The laminitic pony given 10% L-arginine i.v. was treated with GTN 'patches' 12 h after the L-arginine infusion. Patches were applied to 3 limbs only - right and left fore- and the left hindlimbs. The amounts given were as follows: on the first week a total of 60 mg GTN/day (0.3 mg/kg bwt/day) was applied. This amount was reduced to 40 mg GTN/day (0.02 mg/kg bwt/day) for 5 days, which was further reduced to 20 mg GTN/day (0.01mg/kg bwt/day) for 2 days before the end of the treatment.

Effect of transdermal application of glyceryl trinitrate patches: Glyceryl trinitrate was usually administered as an initial dose of 60 mg (0.3 mg/kg bwt/day) for 2 days (dose a). If blood pressure decreased and lameness improved, it was reduced to 40 mg

TABLE 4: Qualitative changes in haemoglobin and cytochrome oxidase in the hoof determined by near infrared spectroscopy. (i) unsedated normal (ii) sedated normal (iii) sedated normal during i.v. infusion of 0.9% saline (iv) sedated normal during i.v. infusion of l-arginine (v) sedated normal during i.v. infusion of 0.9% saline and showing responses to manoeuvres designed to alter blood flow (vi) lack of responses of an acute laminitic pony to manual occlusion of digital vessels or lifting of contralateral limb (vii) sedated acute laminitic during i.v. infusion of l-arginine

		HHb	O ₂ Hb	cyt aa3	tHb
(i) ¹	unsedated normal	(↓)	(↓)	(↑)	(↓)
(ii) ¹	sedated normal	(↓)	(↓)	(slight ↑)	(↓)
(iii) ¹	sedated normal (0.9% saline i.v.)	(↓)	(↓)	(↑)	(↓)
(iv) ¹	sedated normal (l-arginine i.v.)	↓	↑	=	↓
(v) ¹	sedated normal (0.9% saline i.v.)				
	occlusion/lift release	↑ ↓	↓ ↑	= =	↑ ↓
(vi) ²	acute laminitic pony				
	occlusion/lift release	= =	= =	= =	= =
(vii) ²	acute laminitic pony l-arginine infusion	↓	↑	↓	↓

Key: ↑ increase above baseline, ↓ decrease below baseline, = no change, (↑)(↓) indicate slow changes in oxygenation over a period of time which are basal. O₂Hb - oxyhaemoglobin, HHb - deoxyhaemoglobin, cyt aa3 - cytochrome oxidase, tHb - total haemoglobin. ¹Critikon Redox Monitor Model 2001, ²Critikon Redox Monitor Model 2000.

glyceryl trinitrate/day (0.02 mg/kg bwt/day) for 2 days (dose b) and then to 20 mg (0.01 mg/kg bwt/day) for 2 days until the end of the treatment (dose c). This regime varied slightly according to individual responses and severity of the disease.

Statistical analyses

Data are presented as means ± s.e. A 2 sample Students *t* test was used to assess statistical significance, assuming unequal variance and 2-tailed probability. Results were considered statistically significant when *P* was <0.05.

Drugs and other agents

The materials used and their sources were as follows: L-Arginine hydrochloride (Sigma, Poole, Dorset, UK), glyceryl trinitrate ointment (2%) (Pericutol, Cusi (UK) Ltd., Haslemere, Surrey,

UK), 0.9% saline (0.1% glucose) drip (Baxter, Thetford, Norfolk, UK), 0.9% saline drip (Baxter, UK), Detomidine hydrochloride (Domosedan, Smith Kline Animal Health Ltd., Stevenage, Herts, UK), Butorphanol (Torbugesic, C-Vet, Bury St. Edmunds, UK), Phenylbutazone i.v. (Tomanol, Intervet UK Ltd, Cambridge, UK), Phenylbutazone-oral (Equipalazone, Arnold, Essex, UK) and Acepromazine (ACP solution, C-Vet, Bury St Edmunds, UK).

A sterile 10% solution of l-arginine was prepared: 40 g l-arginine hydrochloride were as dissolved in 400 ml of 0.9% saline, pH adjusted to 7.36, filtered through a 0.22 µm membrane (Nalgene Filterware, Nalge Co. New York, USA) and stored sealed at 4°C until use. A similar solution of 12% l-arginine was prepared in 0.9% saline, 0.1% glucose drip.

Results

Blood pressures and heart rates of normal and acute laminitic ponies

Heart rates varied between 43 and 45 beats/min for normal ponies (Table 3). Blood pressures and heart rates of animals during acute laminitis were significantly (*P*<0) higher than normal. These changes are summarised in Table 5 which compares blood pressures with Obel Grade of lameness.

Noninvasive assessment of pedal haemodynamics using near infrared spectroscopy

Basal values: Animals were monitored continually by NIRS during the period when they stood in the examination box. The traces of all but one of these animals (n=6) displayed a gradual reduction in concentration of O₂ Hb, HHb, tHb and an increase in cyt aa3 to stabilise after approximately 30 min. The changes were similar in unsedated and sedated animals (Figs 2 and 3 respectively). Sedated animals showed normal responses to manoeuvres designed to alter blood flow to the hoof such as lifting of the contralateral limb and manual occlusion of the

TABLE 5: Summary of blood pressures, heart rates and Obel grades of lameness of individual cases of acute laminitis

Animal code*	Blood pressure (mmHg)			Heart rate (beats/min)	Obel grade
	Systolic	Diastolic	Mean		
Case 1	183	97	138	70	4
Case 2	188	86	123	56	3.5
Case 3	165	75	106	54	3
Case 4	171	88	129	61	3.5
Case 5	155	94	121	75	3.5
Case 6	177	86	125	57	4
Case 7	131	81	98	51	3
Case 8	164	79	115	52	2
Case 9	165	90	118	71	3
Case 10	155	84	117	62	2
Mean	165	86	119	61	
s.e.	5	2	4	3	
No. of cases		10			
No. of tests		23			

*see Table 1.

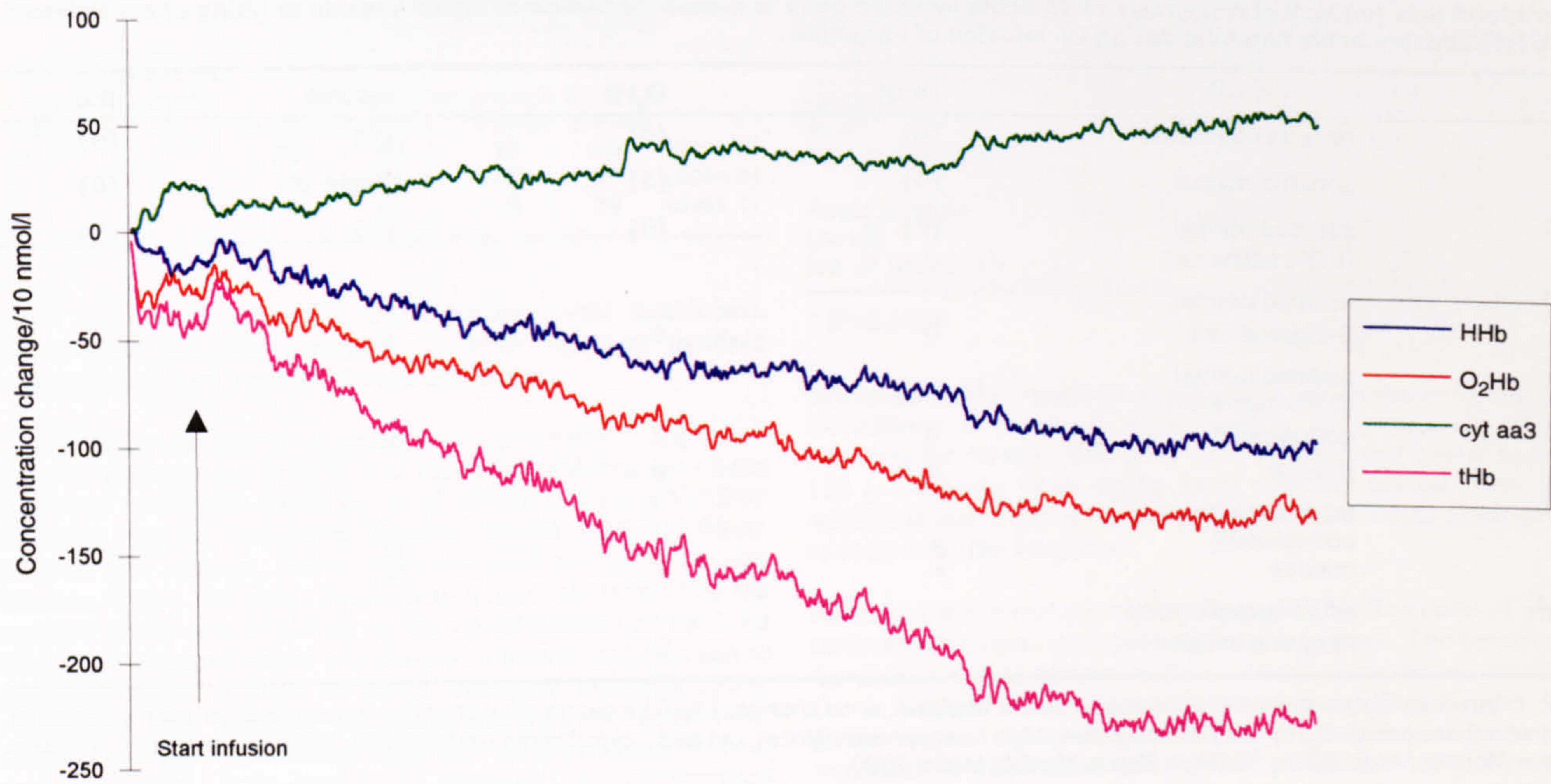


Fig 4: NIRS assessment of pedal haemodynamics of a sedated normal horse during an infusion of 0.9% saline iv.; traces were recorded over a period of 30 min. Key: HHb - deoxyhaemoglobin; cyt aa3 - cytochrome oxidase; O₂ Hb - oxyhaemoglobin; tHb - total haemoglobin.

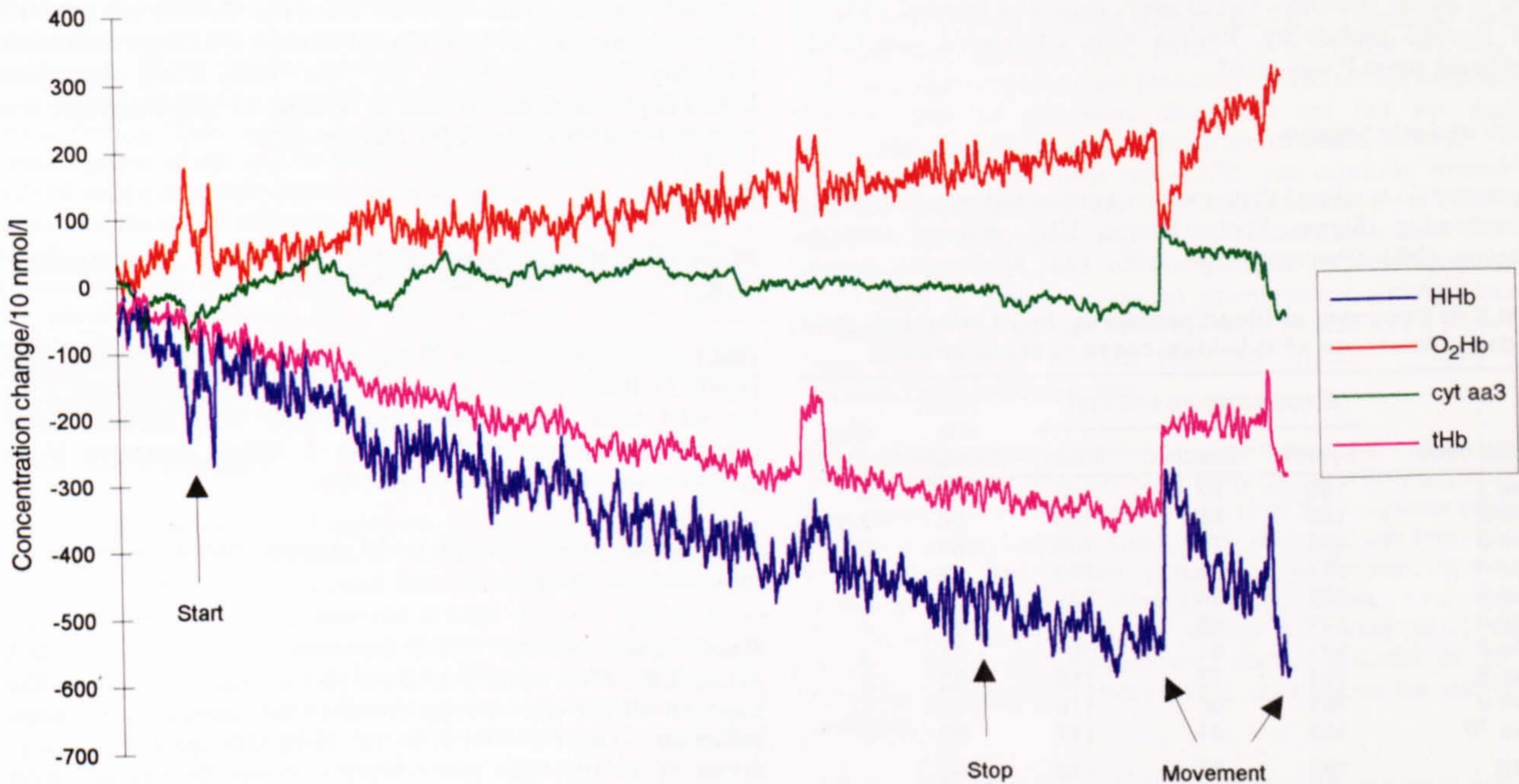


Fig 5: NIRS assessment of pedal haemodynamics of a sedated normal horse during an infusion of l-arginine; traces were recorded over a period of 20 min, the infusion lasted 15 min. Key: HHb - deoxyhaemoglobin; cyt aa3 - cytochrome oxidase; O₂Hb - oxyhaemoglobin; tHb - total haemoglobin.

