Development of respiratory function in the brine shrimp *Artemia franciscana* Kellogg 1908, cultured under different oxygenation regimes.

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Thesis submitted to the University of Sheffield for the degree of Doctor of Philosophy in the Department of Animal and Plant Sciences

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Abstract

The brine shrimp *Artemia* has been a favourite subject of study for molecular biologists and is an extremely important economic resource. Despite this we know little of the physiology of brine shrimp as it relates to their natural environment. While some data are available on physiological responses to temperature and salinity, little is known of respiratory responses to hypoxia, particularly long-term and periodic hypoxia. Consequently the main aim of this thesis was to examine the development of respiratory function in the brine shrimp *Artemia franciscana* Kellogg 1908, cultured under different oxygenation regimes. Not only were developmental patterns documented but particular attention was given to possible underlying physiological mechanisms.

Culture of *A. franciscana* under chronic hypoxia (50 % normoxic saturation) resulted in accelerated development at least in the initial stages (5-9). The weight of individuals of any particular developmental stage was independent of experimental treatment, i.e. hypoxic cultured individuals did not increase developmental rate by producing smaller individuals. By the time they reached sexual maturity there was no difference in growth or development between experimental and control treatments.

Individuals cultured under chronic hypoxia contained considerably more hemoglobin than normoxic controls, with those cultured under periodic hypoxia being intermediate. This hemoglobin was present not only in the hemolymph but was also localised in many of the highly aerobic tissues (appendage muscles). There was very little difference in the pattern of culture mortality between chronic hypoxia and normoxia, with a peak in mortality during the thoracic stage of development. *Artemia franciscana* cultured under periodic hypoxia (8 h, hypoxia, 16 h normoxia) showed a response intermediate to that of chronic hypoxia and normoxia.

Culture under chronic hypoxia resulted in a shift from a mixed oviparity/ovoviviparity to oviparity alone. Although the total number of offspring produced was lower for individuals cultured under chronic hypoxia, the number of cysts produced was the same as for the control treatments.

The relationship between oxygen uptake and dry body weight in *A. franciscana* could be predicted on an allometric basis. The recorded decrease in oxygen uptake with development, between Stage 0 and Stage 3 could be explained by the fact that at this point development was accompanied by a decrease in body weight. the nauplii displayed a marked ability to regulate oxygen uptake when exposed acutely to declining oxygen tensions (critical oxygen tension or $P_c = 6.5 \pm 0.3$ kPa). This ability improved from Stage 0 - 6, with a decrease in Pc to 4.6 ± 0.3. This coincided with beginning of the thoracic

stage of development, which was characterised by the formation of gills and a functional heart. Thereafter there was little change in regulatory ability with continuing development.

Culture under conditions of periodic or chronic hypoxia had no significant effect on rates of oxygen uptake but did result in the improvement in respiratory performance which normally accompanies development being brought forward. Improved regulation under conditions of declining oxygen tension was achieved by Stage 3 and regulatory ability was more highly developed than normoxic controls. Individuals cultured under periodic hypoxia showed a response intermediate to hypoxic and normoxic individuals indicating that it was probably the duration of exposure to hypoxia that was important not the pattern. Regulation of oxygen uptake during conditions of declining oxygen tensions was compromised if the respiratory pigment hemoglobin was inactivated (using CO) in individuals cultured under hypoxic conditions but not in normoxic controls. Whether or not cysts were produced by hemoglobin-rich or hemoglobin-poor parents, made little difference to the respiratory performance of the offspring. Furthermore this respiratory performance was not compromised by CO exposure.

The aerobic capacity of the tissues of *A. franciscana* was improved by culture under chronic hypoxia. This was seen both in the dramatic increase in the total activity of cytochrome *c* oxidase in individual animals, determined enzymatically and by staining for the enzyme in thin sections, and also by the dramatic changes in mitochondria number and morphology observed, particularly for the most aerobic tissues.

It was concluded that respiratory regulation of *A. franciscana* 'improved' early in development and that this 'improvement' could be 'brought forward' by culturing individuals under hypoxic conditions. The development of respiratory regulation in normoxia cultured animals could not be attributed to the presence of hemoglobin, as could its development in hypoxia cultured animals. Furthermore, as well as improving the uptake and transport of oxygen to the tissues hypoxic cultured *A. franciscana* also 'improved' the aerobic capacity of the tissues. The fact that *A. franciscana* cultured under periodic hypoxia showed an intermediate response for most of the features studied suggests that it is the duration not the pattern of hypoxic exposure that is important in modifying respiratory regulation. So *A. franciscana* in response to hypoxic exposure, hurries through early hypoxia-sensitive life stages, but also, and at the same time, develops a respiratory physiology which is better able to cope with hypoxia as an environmental stress. So this study has demonstrated that some animals, early in development may respond to environmental stress by increasing developmental rate to bring the adult pattern of physiological regulation nearer in time and also, if possible, bring that adult pattern of regulation forward in development itself.

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Chapter 1 Introduction

1.1 Aquatic animals and environmental hypoxia

Most animals require the gas oxygen for use in respiration. Reduction in the concentration or partial pressure of oxygen (known as hypoxia) could, therefore, potentially compromise respiratory performance. With some exceptions (e.g. burrowing animals or animals living at, or visiting, great altitude (Bouverot, 1985)) environmental hypoxia is rarely a problem for animals in air (Eckert *et al.*, 1988, page 506). However, due to the fact that oxygen diffuses more slowly (x 10 000) and is less concentrated (x 30) in water than in air, those animals for whom water is their respiratory medium are more likely to experience environmental hypoxia than land-dwellers (Schmidt-Nielsen, 1990, page 14). In fact many aquatic animals frequently encounter exposure to periods of low oxygen, or hypoxia, in their natural environment (Hochachka *et al.*, 1993; Grieshaber *et al.*, 1994).