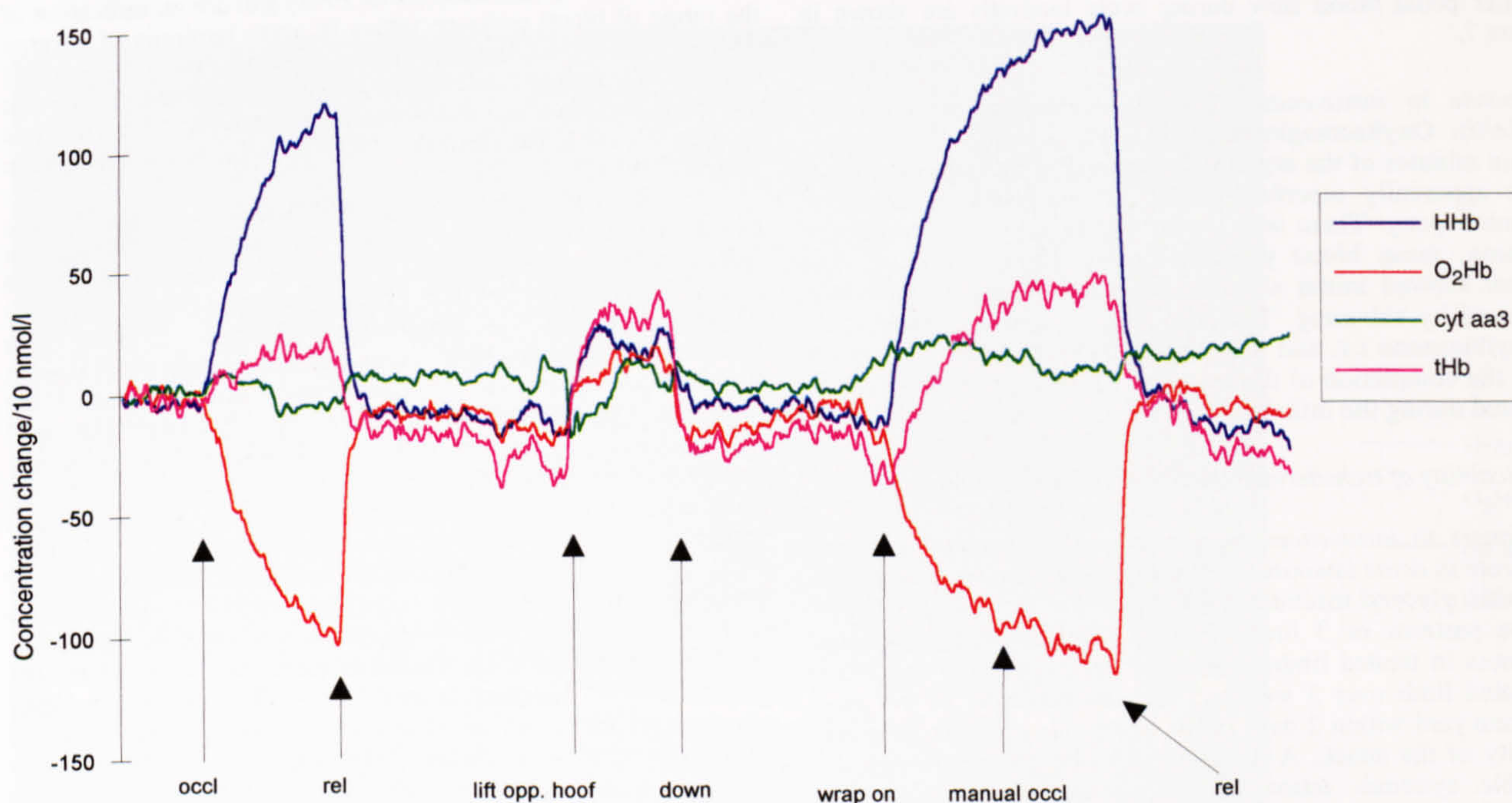


Fig 6: Near infrared spectroscopy assessment of pedal haemodynamics of a sedated normal horse during infusion of 0.9% saline; traces were recorded over a period of 10 min. Each manoeuvre to alter blood flow lasted for 1 min. Key: HHb - deoxyhaemoglobin; cyt aa3 - cytochrome oxidase; O₂Hb - oxyhaemoglobin; tHb - total haemoglobin.

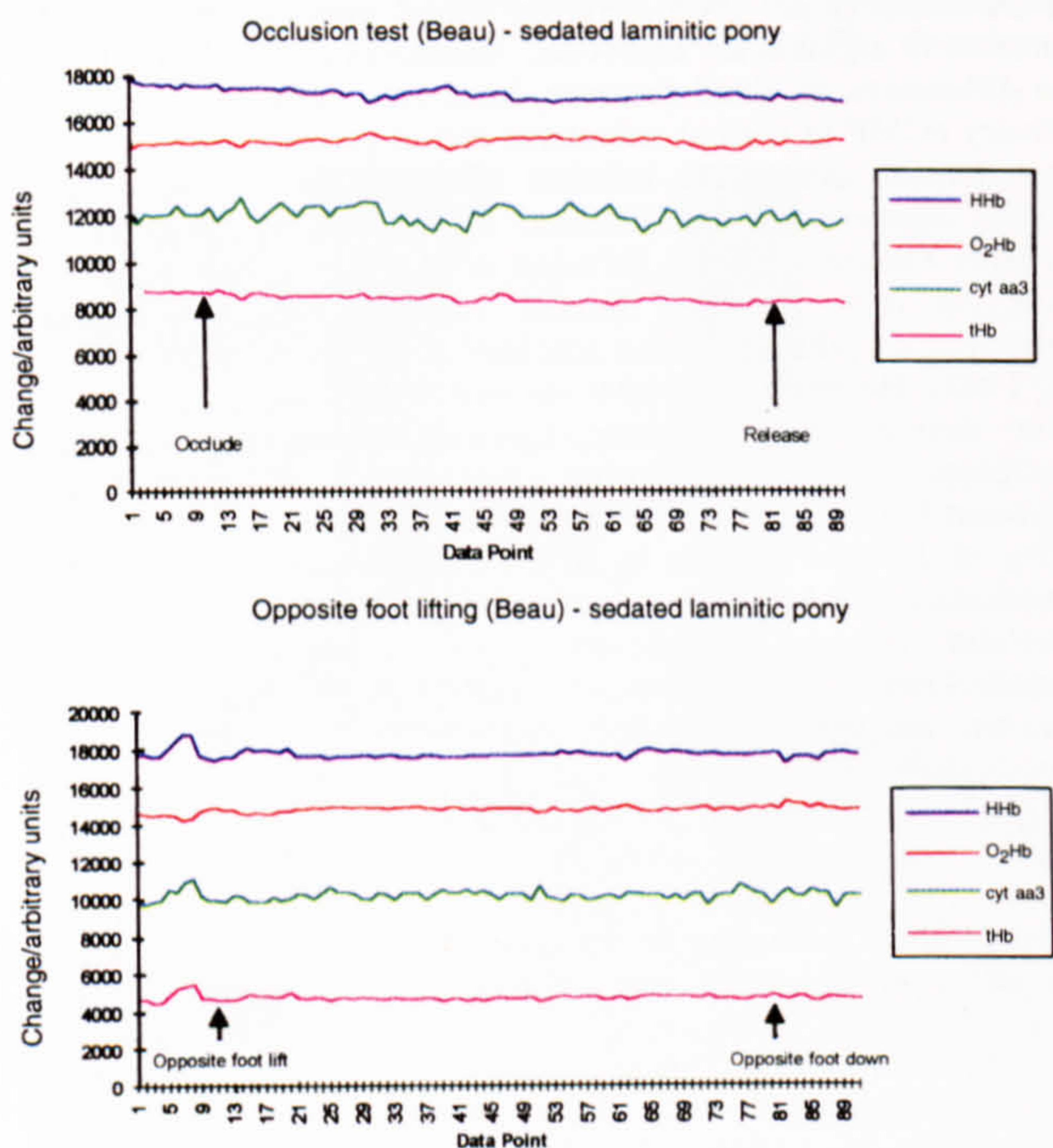


Fig 7: Near infrared spectroscopy assessment of pedal haemodynamics of a case of acute laminitis (sedated) - no changes are seen in response to manoeuvres designed to alter blood flow. Traces were recorded over a period of approximately 2 min. Each manoeuvre to alter blood flow lasted for 1 min. Key: HHb - deoxyhaemoglobin; cyt aa3 - cytochrome oxidase; O₂Hb - oxyhaemoglobin; tHb - total haemoglobin

digital vessels (Fig 3).

Responses of a normal pony to intravenous 0.9% saline: Traces showed the typical pattern during the i.v. infusion of 0.9% saline to a normal sedated pony (Fig 4). Saline infusion had no obvious effect on pedal haemodynamics.

Responses of normal horses to intravenous L-arginine: Increased concentrations of O₂Hb were seen during the infusion of L-arginine (Fig 5). This reflects increased perfusion of the pedal vasculature when L-arginine is administered compared with the 0.9% saline vehicle alone. Heart rate fell to 30 beats/min during the infusion and cardiac arrhythmia was observed. L-arginine 12% infused into another normal horse produced transient hypotension, but this was followed by a slight but sustained increase in blood pressure. Heart rate fell to 30 beats/min and cardiac arrhythmia was also observed, but other side effects in either instance were not apparent.

Vascular responses to digital manoeuvres in normal sedated animals and during an infusion of 0.9% saline: Standard responses were seen in sedated normal horses when manoeuvres (lifting of the contralateral limb and manual occlusion and release of the digital vessels) were performed to alter blood flow. These responses are shown in Figure 3 and, during the saline infusion, in Figure 6.

Vascular responses in acutely laminitic ponies: Baselines for oxyhaemoglobin, deoxyhaemoglobin, total haemoglobin and cytochrome aa3 were obtained (Hinckley *et al.* 1995) but these showed no responses to manual occlusion of the digital arteries, to lifting of the contralateral limb, or to general movement in the acute laminitic pony. The complete absence of responses to these manoeuvres suggests haemostasis. The qualitative changes in

baseline values for normal ponies and an acute laminitic pony are given in Table 4. The responses (or lack of them) during attempts to alter pedal blood flow during acute laminitis are shown in Figure 7.

Response to intravenous l-arginine solution during acute laminitis: Oxyhaemoglobin, HHb, cyt aa3 and tHb changed within minutes of the arginine infusion (Fig 8). Laminal tissues were apparently reperfused after i.v. l-arginine in an acute laminitic pony. There was a transient hypotension during the infusion, mean blood pressure falling 12 ± 2 mmHg. The animal showed initial signs of pain and sweating which was followed by shivering. The pony was rugged and given 5 ml phenylbutazone i.v. and 0.05 mg/kg bwt i.v. acepromazine 1 h after the completion of the infusion. Movement responses were restored during the infusion.

Applicability of transdermal glyceryl trinitrate in acute laminitis

Responses to intravenous l-arginine and transdermal glyceryl trinitrate in acute laminitis: Following i.v. l-arginine during acute laminitis, glyceryl trinitrate (GTN) paste was applied as 'patches' to the pasterns of 3 limbs (Fig 9). This markedly improved lameness in treated limbs, but some lameness continued in the untreated limb over 3 weeks. The pony was able to trot on a concrete yard within 2 days of the acute phase despite the initial severity of the attack. A lowering of blood pressure suggests a possible systemic action of the transdermal treatment. No analgesics or other medication were necessary. The pony continued to improve and there were no apparent secondary changes or sepsis in the hooves.

Topical transdermal application of glyceryl trinitrate in acute laminitic ponies: Topical application of GTN patches to the shaved pasterns of acute laminitic ponies reduced systemic blood pressure, alleviated bounding digital pulses and, after an initial worsening of lameness during the first day of treatment, improved lameness in treated limbs. Figure 10 summarises the data from individual cases. Clinically, the ponies seemed more alert and relaxed when the blood pressure was reduced and lameness improved markedly after the first day of treatment. Where animals had been treated on 3 limbs only, some slight lameness and digital pulses remained in the untreated limbs (Cases 1 and 4). However, Case 8 which received transdermal treatment on only 2 front limbs did not have such a rapid reduction in blood pressure or improvement in lameness; this animal had only Obel Grade 2 lameness at the onset of the disease and, therefore, was only slightly more severe a case than the untreated Cases 9 and 10. Untreated mild cases did not show such a dramatic improvement as those that were treated (Fig 11). There seemed to be a direct relationship between the dose of GTN and mean blood pressure. In all cases, no special shoeing was needed at any stage and gross morphological changes, necrosis or abscessation were not seen in the hooves in the following 9 months. All treated ponies improved steadily and were turned to grass at the end of the treatment.

Discussion

The present study presents data on the treatment of cases of acute laminitis arising spontaneously at grass, a common aetiology of laminitis in native breeds of ponies. The pony herd in this study was kept on unfertilised permanent pasture; animals were accustomed to being handled regularly by the investigators and were relaxed about the procedures. The success of the management regime is reflected in the narrow ranges of normal blood pressures and heart rates within the group.

The blood pressures of acute laminitics were related closely to the Obel Grade of lameness (Obel 1948) and are an indication of the range of blood pressure values in acute laminitis of varying degrees of severity (Table 5). The values can be used to gauge the efficacy of treatment (*vide infra*). Possible causes of hypertension are pain, alongside stress activation of the sympathetic nervous system (Hood 1979; Stashak 1987) and the renin-angiotensin system (Miller 1981; Clarke 1982).

Central to an understanding of laminitis is an insight into blood flow and distribution within the hoof. Methods used to assess pedal haemodynamics during the development of the disease or to ascertain the efficacy of treatments inevitably introduced artefacts in measurement (Robinson 1990). Near Infrared Spectroscopy (NIRS) overcomes these difficulties since it noninvasively determines changes in pedal haemodynamics and oxygen utilisation. Validation for this new has been published (Hinckley *et al.* 1995). Although sedation with detomidine and/or butorphanol has some effect on blood pressure and heart rate, there were no apparent differences between unsedated and sedated normal controls; these aspects are considered in Hinckley *et al.* (1995). Near infrared spectroscopy revealed a number of important differences in the pedal haemodynamic responses to occlusion of the digital vessels of normal and laminitic ponies.

The pony suffering from acute laminitis showed no responses to manoeuvres designed to alter blood flow within the hoof although normal baseline values were present. Normal horses show some movement artefacts (Hinckley *et al.* 1995) and these were absent in the laminitic pony (Table 4). Failure to respond to a compromised arterial supply, together with no alteration of haemodynamics during lifting of the contralateral limb, indicates haemostasis within the dermal laminae and possibly the terminal and circumflex arteries.

Arginine is a known secretagogue for growth hormone, insulin and other hormones but the modes of action are uncertain (Rabinowitz *et al.* 1968). Human blood pressure responses to infusion of arginine are equivocal. Baudouin *et al.* (1993) found no differences in blood pressure, heart rate, skin temperature or urinary cGMP in normal volunteers and in patients with systemic hypertension during i.v. infusion of l-arginine. Nakaki *et al.* (1992) reported hypotension and tachycardia in normotensive patients following an i.v. infusion of 0.5 mg/kg bwt l-arginine over 30 min. Diseased human forearm vessels vasodilate following an infusion with a solution of l-arginine (Creagher *et al.* 1992). Böger *et al.* (1994) showed a 43% increase in blood flow through normal human forearm vessels, together with decreased blood pressure during administration of l-arginine. The transient hypotension and a slight pressor response of a normal horse in the present study to an i.v. infusion suggests either that homeostatic cardiovascular mechanisms are compensating for a transient decrease in blood pressure and/or that slight hypervolaemia elicits neuroendocrine reflex regulation of blood pressure and/or that the slight stress of the procedure masked vasodilatory responses.

The i.v. infusion of 10% l-arginine initiated reperfusion of digital tissues of the acute laminitic pony. The pattern of reperfusion was similar to reperfusion seen after deliberately induced acute ischaemia in the hoof of a sedated normal horse (Hinckley *et al.* 1995) and to that seen in human patients (Thorniley 1988). These changes were consistent with reperfusion patterns in human and equine subjects (Hampson and Piantodosi 1988; Hinckley *et al.* 1995). This pattern is normally associated with hyperaemia after deliberate tourniquet induced forearm ischaemia (Hampson and Piantodosi 1988). The marked decrease in cyt aa3 in the presence of an increased O₂Hb is inconsistent with the classical view of a simple oxygen dependence of the enzyme. Postulated explanations include i) rapid oxidation of the enzyme in response to oxygen inflow followed by return to

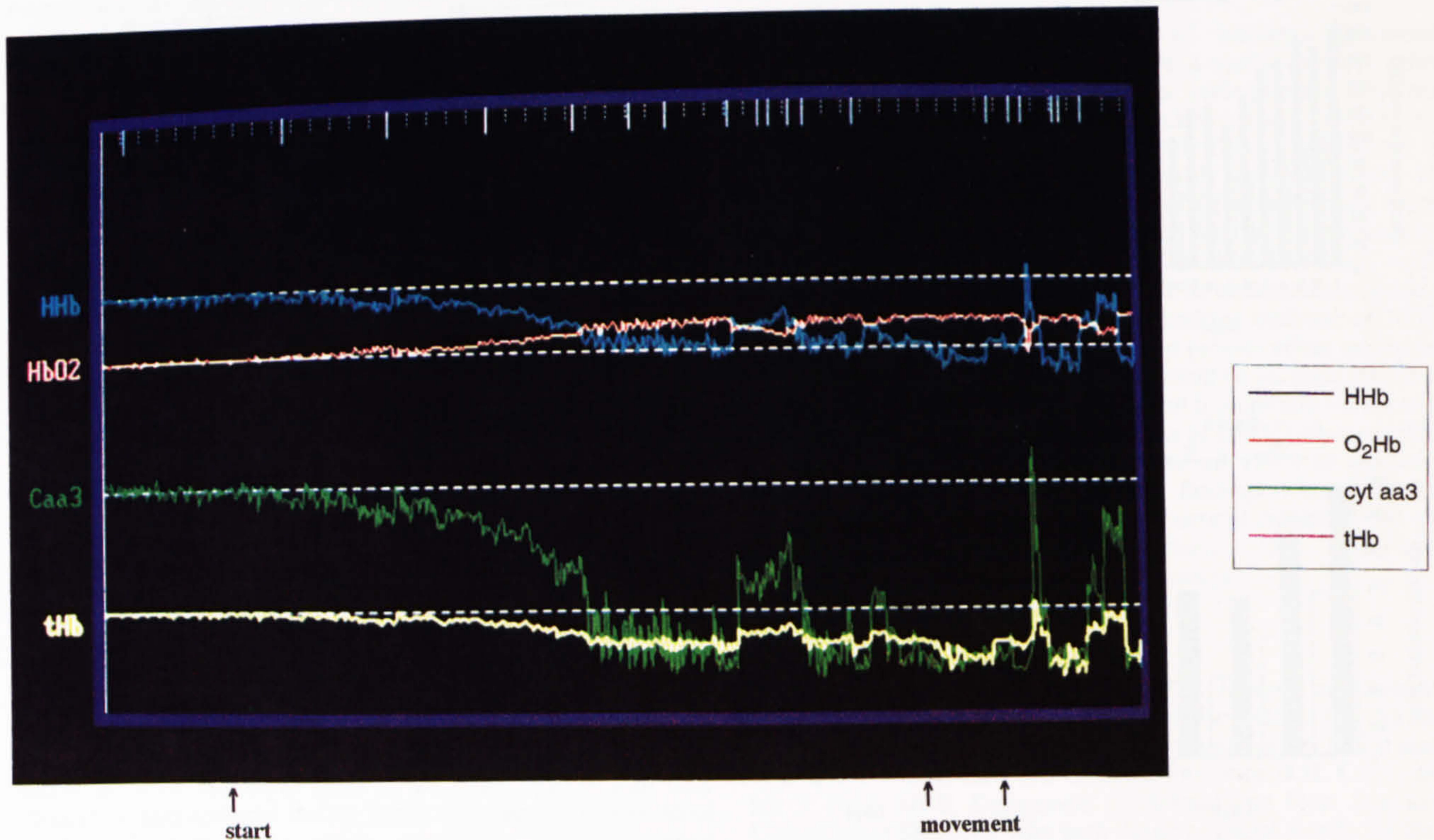


Fig 8: Near infrared spectroscopy recorded responses of pedal haemodynamics of an acute laminitic pony (sedated) to an i.v. infusion of *l*-arginine; traces were recorded over a period of 20 min. Key: HHb - deoxyhaemoglobin; cyt aa3 - cytochrome oxidase; O₂Hb - oxyhaemoglobin; tHb - total haemoglobin.



Fig 9: Glyceryl trinitrate 'patches' in place on the pasterns of a pony.

baseline. This would be in accordance with the known kinetics of cyt aa3 oxidation (Brunori *et al.* 1981) and ii) binding of NO to the copper ion associated with cyt aa3 resulting in inhibition of electron transfer and accumulation of cyt aa3 in its reduced state. The latter mechanism is consistent with the known reactions of cyt aa3 with NO (Brunori *et al.* 1981), but at this stage, the physiological model and effect on NIRS measurements are speculative.

The pony suffering acute laminitis showed a transient decrease in blood pressure when infused with *l*-arginine. Clinically, the pony experienced cold and shivering suggesting peripheral vasodilatation and cutaneous heat loss. Increased pain was evident during reperfusion. The pain on reperfusion may be compared to the phenomenon experienced by anyone who has felt leg pain on restoration of circulation after sitting awkwardly. Such pain accompanying reperfusion has been shown in horses after 2 h of induced pedal ischaemia (Hood 1995). Pain responses may account for elevated blood pressure 12 h after the *l*-arginine infusion; there are obviously many potential neuroendocrine mediators of this response. The hypertension was not the result of decreased substrate availability for NO synthesis since arginine is only slowly cleared from plasma, remaining elevated for 24 h (K. A. Hinckley and I. W. Henderson, unpublished observations). These abnormally high values of circulating *l*-arginine probably provided maximal substrate for NO synthesis, which was limited only by availability of nitric oxide synthase (NOS) itself. If reperfusion was the result of NO synthesis then it may be that synthesis of nitric oxide was submaximal.