Aquatic organisms inhabiting the intertidal zone are periodically left emersed by the receding tide. While many of these animals have some ability to utilise atmospheric oxygen, they must balance hydration requirements with aerial oxygen uptake (conditions 'ideal' for oxygen uptake are also 'ideal' for water loss or desiccation) (Newell, 1979; Little & Kitching, 1996). Consequently some of these animals when emersed can become hypoxic, e.g. limpets that tightly adhere to their rocks or mussels that close their shells (de Zwaan, 1977). Even animals inhabiting intertidal rock pools can be exposed to hypoxia, as the respiration of plants and animals in a pool uncovered by the tide at night, can, within a short space of time, dramatically decrease the oxygen tension of the water in that pool (Morris & Taylor, 1983).

Similarly animals inhabiting rock or brine pools far from the sea can be exposed to periodic or sustained hypoxia, generated by the respiration of the organisms within those pools (see Section 2.3.2.3).

The formation of thermoclines in inland and coastal waters, can often result in large bodies of water being cut off from the overlying air. Consequently respiration by organisms within

those bodies of water results in the generation of hypoxic conditions, as oxygen cannot be replenished from the atmosphere or adjacent bodies of water which have a high oxygen content (Theede, 1984; Rosenberg *et al.*, 1991). This problem can often be made much worse by eutrophication (in fresh, estuarine and marine waters) (Larsson *et al.*, 1985; Rosenberg, 1985; Rosenberg & Loo, 1988). While many animal species are wiped out by such hypoxic events some are not (Theede *et al.*, 1969; Theede, 1984; Rosenberg *et al.*, 1991). The severity of the 'damage' seems to depend on the severity of the hypoxic event. However, most of our best information comes from situations where the hypoxia is so bad and so sustained that environmental oxygen is totally depleted (i.e. the environment becomes anoxic) that there are mass mortalities (e.g. Stachowitsch, 1984). We actually know very little about the effect of marginal environmental hypoxia on animal ecology and physiology.

Hypoxic conditions can also occur in river (freshwaters) or estuarine environments as a result of heightened biological oxygen demand as a result of organic pollutants being added to these water bodies (Hynes, 1960). In such cases the animals inhabiting such environments are potentially exposed to both hypoxic and pollutant stress simultaneously.

Throughout many of the world's oceans there would appear to be, approximately 0.1 - 1 km below the surface, a water layer, which although inhabited by many different animal species, is permanently hypoxic. This has been termed the oxygen minimum layer and its existence is attributed to incomplete mixing of water bodies and the respiration of its inhabitants (Sverdrup, 1938; Childress, 1975, 1995).

One further environment that is subject to hypoxic and even anoxic stress is the burrow environment (Dales, 1958; Pörtner *et al.*, 1985; Atkinson & Taylor, 1988). Many freshwater, estuarine or marine animals construct temporary or permanent burrows, and, as a result of respiration by the constructor of the burrow (e.g. fish, crustacean, bivalve, annelid worm) and the micro-organisms associated with the burrow wall (together with inadequate flushing of burrow water) can be exposed to periodic or chronic hypoxia.

So we see that under many different sets of circumstances, and in many different environments, periodic and chronic exposure to hypoxia or even anoxia can be a problem for aquatic animals.

1.2 Physiological responses to chronic hypoxia

In very few of the examples, given above, is exposure to hypoxia an acute (short duration) or a pulse ('one-off') event. The hypoxia can occur for sustained periods, such as in the case of inland lochs and seas, and more often that not the pattern of occurrence is also periodic, e.g. tide pools and brine pools where low oxygen only occurs at night. And yet nearly all of the information we possess for the effect of hypoxia of the physiology of aquatic invertebrates has been derived from studies of short term effects. In many of the major physiological textbooks responses to hypoxia are characterised using the acute respiratory response of an animal to declining oxygen tensions over a period of hours or, more exceptionally, days (Calow, 1981; Eckert *et al.*, 1988; Schmidt-Nielson, 1990; Barnes *et al.*, 1994). These animals are then divided between one of two categories (or two ends of a spectrum) using this information; those that regulate their oxygen uptake when exposed acutely to declining oxygen tensions, and those that do not (Jones, 1972; Mangum & Van Winkle, 1973; Bridges & Brand, 1980; Herreid, 1980; Schmidt-Nielson, 1990).

Even when 'long-term' studies are carried out, these only last for a few days and they often discover patterns of response that are quite different from the acute response. To take one example, the common lobster, like many other aquatic animals (including fish), when exposed to acutely declining oxygen tensions responds principally by increasing its ventilation rate (the amount of water it passes over its gills) and increases the efficiency at which oxygen is extracted from the hypoxic water. However, if the lobster is held under continuous low oxygen (hypoxic acclimation), it does not maintain the 'energetically expensive' hyperventilation that is the characteristic of acute exposure. Instead, it modifies the oxygen

transporting properties of the hemolymph, to ensure efficient oxygen uptake and delivery. In this case the acute response to hypoxia, hyperventilation is quite different from the more long term response (more efficient hemolymph oxygen binding and delivery) (see Butler *et al.*, 1978; McMahon *et al.*, 1978 for details, cf. also McMahon *et al.*, 1974; Taylor, 1976).