Nitric oxide production may be limited by disruption of physiological supplies of *l*-arginine or by inhibition of NOS by

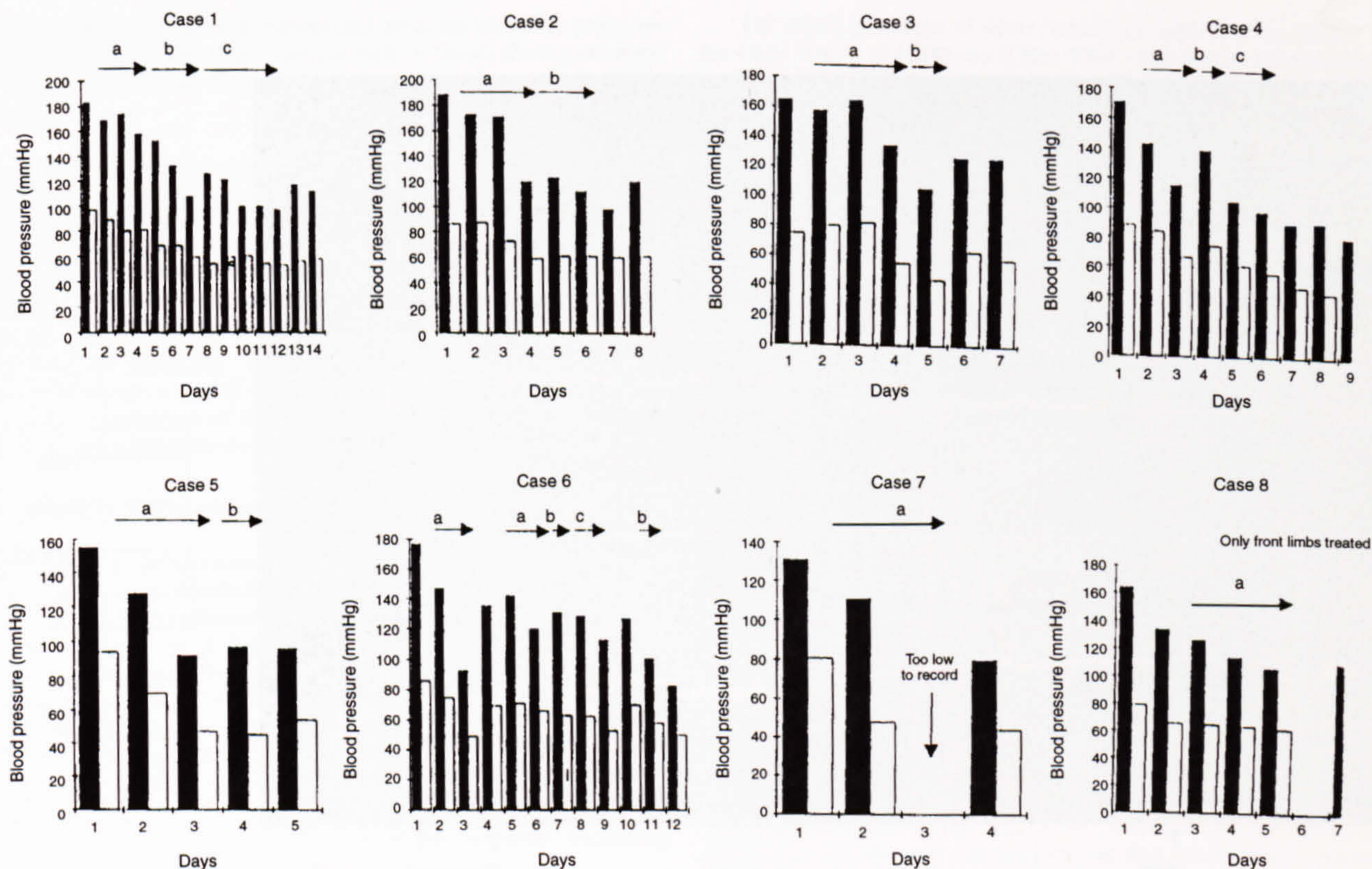


Fig 10: Case reports of acute equine laminitis treated with transdermal application of glyceryl trinitrate 'patches'. Doses (a, b and c) of glyceryl trinitrate are given in Table 2. Key: ■ Systolic blood pressure □ Diastolic blood pressure.

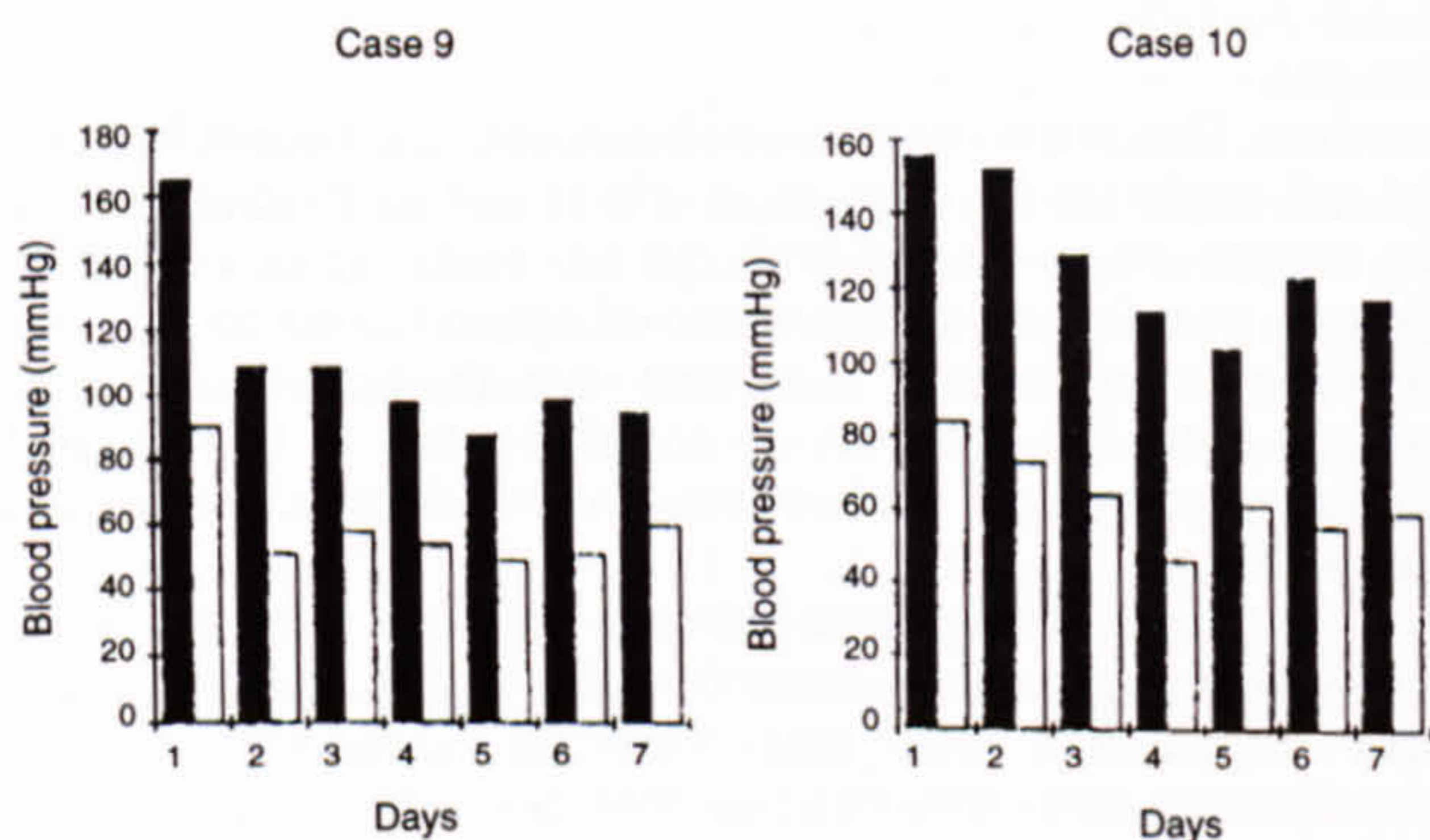


Fig 11: Case reports of mild acute equine laminitis left untreated. Key: ■ Systolic blood pressure □ Diastolic blood pressure.

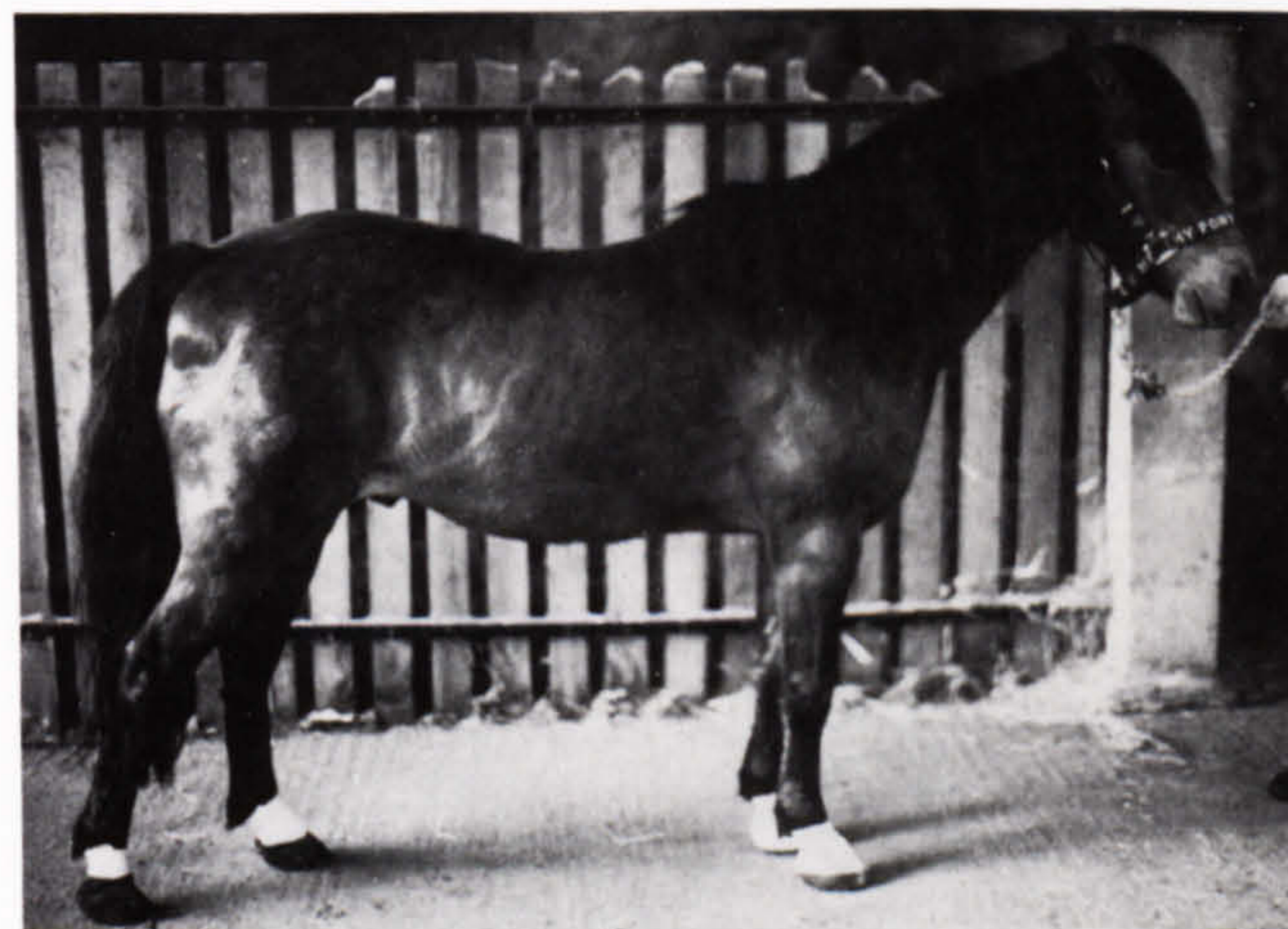


Fig 12: Case 1. Pony that received 10% l-arginine i.v. with glyceryl trinitrate 'patches' in place 2 days after onset; this pony was Obel Grade 4 of lameness on the first day of acute laminitis, was Obel 2 on the second day of treatment and able to trot on concrete without analgesics.