When animals that live in chronically hypoxic or anoxic environments have been the subject of study, it has nearly always been their ability to resort to anaerobiosis that has been examined. We actually know quite a good deal about recourse to anaerobic metabolism during exposure to anoxia, of such animals (Hochachka *et al.*, 1973, 1993; de Zwaan 1977; Hochachka, 1980; Ellington, 1983; Bryant, 1993; Grieshaber *et al.*, 1994) . However, the number of studies which have considered recourse to anaerobiosis under different degrees of 'realistic' hypoxic stress are comparatively small. They consist almost entirely of studies of tide pool species, such as prawns (Taylor & Spicer, 1986, 1988) crabs (Teal & Carey, 1967; Hill *et al.*, 1991) and echinoderms (Spicer, 1995a) and the hypoxia they are exposed to, is of comparatively short duration.

Despite the fact, that as already noted above, most aquatic hypoxia is prolonged and/or periodic in occurrence we actually know very little about the long term physiological consequences of prolonged hypoxic exposure in aquatic animals and virtually nothing of long term exposure to periodic hypoxia (for reviews of what we do know see the Introductions to chapters 3, 4 & 5). Part of the reason for this may be that it is assumed that long term exposure to hypoxia or anoxia, with very few exceptions, is lethal to animal life. This is almost certainly true in the case of anoxia (Hochachka *et al.*, 1993; Grieshaber *et al.*, 1994). But, for animals inhabiting many of the environments, described above, that are exposed to continuous or long term periodic hypoxia this is plainly not the case. This gap in our current knowledge and understanding is clearly unacceptable. Particularly as many of the biological consequences of sustained or chronic hypoxia also have economic repercussions, e.g. the effect of coastal eutrophication and hypoxia on fisheries, and the potential problems with diurnal and/or sustained hypoxia in small ponds/enclosures used for aquaculture (crustaceans and fish).

1.3 Aim of thesis

As we know so little about the effect of sustained or chronic hypoxia on the physiology of aquatic organisms, this has been the focus for my thesis. In particular I wanted to know what effect chronic hypoxia would have on the key respiratory functions of oxygen uptake, transport and tissue respiration, the rational being that if these features were compromised it would have implications for the oxygenation status of the whole animal and therefore the amount of energy currency (ATP) that could be generated to do biological work (maintenance growth and reproduction).

The specific questions I have addressed are:-

(1) How does oxygen uptake, during normoxia and during exposure to acutely declining oxygen tensions, change during development in animals cultured under periodically and chronically hypoxic conditions (Chapter 4)

(2) To what extent can any changes observed be related, or attributed, to the presence of a respiratory pigment (Chapter 4)

and

(3) What effect does culture under hypoxic conditions have on respiration at the level of the tissues (*via* an examination of mitochondria structure, density and distribution, and cytochrome *c* oxidase activity and concentration) (Chapter 5).

As a backdrop to these key questions I have also examined the effect of culture under periodic and chronic hypoxia on growth and reproduction (Chapter 3).

The animal species studied, the brine shrimp *Artemia franciscana*, was chosen because:-

(1) it inhabits brine pools that can be subject to periodic or chronic hypoxia (Section 2.3.2.3)

(2) it is readily and easily cultured in the laboratory and has a relatively short life cycle (Sections 3.1.1 and 3.2.1).

(3) it is possible to generate relatively large numbers of individuals at different developmental stages for physiological studies (Section 3.2.1).

(4) it possesses a respiratory pigment, hemoglobin, that can be 'deactivated' using carbon monoxide (Section 4.2.2)

(5) we already have some good physiological data for the adults of this species (Sections 2.3.2, 4.1.2 and 4.1.3).

(6) brine shrimp and their culture are of tremendous economic interest (Section 2.4).

The chapter that follows (Chapter 2) is given over to a brief consideration of the morphology, systematics and ecology of brine shrimp, as well as their economic importance. It acts as an introduction to the studies that follow on growth, reproduction (Chapter 3) and respiratory performance (Chapters 4 & 5).

The thesis concludes by drawing together the conclusions of each of the chapters to answer the questions, what effect does culture under periodic and chronic hypoxia have on the functional biology of brine shrimp during development and why (Chapter 6).

Chapter 2 General Biology of Brine Shrimp

2.1. Introduction

Presented in this chapter is an account of some of the basic biology of brine shrimp that is required as a basis for the experimental work that follows. First there are some brief notes on the general morphology and systematics of brine shrimp, paying particular attention to the species used in the experimental work, *Artemia franciscana*. The next section considers the basic ecology of brine shrimp, with particular emphasis being given to changes in environmental physico-chemical parameters. Finally there is an account of the commercial importance of brine shrimp which shows just how critical it is for us to understand the ecological physiology of this interesting animal.

2.2. General morphology and systematics

Brine (or fairy) shrimps are branchiopod crustaceans that belong to the order Anostraca (Kuenen, 1939; Linder, 1941) Members of this order are characterised by the possession of an elongated body, comprising of twenty or more segments (with no carapace), with at least half of those segments bearing swimming appendages, and the possession of stalked compound eyes (Figures 2.1 and 2.2) They are active swimmers, and orientated according to the light source: normally they will swim upside down although this can be reversed by illuminating the aquaria they are in from below. The limbs used for swimming are also used for suspension feeding.