endogenous competitors such as ADMA (Vallance *et al.* 1992; Fickling *et al.* 1993; Hinckley *et al.* 1994). An alternative source of NO, glyceryl trinitrate (GTN), is endothelium independent and is neither regulated by NOS nor by the integrity of the endothelium, which is damaged during the acute phase of laminitis (Roberts *et al.* 1980; Templeton *et al.* 1985) even prior to the appearance of clinical signs (Hood *et al.* 1993). Glyceryl trinitrate can, therefore, provide vasodilatation even when the integrity of the endothelium is severely compromised. Treatment with GTN has been used for angina since the 19th century (see Vane *et al.* 1990) and, more recently, as a safe prophylactic agent to prevent preterm labour (Lees *et al.* 1994). Its vasodilatory action results from the molecule 'donating' nitric oxide which

relaxes smooth muscle (Katsuki *et al.* 1977). The patches of glyceryl trinitrate appeared to have both local (improving lameness and reducing the typical bounding pulse) and systemic effects (lowering blood pressure) (Fig 10). It is not known whether lowering of blood pressure was a direct systemic effect of GTN or because pain was relieved. Clinically, the ponies showed rapid improvement in lameness and reduced pain, distress and depression. Such assessments are subjective and

further NIRS studies which investigate haemodynamic changes following GTN application to digital vessels are underway. Side effects of transdermal GTN were minimal; slight skin irritation sometimes occurred at the site of application and sometimes low blood pressure was associated with an increased heart rate. In both instances, the treatment was discontinued without recurrence. Treatment with GTN showed no sedative effects and clinical assessment of improvement was much easier than with acepromazine treatments. Further, GTN lowered blood pressure and this was usually accompanied by a marked improvement in lameness but individual variation must be taken into account. If GTN was reduced or stopped prematurely then blood pressure increased and lameness worsened (Fig 10). At the onset of GTN treatment, blood pressure could show a transient elevation during the first 24 h, concurrent with some worsening of lameness, possibly resulting from reperfusion pain equivalent to that seen during the infusion of *l*-arginine. The observation that pain occurs during reperfusion supports the theory that laminitis is primarily a vasoconstrictive condition (Hood *et al.* 1993). Concurrent adjunct therapy with phenylbutazone may be needed for the first 24 h of the disease to relieve the pain of reperfusion; otherwise analgesic therapy is unnecessary. Transdermal GTN has been reported as having direct anti-inflammatory and analgesic actions (Berrazueta *et al.* 1994) when used as treatment for thrombophlebitis. Therefore, one advantage of the GTN treatment is that the need for analgesics is markedly reduced and the side effects of analgesia, particularly renal damage will be largely overcome. The GTN patches are very easy to apply and the problems of oral or invasive administration of therapeutic agents are obviated. Mild cases of laminitis recover with only changes in management but no doubt they would recover more quickly if given nitric oxide vasodilator treatment when symptoms first appear. The treated ponies were clinically normal after the treatment ended and there was no increase in blood pressure, or return of lameness after therapy. The ponies were turned out to grass. However, treated ponies were subject to further episodes of acute laminitis weeks or months after the initial attack. Subsequent bouts of acute laminitis received the same regime as before and none of the ponies had any permanent disability, sepsis in the hooves or apparent changes in the following 9 months.

Although the aetiology of equine laminitis is multifactorial and many hormones are certain to be involved in the cascade of vascular events that occur during the acute stage of the disease, the paracrine homeostatic control of vascular tone governed by nitric oxide may be involved. The homeostatic mechanisms of normal horses are usually capable of overcoming potential cardiovascular disruption, whilst in disease such mechanisms may be impaired. Attenuation of vascular responses by glycation, competitive inhibition of NO synthesis, or by vasoconstrictor agents (such as endothelin, angiotensin and catecholamines), may predispose horses to laminitis. During acute grass induced laminitis, vasodilatation is enhanced using NO donors to overcome haemostasis. The mechanisms involved are complex. NO may have a direct action on vascular smooth muscle or the vasodilatory effect may be mediated by inhibited vasoconstrictor activity. GTN reduces both plasma endothelin and angiotensin II concentrations during acute laminitis (K. Hinckley and I. W. Henderson 1995; unpublished observations). Many other mechanisms are certain to be involved. This preliminary study suggests that grass induced laminitis can be treated in the acute stage with precursors of nitric oxide, such as *l*-arginine or by synthetic donors of nitric oxide. Clearly, more studies are required to assess nitric oxide donors as a possible therapy for grass induced acute laminitis and to evaluate pharmacokinetics and GTN tolerance, which is well documented in man (Goodman and Gilman 1970). Tolerance to GTN in man usually occurs within a

few days of chronic administration but is completely lost after 10 days without treatment. Mechanisms of tolerance are likely to vary with species.

This study addresses laminitis of metabolic/nutritional aetiology where nitric oxide donors are a useful therapy; other types of laminitis of endotoxic/sepsis pathogenesis may not behave in the same way. Endotoxic shock is a major concern and administration of nitric oxide donors may be contraindicated in case the inducible form of nitric oxide is synthesised by macrophages to induce shock. However, if synthetic nitric oxide donors were given together with an analogue of *l*-arginines this strategy would inhibit the production of inducible nitric oxide from macrophages; vasodilatation would be enhanced alongside a reduced risk of shock. A specific competitive inhibitor of NOS, such as L-NAME, which has a sustained pressor effect on equine blood pressure (Hinckley *et al.* 1994) would be an ideal choice in such circumstances. It may also be useful to combine nitric oxide therapy with free radical scavengers such as DMSO (Baxter 1992) and calcium channel blockers (Hood *et al.* 1993) to minimise reperfusion injury. These preliminary findings warrant further investigation, but if found to be a successful treatment for the devastating disease of equine laminitis, they will have considerable and obvious welfare implications.

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Near infrared spectroscopy of pedal haemodynamics and oxygenation in normal and laminitic horses

K. A. HINCKLEY, S. FEARN*, B. R. HOWARD† and I. W. HENDERSON

Institute of Endocrinology, Department of Animal and Plant Sciences and †Field Laboratories, University of Sheffield, Sheffield S10 2TN, UK.

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Summary

The present study applies near infrared spectroscopy (NIRS) to the haemodynamics of the pedal circulation in normal and laminitic horses. NIRS is a noninvasive technique which uses changes in light absorption at 4 wavelengths to provide information on the changes in cytochrome aa3 (cyt aa3) reduction-oxidation (redox) status, and changes in the tissue concentration of oxyhaemoglobin (O₂Hb), deoxyhaemoglobin (HHb) and therefore total haemoglobin (tHb). Other studies have shown NIRS to be sensitive to changes in tissue oxygenation and perfusion in human cerebral and limb circulation. In this study, the NIRS sensor was applied to the dorsal surface of horses' hooves. Normal and laminitic animals (acute and chronic) were subjected to manoeuvres (cuff tourniquet; digital vessel occlusion at the palmar surface of the pastern; lifting of contralateral limb) predicted to change pedal haemodynamics. The procedures produced changes in pedal haemodynamics and oxygenation, which were similar to those observed in the ischaemic/reperfused human forearm. Laminitic differed from normal horses: return of HHb to baseline was slower and the change in cyt aa3 more rapid than normal in cases of chronic laminitis, taken to indicate reduced O₂ stores as a result of compromised basal perfusion. In acute laminitis, baseline values did not fluctuate following any of the manoeuvres, suggesting haemostasis in the diseased hoof. NIRS is a useful noninvasive method to assess pedal vascular function in normal and laminitic horses.

Introduction

The equine hoof contains an elaborate and complex vasculature. The digital vascular system is robust enough to tolerate the obvious mechanical stresses of weight bearing and to dissipate concussive forces during movement while also being highly sensitive for haemodynamic regulation of normal tissue perfusion. Equine laminitis is a crippling disease of the delicate microvascular laminae bed which lies between the pedal bone and the hoof wall. There is no known cure and, although many factors are implicated, the precise mechanisms inherent in the aetiology are unclear. Haemostasis and ischaemia are widely accepted as central features but the reasons for the altered blood flow are

debated (Robinson 1990; Hunt 1991). Inappropriate opening of arteriovenous shunts (Molyneux *et al.* 1994), local vasoconstriction, or microvascular thrombosis (Weiss *et al.* 1994) are all possible candidates. Prolonged ischaemia in dermal tissues produces secondary (and often permanent) changes in hoof morphology. Recent studies of the disease have concentrated on circulatory differences between diseased and normal animals.

The gross anatomical arrangements of the digital arterial tree and venous drainage (Kruger 1934; Schummer 1951; Mishra 1982) alongside the microanatomy of the smaller digital vessels have been described, using a combination of angiographic perfusion and electron microscopy (Mishra and Leach 1983; Pollit and Molyneux 1990). Together, these studies give details of the characteristics of the dermal laminae tissues and also reveal the presence of arterio-venous anastomoses which potentially could shunt blood away from the terminal portions of the hoof vasculature. Electron microscopy of perfusion casts have revealed a number of macroscopic vascular lesions within the laminitic hoof. These descriptions have all employed disarticulated hooves and clearly may not reflect the arrangement *in situ*.

To place the anatomical arrangements into a functional context, it is obviously necessary to determine blood flow, both qualitatively and quantitatively, within the hoof and thereby arrive at definitions of local and systemic factors that influence pedal haemodynamics. With varying success, a variety of invasive and noninvasive techniques have been applied to determine blood flow to, and its distribution within, the hoof. The techniques and approaches applied have included radiography/angiography using radioactively labelled materials and contrast media, electromagnetic flow probes, dye dilution and extrapolation of data obtained from the measurement of vascular resistances (Coffman *et al.* 1970; Robinson 1976; Hood *et al.* 1978; Scott and Sandler 1978; Trout *et al.* 1990). Most recently, Hood *et al.* (1994) and Adair *et al.* (1994) have used radioactively labelled platelets and Laser Doppler Flow Probes respectively to assess pedal blood flow with some success.

The Doppler system, successfully applied in human clinical practice, involves subcutaneous implantation of the probe and has been used in horses to assess muscle blood flow (Sertyn *et al.* 1986). Finally, 2 other noninvasive methods have been applied: thermography (Turner 1991) and nuclear magnetic resonance imaging on disarticulated limbs (Denoix *et al.* 1993); the thermographic study provided information on coronary band and digital arterial flows but details of flow within the hoof were not accurately observed. It follows, therefore, that this is a useful manner in which to measure sizes of larger vessels and gross estimates of blood to and from, but not within, the hoof (K. A. Hinckley and I. W. Henderson, unpublished observations).

*Present address: c/o Johnson and Johnson Medical Ltd., Coronation Road, Ascot, Berkshire SL5 9EY, UK.

TABLE 1: Factors causing signal changes in near infrared spectroscopy (after Brazy 1991)

Increased deoxyhaemoglobin Decrease in oxygen saturation Obstruction to venous return Increase inflow of desaturated blood Increase in concentration of deoxyhaemoglobin	Increased total haemoglobin Increase in blood flow Obstruction to venous return Increase in concentration of haemoglobin
Increased oxyhaemoglobin Increase in oxygen saturation Increase in blood flow Increase in concentration of oxyhaemoglobin	Increased cytochrome aa3 oxidation Increase in oxygen delivery to cells Increase in metabolic activity of cell with oxygen sufficiency Decrease in supply of electrons to respiratory chain

Magnetic resonance is potentially of greater use but is expensive and not presently available for *in vivo* studies.

An ideal method is not yet available but clearly noninvasive methods are preferred especially since invasive procedures may produce artefacts resulting from secondary cardiovascular responses to the procedure itself. Similarly the injection of exogenous agents may also produce secondary vascular responses in a physiological sense or may produce mechanical alterations within the pedal microvessels (Robinson 1990). It is, at least in part, for these reasons that there is not a consensus as to the precise nature of pedal vascular perfusion and more especially what occurs in either the acute or the chronic laminitic hoof.

In human clinical medicine, near infrared spectroscopy (NIRS) is now used successfully to monitor cerebral haemodynamics following vascular episodes (Jöbbsis-Vandervliet *et al.* 1987; Bucher *et al.* 1993). Near infrared spectroscopy has an obvious potential for application to the study of laminitis. The present investigation assessed the technique's applicability to the study of pedal microvascular blood flow and oxygenation in laminar tissues, with the aim of establishing a noninvasive method for determining pedal haemodynamics under normal and pathological circumstances.

Materials and methods

Animals

Six adult cross-bred ponies, 4 mares and 2 geldings age 4–18 years, were kept on identical management systems of diet, exercise and routine veterinary care for 6 months. Four animals were normal and 2 animals had suffered laminitis at least 12 months before the study and prior to joining the group. These animals exhibited signs of chronic laminitis of Obel Grades of lameness 1 and 2 (Obel 1948), divergent growth rings and some degree of solar prolapse although no special shoeing was necessary. One of the chronic laminitic animals developed acute laminitis, and Obel Grade 4 lameness, spontaneously when at grass. Three animals were studied sedated and unsedated.