Despite the fact that much effort has been expended on investigating the molecular biology, aquaculture and the physiology as it relates to cryptobiosis, of these animals (see the multivolume works edited by Persoone *et al.*, 1980 and Decleir *et al.*, 1987), the ecology of brine shrimp in nature is relatively poorly known (see Section 2.3, below).

Figure 2.1 Photograph of male and female (gravid) brine shrimp *A. franciscana*. a = antenna; ab = abdomen; cc = caudal cirrus; ce = compound eye; th = thoracic appendages; tho = thorax. Scale is indicated on the photograph.

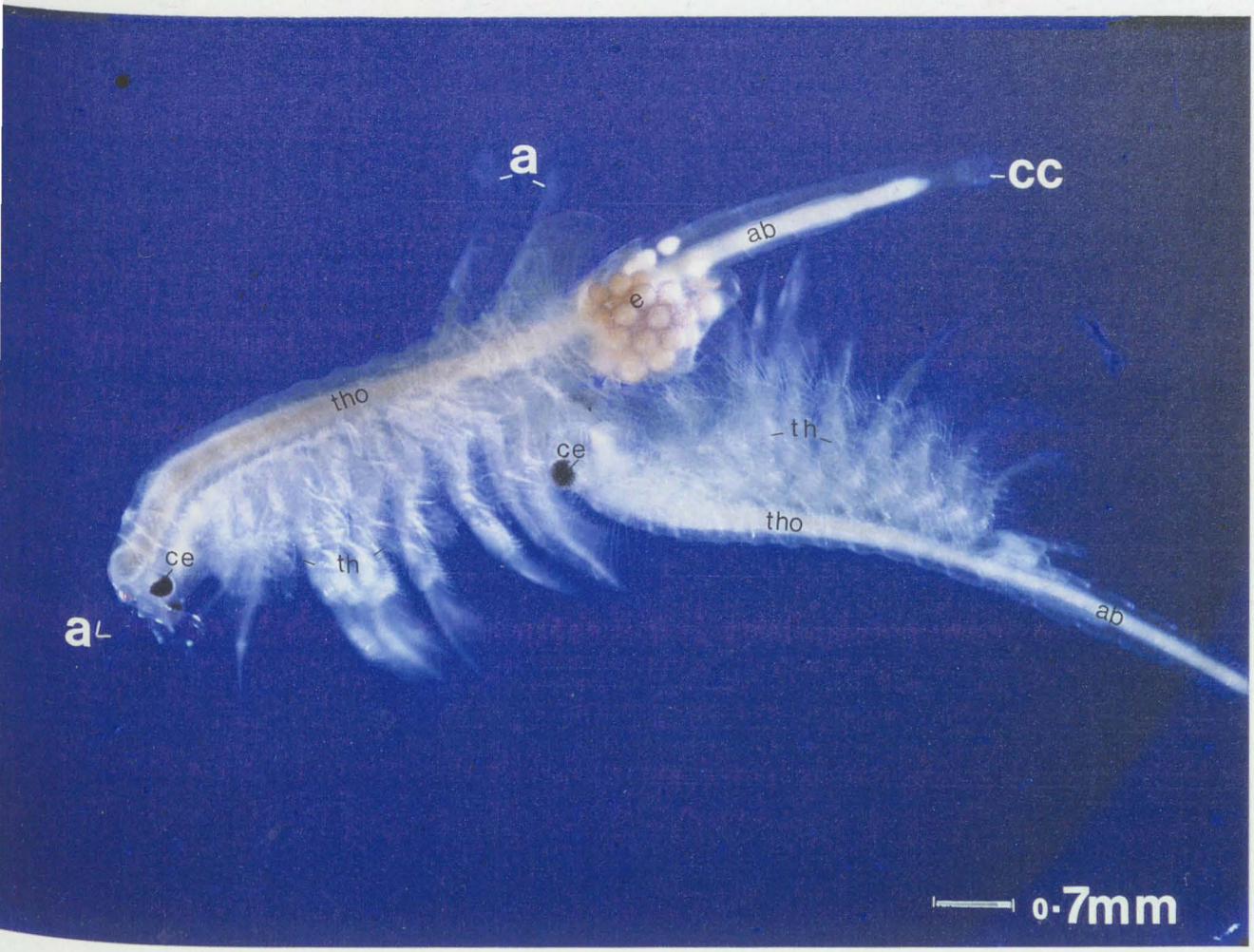
a
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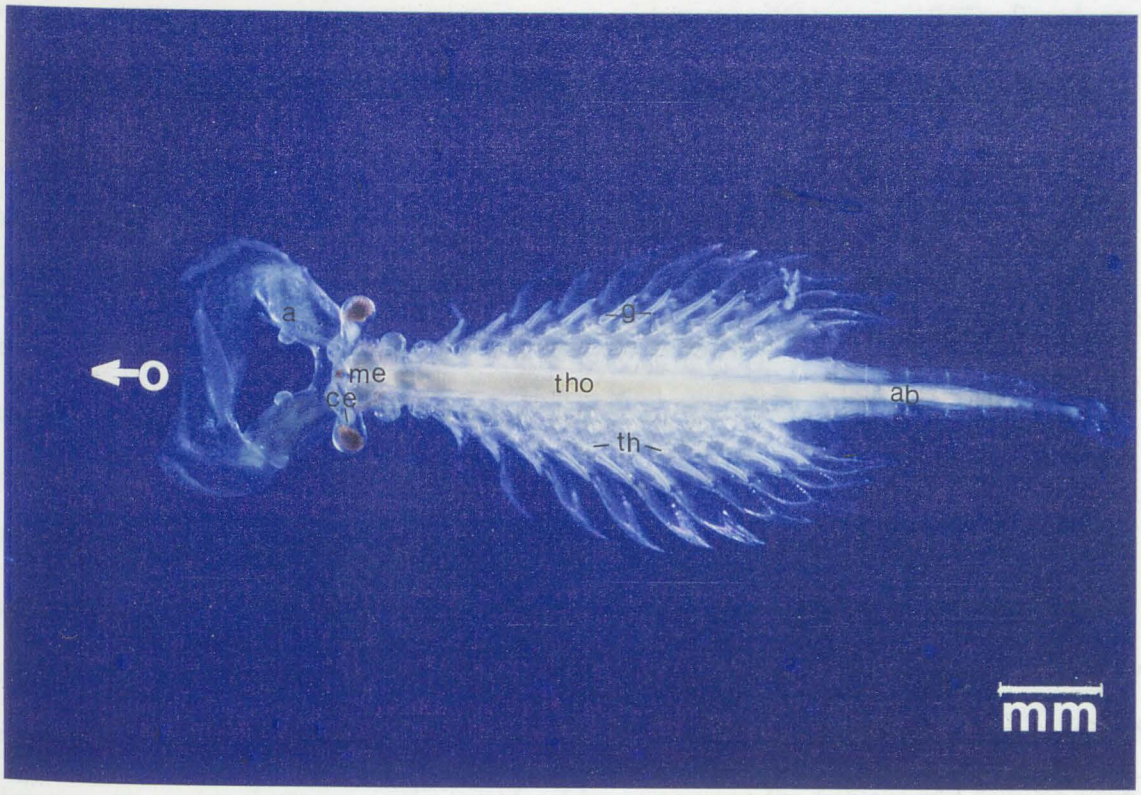
Figure 2.2 Photographs of male (A) and female (B) brine shrimp *A. franciscana* illustrating the principle morphological features of the adult. a = antenna; ab = abdomen; ce = compound eye; e = egg mass; g = gills; me = medial (nauplius) eye; th = thoracic appendages; tho = thorax. Scale is indicated on the photograph.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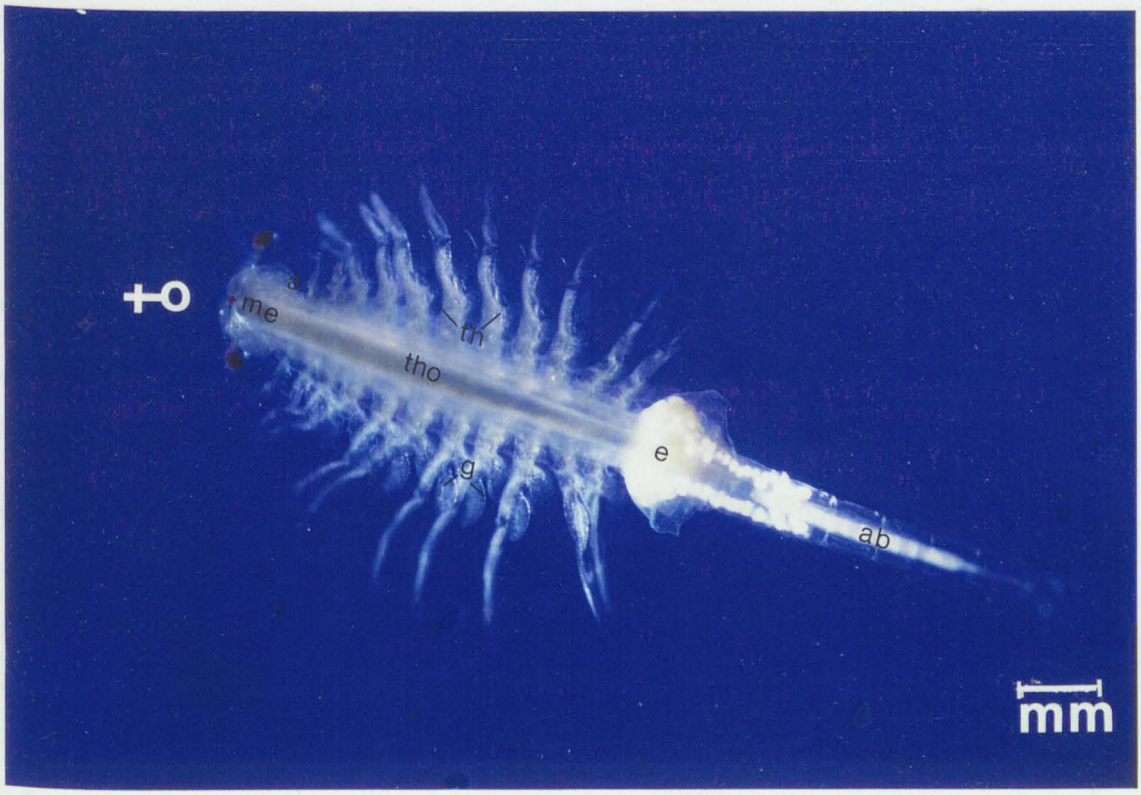
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The first scientific description of *Artemia* was given by Schlosser (1756), based on animals collected from salt pans at Lymington, in the south of England. The genus name of *Artemia* was given by Lamarck (1818) and a short time after many 'new' species were being assigned to this genus. However, even early on Schmankewitsch (1875, 1877) brought to the attention of the scientific world, the fact that many, if not all, of the characters used in the systematics of this group were variable under the influence of the external medium. Consequently he postulated, rightly, that many of the species described would have to be taken together as varieties of one species. Bateson (1894) disagreed with this view and suggested that many of the key differences between *Artemia* species could not be attributed to salinity. Slightly later Daday (1910) brought all *Artemia* species, found in salt waters, together into one species but designated four varieties. The first cytological studies of *Artemia* were carried out by Artom (1906). He tried to useof microscopy to solve the 'systematics problem' by comparing the nuclear and cellular sizes of different tissues in *Artemia* collected from widely different localities, although it was 15 years before he succeeded in bringing individuals from 20 localities together into two categories (Artom, 1920).