Basis of near infrared spectroscopy (NIRS)

The basis of NIRS relies on the transparency of bone and other tissues to electromagnetic radiation in the near infrared spectrum, allowing transmission of these wavelengths through several centimetres of tissue. Changes in light absorption at selected NIR wavelengths allow inferences to be drawn regarding changes in haemodynamics, oxygen delivery and intracellular oxygen utilisation within the optical field. Changes in cytochrome aa3 (cyt aa3) reduction-oxidation (redox) status, together with changes in concentrations of oxyhaemoglobin (O₂Hb), deoxyhaemoglobin (HHb) and total haemoglobin (tHb) are monitored as deflections of a trend waveform from a baseline. Total haemoglobin is obtained by summing the O₂Hb and HHb signals and reflects local changes in blood volume. Cytochrome

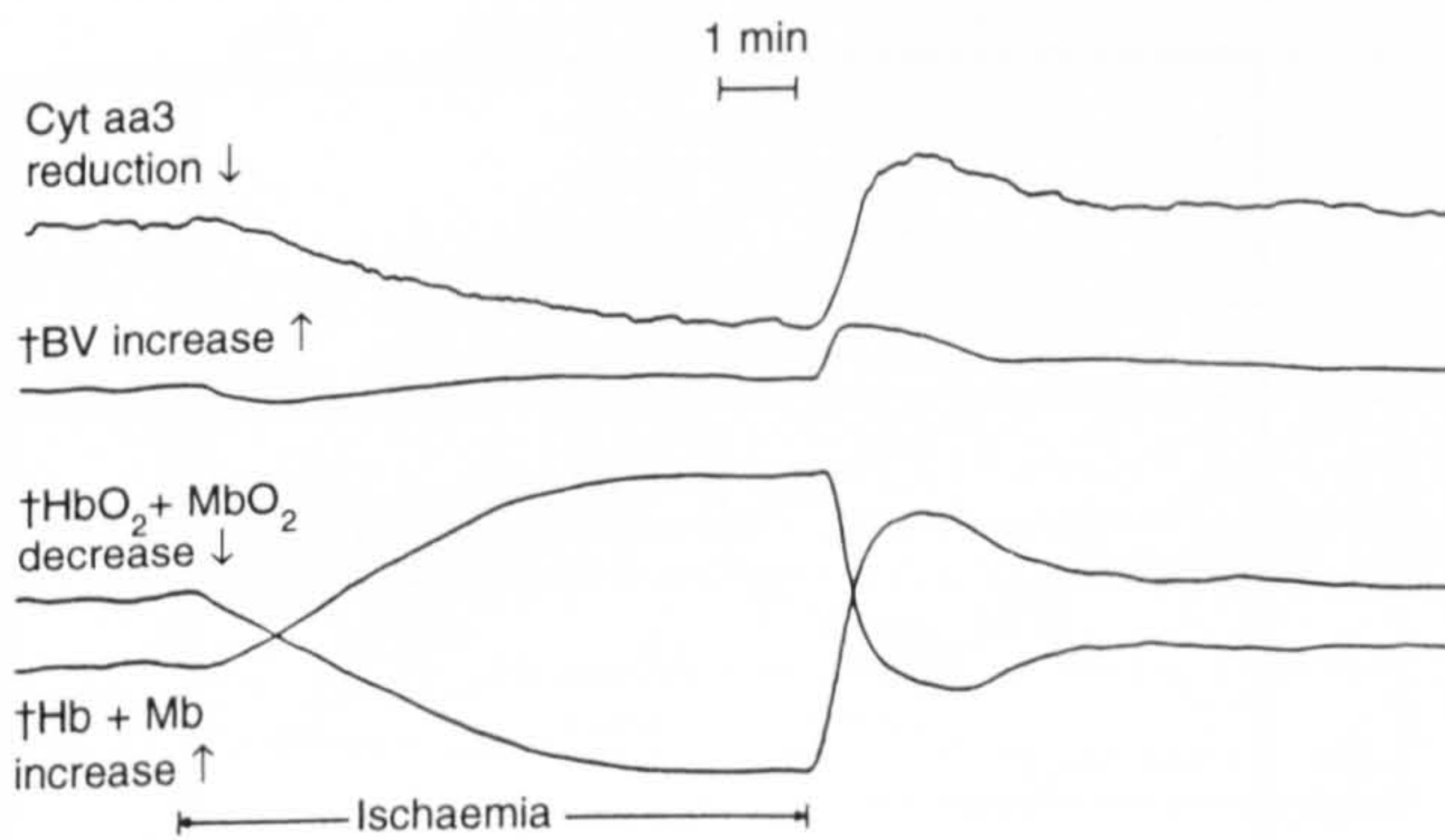
aa3, the terminal enzyme in the mitochondrial electron transport chain, accounts for greater than 95% of oxygen utilisation in aerobic metabolism and indicates intracellular oxygen sufficiency. The factors causing signal changes are outlined by Brazy (1991) and are summarised in Table 1.

Both O₂Hb and HHb exhibit weak absorption throughout the NIR range of 700–1300 nm. Deoxyhaemoglobin has a peak at 760 nm which disappears on oxygenation, while O₂Hb has a broad band around 900 nm which is not present in the HHb spectrum. Oxidised cyt aa3 has a weak absorption band between 780–870 nm which disappears upon reduction, hence cyt aa3 absorption in this range is due to the presence of the oxidised form. This enzyme may indicate mitochondrial oxygen availability as it changes its absorption characteristics in parallel with the availability of oxygen for its function. A downward deflection of the cyt aa3 trace may be caused by an increased amount of electrons on the respiratory chain, due to a decrease in oxygen availability or decreased metabolic activity.

Real time monitoring of these parameters provides information on arterial delivery, venous return and cellular utilisation of oxygen, alongside an insight into potential venous pooling. Hampson and Piantadosi (1988) have successfully applied and validated NIRS in a study of human forearm undergoing ischaemia and reperfusion. The same study showed venous occlusion to be characterised by an increase in HHb and tHb while O₂Hb and cyt aa3 remained constant. In contrast, total arterial and venous occlusion was associated with a decrease in oxygenated haemoglobin and a downward shift in the cyt aa3 trace. These changes were reversed during reperfusion, with an overshoot from the baselines indicating a period of hyperaemia (Fig 1).

Equipment

A Critikon Cerebral RedOx Monitor Model 2000 (Critikon, Johnson and Johnson, Ascot, UK) was used to assess vascular function within the hoof. An electro-optic cable connected the monitor to the sensor which was placed on the dorsal surface of the hoof. Adult or neonatal sensors were chosen according to hoof size and to give an optimal strength of signal. The placement of adult sensors on the hooves, are shown in Figure 2. A 2 cm square area of the coronary band was clipped dorsally mid-line to ensure good neonatal surface contact of the sensor. The emitter section of the sensor was placed on the dorsal surface of the hoof wall of one front foot. On horses with an uneven hoof wall, contact was aided by placement of a small amount of cotton wool under the emitter section of the sensor. Exclusion of ambient light and constant sensor to hoof coupling were achieved by the application of adhesive tape (Treatplast, Animalcare Ltd., York, UK) or polythene adhesive tape (Gaffatape). Real time changes in deoxyhaemoglobin (HHb), oxyhaemoglobin (O₂Hb), cytochrome aa3 (cyt aa3) and total haemoglobin (tHb) were displayed on the monitor cathode ray screen as coloured traces and data were recorded directly from the monitor RS232 port on a portable computer (Thinkpad, IBM, UK).



O_2Hb =oxyhaemoglobin, MbO_2 =oxymyoglobin, Hb =deoxyhaemoglobin, Mb =myoglobin, $cyt\ aa3$ =cytochrome oxidase, BV =total haemoglobin and myoglobin.

Fig 1: Near infrared spectroscopy (NIRS) traces of human forearm skeletal muscle during 8 min of tourniquet induced ischaemia (from Hampson and Piantadosi 1988).

Medication

Animals were restrained in a standard crush unless sedated. Sedation involved i.v. administration of 0.006 mg/kg bwt detomidine hydrochloride (Domosedan, Smith Kline Animal Health Ltd., Stevenage, Herts, UK) and 0.012 mg/kg bwt butorphanol (Torbugesic, C-Vet, Bury St. Edmunds, Suffolk, UK); a pony of 250 kg received a dose of 0.15 ml of detomidine and 0.3 ml of butorphanol i.v. The pony suffering acute laminitis was sedated as above.

Assessment of pedal haemodynamics

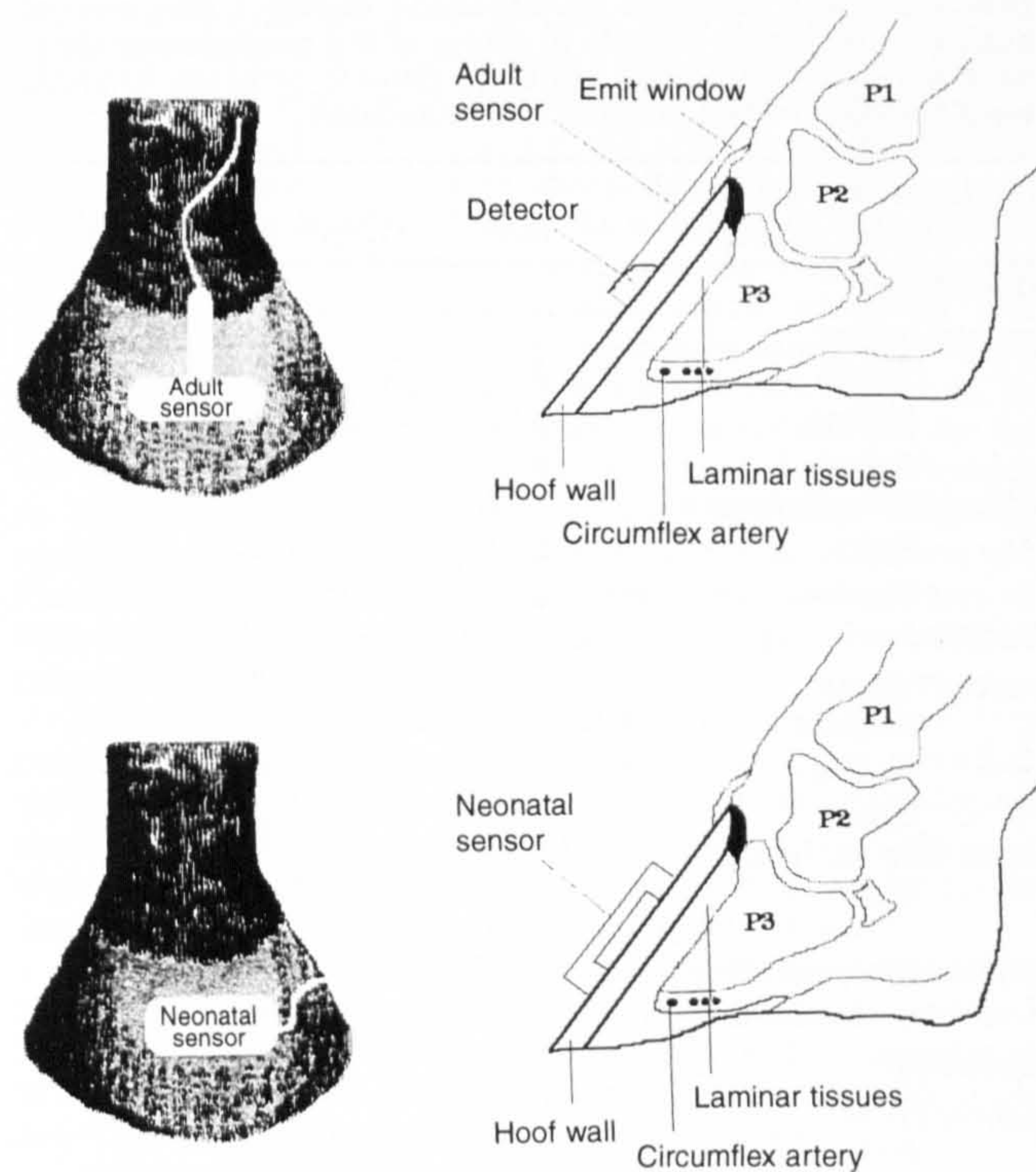
(i) *Cuff inflation*: A human adult blood pressure cuff attached to an aneroid manometer (Perimed, Norfolk) was placed over a cotton wool pad placed medially on the animals left foreleg, proximal to the carpal joint. Points were marked on the monitor traces when the cuff was inflated to a pressure of 280 mmHg to occlude the brachial artery and vein for 1 min, and marked again when deflated rapidly. This method was performed only on unsedated animals. The start of cuff inflation for a given animal was marked on the monitor trace with the built in 'event mark' function. Release of the cuff and movement artefacts were similarly identified, each event mark being automatically assigned a unique reference number. The event reference and related intervention were recorded by a separate observer.