Kellogg (1906) described a form as *Artemia franciscana* from the salt works at Redwood City, on San Francisco Bay, California although he does not seem to have noticed that the description of abdomen that he gave, while it agrees with what is found in European *Artemia*, is different from *A. franciscana*, from the same location, as described by Martin & Wilbur (1921). (It is this species that has been chosen for investigation in the present study). In 1933 Bond (1933) found that Californian *Artemia* strains differed from their European counterparts in their reaction to widely different experimental media; the Californian *Artemia* did not display any differences in abdomen length to the rest of the body when exposed to high concentration medium as was found for the European species and so Bond called his animals *Artemia franciscana* Kellogg.

This species not only differed in its reaction to the external medium but also differed in cytology (cellular and nuclear size).

So *Artemia* biotopes are geography-isolated from one another. The study of the strains from a genetic point resulted in the genus *Artemia* being split into several sibling species which are isolated from the reproductive point of view (Barigozzi & Tosi, 1959; Clark & Bowen, 1976). Presently five species are referred to the genus *Artemia*; *Artemia monica*, *A. tunisiana* , *A. urmiana*, *A. persimilis* and *A. franciscana*. However, the systematics of *Artemia* are still far from being resolved (Editorial note on the taxonomy of *Artemia* in, Persoone *et al.*, 1980) and are likely to remain so until we understand more about speciation in this genus and in brine shrimp in particular. Recent studies, examining speciation in the genus *Artemia* using mitochondrial DNA analysis look as if they may prove particularly fruitful in this respect (Luz-Perez *et al.*, 1994).

2.3 Ecology

2.3.1 Distribution and habitat characteristics

All extant species normally inhabit isolated inland saline ponds, lakes and coastal salterns, and typically ephemeral habitats (Persoone & Sorgeloos, 1980; Browne & Macdonald, 1982; Vanhaecke *et al.*, 1987). Despite the fact that brine shrimp were recorded to have a world-wide distribution at the turn of the century, i.e. at least eighty salt water habitats in a number of different countries on each of the five continents (Abonyi, 1915; Artom, 1922; Stella, 1933; Mathias, 1937), many of the ancient salt lakes or pans where they have occurred have been either destroyed or abandoned. On this basis Persoone & Sorgeloos (1980) reported that brine shrimp are no longer found in the United Kingdom, Germany or Yugoslavia. Yet the comparatively recent inventory by these two authors (Persoone & Sorgeloos, 1980) has presented a very impressive list of

current '*Artemia*' sites, with the written proviso that for most countries systematic survey work looking for brine shrimp has not been undertaken. Consequently the list of 'brine shrimp sites' is growing all the time (e.g. Herbert & Hann, 1986 (Canadian Arctic); Pereira & Belk, 1987 (Central and South America). It should not be too surprising for us to find, therefore, that since 1980 brine shrimp have been 'rediscovered' in the UK (Bratton & Fryer, 1990), Germany (Theunert *et al.*, 1987) and Yugoslavia (Petrov & Marincek, 1991).

Typically the physico-chemical conditions of waters where brine shrimp occur are extreme, i.e. they contain a very high salt content (often significantly different in its composition from sea water, e.g. they can be rich in the ion potassium (Cole & Brown, 1967), sulphates (Hammer *et al.*, 1975) and carbonates (Mason, 1967). So much so that only a very few highly specialised species, e.g. bacteria, alga and brine shrimps, can survive in such environments. Such highly specialised species can often occur in huge numbers (e.g. 400 individuals.l^{-1} in Mono Lake, California (Lenz, 1980)). At certain times of the year, large quantities of tiny brineshrimp cysts can be found either floating on the water surface or driven ashore by waves and/or wind (see Section 3.1.1 and 3.1.2 for accounts of reproduction and development of brine shrimp). Even although there are no, or few, aquatic predators, this 'food source' is exploited by birds such as the flamingo and the shelduck, which can feed on whole animals or on cysts, and the latter in particular when they are concentrated as 'scum' by the wind (Savage, 1967; MacDonald, 1980; Verkuil *et al.*, 1993).

There are some excellent reviews of some general aspects of the ecology and biogeography of *Artemia* (that have already been referred to above) (Persoone & Sorgeloos, 1980; Browne & McDonald, 1982; Vanhaecke *et al.*, 1987). One of the main conclusions they all come to is that although we have a remarkably detailed database for the biology of *Artemia* we know very little about the animal in its natural environment, i.e.

its ecology and its ecological physiology are actually poorly studied. Persoone & Sorgeloos, (1980) point out that of the 2700 papers published on *Artemia* by 1980 , less than 50 of them are 'strictly ecologically orientated'.

While salinity is obviously a key ecological factor, so too are temperature and oxygen. We will now briefly consider what we know of natural fluctuations in each these ecological parameters and the general responses of brine shrimp to them.

2.3.2 Ecological physicochemical characteristics

2.3.2.1 Salinity and ionic content

Brine shrimp typically inhabit ephemeral pools that, by their very nature, show tremendous fluctuations in salinity. Such pools seasonally are subject to cycles of drying out and refilling by means of rainfall or freshwater run-off. Loss of water from bodies of saline water will result in a concentration effect, where in some cases, the end point is crystallising seawater brine (> 2 000 $mmol.l^{-1}$ NaCl).

Both larval and adult *Artemia* are very effective hypoosmotic regulators (i.e. they can maintain their body fluids at a much lower osmotic concentration than is found in the surrounding water) over an extremely wide range of salt concentrations, e.g. from 0 - 2000 $mmol.l^{-1}$ NaCl for animals from Great Salt Lake, Utah (Croghan, 1957; Conte *et al.*, 1973). This salt tolerance can be reduced, however, by exposure to other stresses. For example, *Artemia* from the more alkaline Mono Lake in California could not survive exposure to the greatest concentration tolerated by the Great Salt Lake population (Herbst & Dana, 1980). The natural range of salt concentrations of waters that contain *Artemia*, as reviewed by Cole & Brown (1968) is from 61 - 258 ‰, with one exceptionally low reading of 31.3 ‰ for a salt lake in Iran. Even for individual lakes or salt pans, the seasonal or annual range can be substantial, e.g. from 80 - 105 ‰ in Alviso Salt Ponds in

California (Carpelan, 1957) and exceptionally 150 to 207 ‰ in one particular large Australian salt lake that is used commercially for salt extraction (Geddes, 1980). Populations of *Artemia* in S. India occur in coastal inland waters where S = 151 - 105 ‰, but when the monsoons come and the salt concentration is reduced to 15.9 ‰, the populations disappear (Ramamoorthi & Thangaraj, 1980) although temperature also falls slightly (see Section 2.3.2.2 below).

Ecologists consider that the ability to inhabit highly saline waters is what enables brine shrimp to avoid predation. Unlike some other anostracans such as *Daphnia*, brine shrimp do not possess any structural defence mechanisms against predation (Persoone & Sorgeloos, 1980). The lowest salinity at which brine shrimparefound in nature varies from place to place and it appears to be determined (or at least related) to the upper salinity tolerance level of the local predators (Persoone & Sorgeloos, 1980).

Environmental salinity levels are critical with regards to the hatching of brine shrimp eggs or cysts, as, for many populations there is a lower threshold level below which embryonic development will not proceed (Royan *et al.*, 1978).

2.3.2.2 Temperature

In a receding lake or salt pan, concentration of salts may also be accompanied by increases in temperature fluctuations. It has been suggested that salinity and temperature, acting synergistically, lowered the lethal tolerance of Australian *Parartemia* (Geddes, 1975) and Californian *Artemia* (Herbst & Dana, 1977 quoted in Herbst & Dana, 1980) (cf. also Vanhaeke *et al.*, 1984). Certainly sudden mortality in natural Australian populations of brine shrimp has coincided with concomitant decreases in both salinity and temperature (Geddes, 1980). Interestingly brine shrimp seem able to tolerate very low temperatures as long as the salt content is high, e.g. populations from the Great Salt Lake, Utah, survived temperatures as low as 10°C but only at a salt concentration of 259 ‰

(Stephens & Gillespie, 1976). It is thought that *Artemia* 'does best' between 20 and 30°C (Carpelan, 1957, Von Hentig, 1971, Sorgeloos *et al.*, 1976, Geddes, 1980). Above this higher temperature, in conjunction with concentration of the brine, there is normally substantial brine shrimp mortality, e.g. in Boca Chica salt lake, Venezuela substantial mortality was recorded at a temperature of 36°C and a salt concentration of 320 ‰ (Scelzo & Voglar, 1980).

It is interesting that the thermal tolerance of brine shrimp from the Great Salt Lake does not encompass the seasonal range in temperature from 0 to 40°C, meaning that although there may be physiological mechanisms for dealing with temperature fluctuations, the range is such that mortalities may be inevitable.

2.3.2.3 Oxygen

Brine shrimp could potentially find themselves exposed to low oxygen as a result of two factors, one physico-chemical, the other biological. As either the concentration or the temperature of a salt solution increases the solubility of oxygen in that solution decreases (Herbst & Dana, 1980). Certainly the total oxygen content of many saline lakes is as much as a third or quarter (see Sclezo & Voglar, 1980; Ramamoorthi & Thangaraj, 1980 for examples) that one might expect to find in well -oxygenated coastal waters, just as a result of these physico-chemical differences (Herbst & Dana, 1980). This means that potentially brine shrimp can, and perhaps do, carry out their whole life cycle in permanently hypoxic waters. It should be noted, however, that this may be relatively unimportant in very large bodies of water, as the partial pressure of oxygen in the water (which is the driving force of gas exchange) can remain high. However, the biological oxygen demand of brine shrimp and algae (during the night) could result not just in pools, but even lakes becoming periodically hypoxic. This is a feature that is found in intertidal rock pools (Morris & Taylor, 1983), in enclosed seas (Larsson *et al.*, 1985), marine basins (Rhoads & Morse,

1971) and even in eutrophic coastal waters (Rosenberg, 1985). While many studies mention that hypoxia is a stress that brine shrimp face, most of the studies that have been carried out have only measured total oxygen, and not the partial pressure, e.g. Sclezo & Voglar (1980) and Persoone & Sorgeloos (1980).