(ii) *Manual occlusion of digital vessels*: Branches of the digital arteries and veins were palpated and occluded manually for periods of up to one minute. Moments of occlusion of digital vessels and subsequent release were recorded on the traces. This procedure was performed on sedated and unsedated animals.

(iii) *Lifting of contralateral limb*: One forelimb was raised and held up and changes in blood flow during a period of extra weight bearing were assessed. Traces were marked when the contralateral forelimb was raised and returned to the ground. This procedure was performed on unsedated and sedated animals.

Results

The Critikon Cerebral RedOx Monitor identified changes in oxyhaemoglobin (O_2Hb), deoxyhaemoglobin (HHb) cytochrome aa3 ($cyt\ aa3$) and total haemoglobin (tHb) within the hooves of normal and chronic laminitic horses (unsedated or sedated) in



P1=proximal phalanx, P2=middle phalanx, P3=distal phalanx.

Fig 2: Diagrams of the near infrared spectroscopy adult and neonatal sensors (emitter/detector) in place (before taping) on the hoof exterior and shown in cross section.

field conditions. However, the pony suffering acute laminitis showed no pedal vascular responses either to occlusion of digital vessels or to weight bearing. Table 2 summarises qualitative changes in parameters monitored under the various protocols.

1. Responses to cuff inflation

Only small changes were seen when the cuff was inflated. When the blood pressure cuff was inflated there was a slight increase in the deoxyhaemoglobin (HHb), an increase in total haemoglobin (tHb) together with a slight decrease in oxyhaemoglobin (O_2Hb). Cytochrome aa3 ($cyt\ aa3$) decreased. After release of the cuff, $cyt\ aa3$ and O_2Hb increased together with a concurrent decrease in HHb and tHb. Those animals with well developed forearm musculature and/or deep set vessels failed to show any significant responses to cuff inflation, suggesting that occlusion of the vessels was incomplete at this site and this method was not continued.

2. Manual occlusion of digital vessels

Sedated normal horses: These showed classic responses to ischaemia and to reperfusion (Fig 3), namely divergence of O_2Hb and HHb and downward deflection of the $cyt\ aa3$ trace. Another sedated horse showed a different response to manual occlusion of digital vessels having an increase in $cyt\ aa3$ and decrease in tHb (Fig 5). Release of occlusion, and hence reperfusion, resulted in HHb and tHb returning to baseline values while $cyt\ aa3$ not only returned to baseline but also 'overshot' previous baseline values.

Unsedated normal horses: These showed a decrease in HHb, tHb and oxidised $cyt\ aa3$, but little change in O_2Hb . Movement artefacts sometimes interfered with the changes shown in trace values (Fig 4) but changes in values were nevertheless apparent. Light sedation prevented movement artefacts and enabled smooth

TABLE 2: NIRS changes in responses during 1 min manual occlusion of digital vessels or lifting of the contralateral limb. Normal horses and those suffering chronic or acute laminitis were assessed when sedated and unsedated

(1) Responses to manual occlusion of vessels	HHb	O ₂ Hb	cyt aa3	tHb
Unsedated				
<i>normal horses</i>				
Albert	↓	=	↓	↓
Marron	↓	=	↓	↓
Selluci	↓	=	↓	↓
<i>chronic laminitic horses</i>				
Beau	↑	↓	↓	=
Domino	↑	↓	↓	=
Sedated				
<i>normal horses</i>				
Despina	↑	↓	↓	↑
Marron	↑	↓	↓	↑
Selluci	↑	↓	↑	↓
<i>acute laminitic horse</i>				
Beau	=	=	=	=
(2) Responses to lifting contra lateral limb				
Unsedated				
<i>normal horse</i>				
Albert	↑	↓	=	=
Sedated				
<i>normal horse</i>				
Marron	↑	↓	↑	↓
Selluci	=	↓	↑	↓
<i>acute laminitic</i>				
Beau	=	=	=	=

↑, increase above baseline, ↓, decrease below baseline, =, no change, O₂Hb, oxyhaemoglobin, HHb, deoxyhaemoglobin, cyt aa3, cytochrome oxidase, tHb, total haemoglobin.

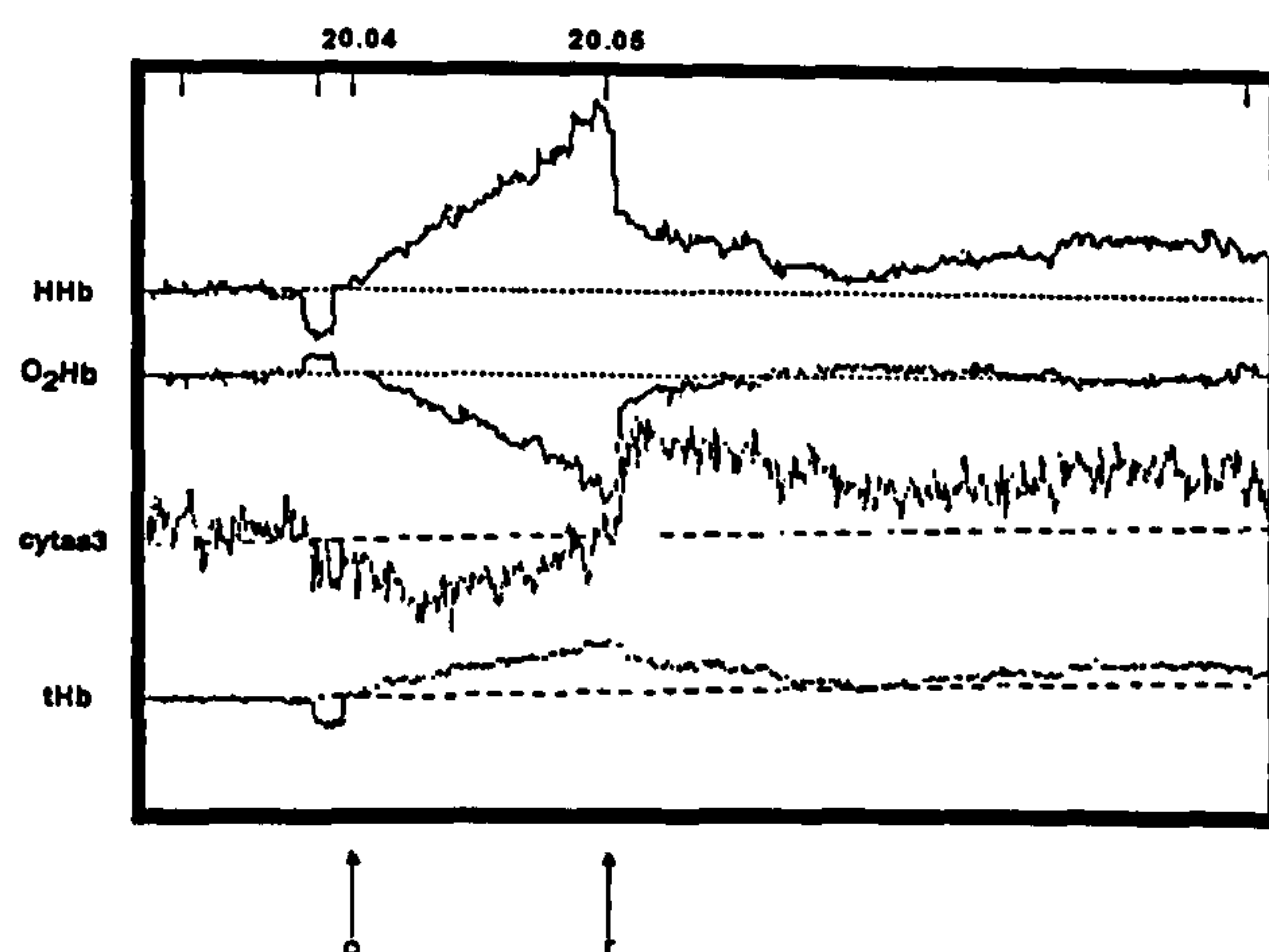
baseline values to be observed, thereby improving the quality of the traces.

Unsedated chronic laminitis cases: These showed a decrease in O₂Hb and oxidised cyt aa3 on occlusion of digital vessels, with a corresponding increase in HHb. Total haemoglobin remained constant. The rates of response differed from normal horses. Laminitis cases showed a more rapid change in cyt aa3 on occlusion. Laminitic individuals also showed a slower change in HHb upon reperfusion. Subjectively, unsedated laminitic ponies seemed to find occlusion of digital vessels uncomfortable and frequently lifted their feet, although it is usual for laminitic animals to move weight from one foot to the other to some extent.

Sedated acute laminitic pony: This gave clear signals and baseline values but occlusion had no effects on the measured parameters.

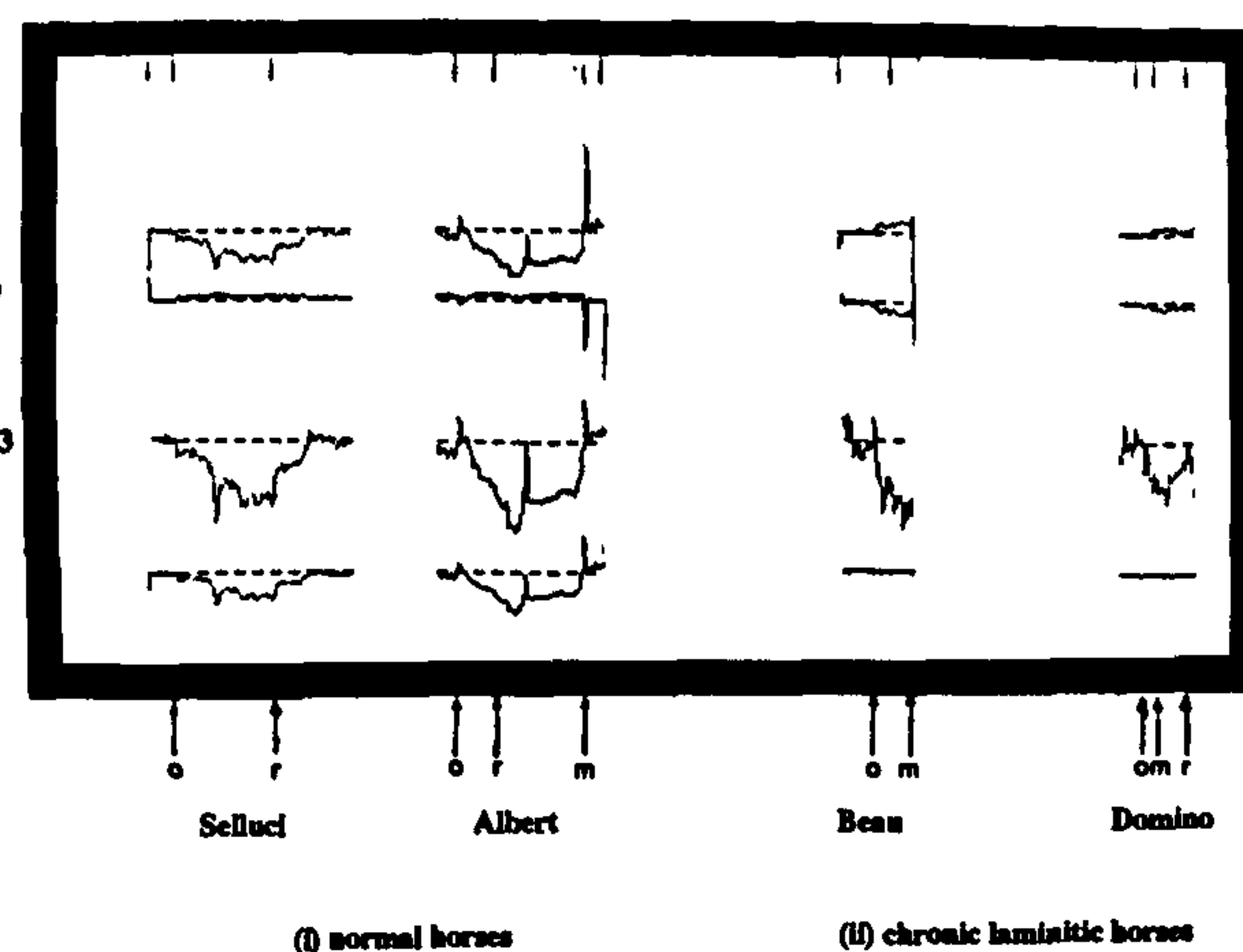
3. Weight bearing

Movement artefacts produced rather erratic traces in unsedated horses; nevertheless a divergence of the O₂Hb and HHb traces was clearly seen, although there was little change in cyt aa3 and tHb following lifting of the contralateral limb. The divergent pattern for O₂Hb and HHb was reproduced in a sedated normal horse, but unusually there was an increase rather than the usual decrease in cyt aa3 for this particular horse and little change seen in the value for HHb until after release of the vessels (Fig 5). No



O₂Hb=oxyhaemoglobin, HHb=deoxyhaemoglobin, cyt aa3=cytochrome aa3, tHb=total haemoglobin.

Fig 3: Changes in near infrared spectroscopy (NIRS) traces of a sedated normal horse upon manual occlusion (o) of digital vessels for 1 min and release (r).



O₂Hb=oxyhaemoglobin, HHb=deoxyhaemoglobin, cyt aa3=cytochrome aa3, tHb=total haemoglobin.

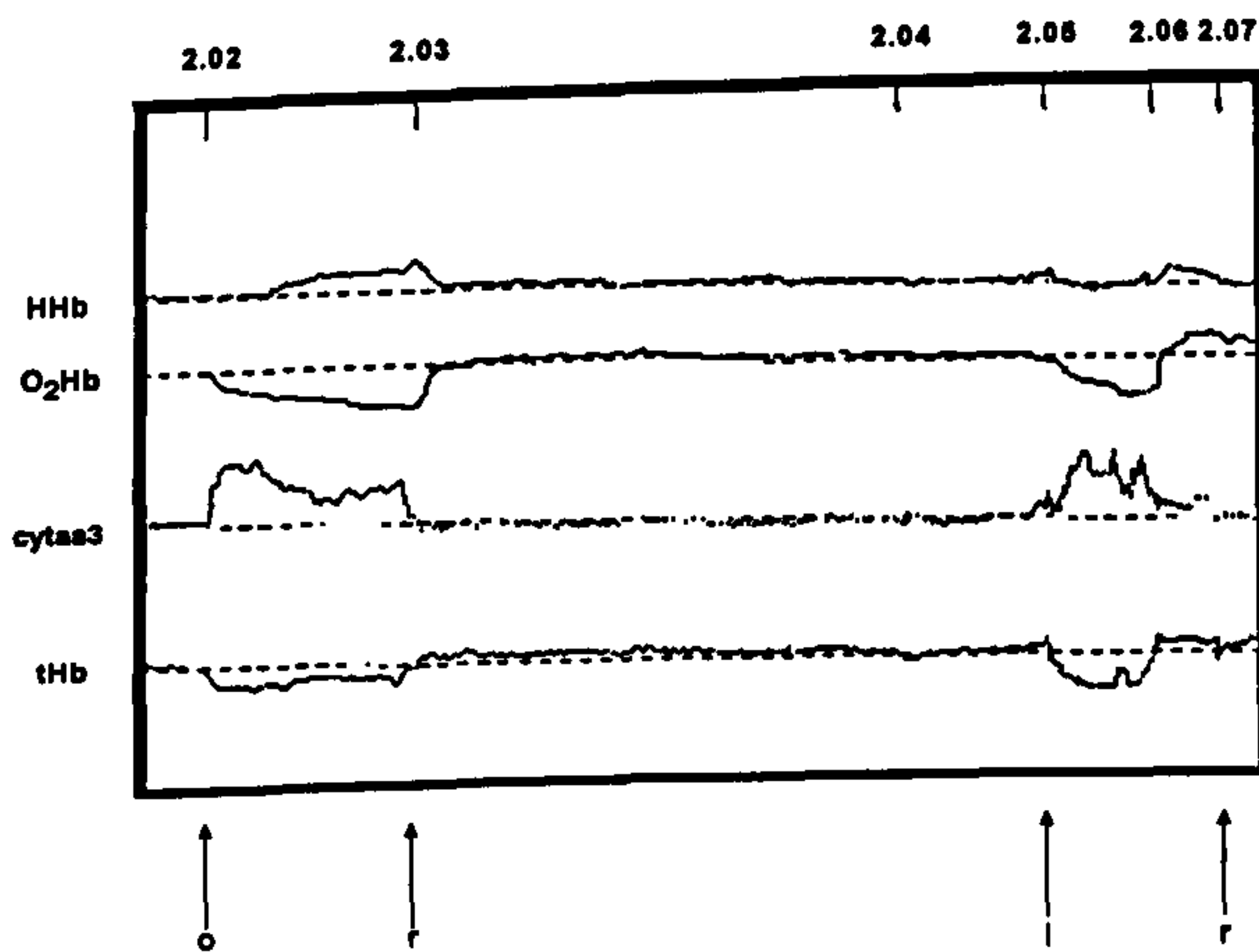
Fig 4: Near infrared spectroscopy (NIRS) traces of unsedated normal horses (i) and unsedated chronic laminitic horses (ii) during 1 min manual occlusion (o) and release (r) of digital vessels. Movement artefacts (m) are also shown.

responses were seen in the case of acute laminitis.

Discussion

Near infrared spectroscopy is a rapidly developing technique used to noninvasively monitor changes in tissue haemodynamics and oxygen utilisation in tissues otherwise inaccessible without surgery. It has been applied to various human and animal systems (Jöbbsis-Vandervliet 1987; Hampson and Piantadosi 1988; Brazy 1991; Bucher *et al.* 1993) but these are the first observations to be made on the equine hoof. It is, therefore, apt to consider the basic features of the monitoring system and to assess in general terms the suitability of the technique for the study of normal and diseased pedal haemodynamics.

The neonatal sensor has one emitter which directs the near



(r)=release of occlusion or return of limb to the ground, O₂Hb=oxyhaemoglobin, HHb=deoxyhaemoglobin, cyt aa3=cytochrome aa3, tHb=total haemoglobin.

Fig 5: Changes in near infrared spectroscopy (NIRS) traces of a sedated normal horse showing abnormal responses of cyt aa3 and tHb during 1 min manual occlusion (o) of digital vessels; and lifting (l) of the contralateral limb; (r) release of occlusion or return of hoof to ground.

infrared light into the tissue and one detector 3.5 cm from the centre of the emit window. The adult sensor consists of one emitter and an array of seven equally spaced detectors on an arc 5.5 cm from the centre of the emit window. It is not possible to describe exactly the path of the photons through the equine foot, but the basic principle of light transmission through the human skull may be used for reference. When NIR light strikes biological tissue it is randomly scattered so that it is not possible to describe with exactitude the path of any one photon between emitter and detector. A mean path length can however be determined. The mean path length is the distance travelled by the 'average' photon and is calculated from the 'Time of Flight' (TOF) - a calculation based on the measurement of the length of time it takes a photon to travel between emitter and detector. Based on TOF, it can be shown that NIR light penetrates up to 4 cm into the human head for the neonatal sensor and 5 cm for the adult sensor.

These values cannot be extrapolated directly to the equine hoof but they give an empirical idea of the tissues that are being monitored: the laminar tissues and to an extent the tissue of the coronary band. Figure 2 diagrammatically shows the arrangement of the sensors on the dorsal surface of the equine hoof. Exclusion of ambient light, and constant sensor to hoof coupling, were achieved by the application of opaque tape.

Baseline traces for the haemodynamic and tissue perfusion indices were obtained in all horses which could, for the most part be predictably modified by experimentally occluding and reperfusing the hoof. In terms of the general methodology applied, it is worthwhile to compare the present data with studies of the human forearm during compromised circulation (Hampson and Piantadosi 1988). Figures 1 and 3 should be compared. The divergence of O₂Hb and HHb upon vessel occlusion (Figure 3) indicates a reduction in arterial input of O₂Hb. The initial fall in the cyt aa3 trace (shift of more cyt aa3 into a chemically reduced state) follows the known response of this enzyme to decreased oxygen availability (Jöbsis-Vandervliet *et al.* 1987).

The divergence of O₂Hb and HHb may also indicate an increase in oxygen extraction to maintain aerobic respiration. The increase in tHb during ischaemia contradicts previous observations (Hampson and Piantadosi 1988) and suggests venous occlusion with incomplete arterial occlusion and/or collateral

input. The greater deflection of HHb than O₂Hb supports this hypothesis. Hampson and Piantadosi (1988) characterised venous occlusion as an increase in tHb and HHb with little change in O₂Hb and cyt aa3. The rise in cyt aa3 after an initial fall also supports the incomplete arrest of arterial input hypothesis.

Upon release of the occlusion, the return of O₂Hb and HHb towards baseline is consistent with reperfusion with oxygenated blood and washout of deoxygenated blood. Hampson and Piantadosi (1988) noted a phase of hyperaemia during reperfusion in which O₂Hb, tHb and cyt aa3 overshoot their baseline values. In Figure 3, O₂Hb and tHb did not behave in this fashion, but the cyt aa3 did, indicating the sensitivity of the cyt aa3 signal to changes in oxygenation. In horses, the cyt aa3 recordings following reperfusion are typical of hyperaemia seen in man (Hampson and Piantadosi 1988; Thorniley *et al.* 1988). The slower return of HHb and tHb to baseline may indicate compromised venous return from the pedal circulation.

The response to arterial occlusion in this animal (Figure 5) is particularly clear and of note is the decrease in O₂Hb preceding the increase in HHb. This is consistent with the physiological model of increased oxygen extraction beginning after, and as a response to decreased perfusion. It also perhaps indicates the great sensitivity of the Critikon Cerebral RedOx Monitor.

Increased weight bearing by the hoof (following raising of the contralateral foot; Fig 5) resulted in an increased cyt aa3 alongside a decreased O₂Hb, i.e. these 2 indices need not change in the same direction. This evidence suggests that changes in cyt aa3 reflect rather more than simple oxygen dependence.

Several interesting features are apparent when the vascular responses to manual digital arterial occlusion of normal and laminitic vessels are compared. The more rapid response of the laminitic hoof in terms of cyt aa3 may indicate a reduced oxygen store, as a result of compromised perfusion and, together with changes in haemoglobin, are consistent with decreased oxygenation of tissues. The more sluggish haemodynamic responses of the laminitic horses may be taken to indicate poor vascular tone.

The digital vessels of the acute laminitic had a 'bounding pulse' and could be palpated easily. Despite repeated manual occlusion of the vessels, no responses on the NIRS traces were seen. No responses to lifting the contralateral limb, or movement artefacts were seen prior to sedation. Together these observations are indicative of haemostasis in this acute laminitis case.

The studies with unsedated normal horses in some cases gave major 'movement artefacts'. Following the occlusion cyt aa3 fell from baseline, but O₂Hb did not change and the fall in tHb was due to a fall in HHb. This may be accounted for by the fact that in the unsedated condition full occlusion was not achieved, such that arterial collaterals sustained the O₂Hb while only venous drainage occurred.

The sedatives employed are known to have (minor) effects upon respiration and blood gases (Clarke and Paton 1988) but it is considered that the magnitudes of such actions are unlikely to be major reasons for the discrepancies.

In conclusion, NIRS applied to the equine hoof provides information on changes in basal haemodynamics and tissue oxygenation under field conditions. The responses seen in the hoof following manoeuvres to interfere with vascular perfusion are broadly similar to those of, for example, the human forearm submitted to ischaemia/reperfusion. Clear differences between laminitic and normal hooves are apparent when arterial flows are interrupted and it has also been demonstrated that oxidation of cyt aa3 is not maximal in the equine hoof, at least at rest. The method described is currently being applied to assess efficacies of various actual and putative therapies for laminitis. It is also possible that NIRS may be used a predictor of laminitis in the pre-laminitic condition.

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