In conclusion what is clear is that it is extremely likely that brine shrimp are likely to encounter either chronic and/or periodic hypoxia in their natural environment, although currently it is difficult to put any values on diurnal or seasonal changes in oxygen partial pressures. Such problems with oxygen are likely to become even more acute when dealing with small 'rock pool' size environments.

2.4 Economic importance

2.4.1 Introduction

There are three main areas in which brine shrimp have special economic importance. The principle one is as a food source for aquaculture, but they are also used for educational purposes and have recently become important in ecotoxicological testing.

2.4.2 Aquaculture

The first significant recognition of the economic importance of live *Artemia* took place when Seale (1933) in the USA and Rollefsen (1939) in Norway discovered that not only did brine shrimp play a predominant role in the plankton of many saline lakes but the nauplius larvae of *Artemia* constituted an excellent food source for newborn fish larvae. Until then using *Artemia* was restricted to use of dry cysts.

Nowadays the newly hatched nauplii of the brine shrimp, due to their high energetic (yolk rich) value, are used in many locations through-out the world as food for marine larval fishes and invertebrates (e.g. coelenterates, polychaetes, squids, marine and freshwater crustaceans), reared for mariculture (Sorgeloos & Persoone, 1975; Manzi & Maddox, 1980; Brendonck *et al.*, 1990; Mura, 1992). The choice of *Artemia* nauplii is not just because of their high energy content but is also due to fact that *Artemia* can be obtained from an inert source (dried eggs) which requires only approximately 24 hours immersion in illuminated sea water to produce nauplii (Sorgeloos, 1972). Kinne (1977) in his treatise on cultivation of marine organisms has found that more than 85% of the marine animals cultivated thus have been offered *Artemia* as food source either together with other food or more often, as a sole diet. Rosenthal (1977) has even suggested that, taken together with all of the advantages outlined above, the fact that brine shrimp can be reared on relatively inexpensive inert diets places them in the top place in rank of importance in the field of aquaculture. Sorgeloos (1980) concurs with this suggestion when he says that *Artemia* should no longer considered as a luxury food in aquaculture but rather as a cheap and high quality source of animal protein.

There is increasing evidence that various geographical strains of brine shrimp differ in their food value due to nutritional composition and /or contaminants and that even individual strains can vary seasonally or even from year to year (Bookhout & Costlow, 1970; Wickins, 1972; Provenzano & Goy, 1976; Johns *et al.*, 1980). As well as this it is also known that the energy content differs between different developmental stages (Morris, 1956). This leads to a key question relevant to the economic importance of this animal - how we can increase the *Artemia* quality but without increasing culture demands? Several techniques have been described for high density culturing of healthy brine shrimp using a batch system (Dohse, 1971; Sorgeloos, 1973, 1980). Sorgeloos (1979, see also Sorgeloose & Persoone, 1975) in particular emphasised the effect of

various biotic parameters on the hatching and suggested the following general features of brine shrimp culture; 1) The *Artemia* cyst should be hatched under strictly controlled conditions (to prevent the wastage of a precious live food) 2) nauplii should be fed to aquaculture species (e.g. fish and crustaceans) as soon as possible after hatching (to ensure these animals get a high energetic value food), 3) The effects of parasites should be minimised as well as contamination by bacteria (which should be treated using antibiotics) and fungi (Coleman *et al.*,1980; David *et al.*, 1980) . 4) The mode of reproduction should be carefully controlled (i.e. it should be ovoviviparity), modifying external 'stress' factors, e.g. temperature, salinity and oxygen. However it is clear that while we are relatively advanced in our understanding of salinity and temperature effects, this is not the case with oxygen.

2.4.3 Education, research and ecotoxicology

As *Artemia* can be cultured easily in the laboratory and is readily available all year around, it has been widely used in education and research. For example Koshida & Hiroki (1980) have developed a large number of practical exercises in which the brine shrimp is utilised as a biomaterial for biology education in various disciplines including osmoregulatory physiology , radiobiology and enzyme biochemistry. This is not new.

For example, in a practical booklet entitled '*Laboratory Exercises in Invertebrate Physiology*' (Welsh *et al.*, 1968) brine shrimp are recommended as useful study material on a number of occasions, namely for examination of feeding using setae (page 14), hemoglobin synthesis (page 40) and osmotic and ionic regulation (page 65). From time to time, in various popular magazines brine shrimp cysts are advertised for sale as educational toys, and marketed as 'living mermaids'.

The fact that brine shrimp are so easy to obtain has made them a favourite 'laboratory animal' on which to base experimental studies: the majority of theses studies have been concerned with molecular biology or physiological questions (Truchot, 1993a on their use in studying metabolic and transport physiology; Reznick, 1993 on their use in addressing the molecular biology of ageing; Valverde *et al.*, 1994 where these workers have determined the complete mitochondrial DNA sequence for *Artemia franciscana*; for other examples peruse the cited literature in the reference list and the multivolumed works referred to in Section 2.1 above).

Artemia is now used routinely in ecotoxicological testing. It has been used for a bioassay and 24 hr LD_{50}'s determined for several heavy metals in Instant Ocean by Trieff (1980) who concluded that although *Artemia* is more resistant to heavy metals than say fish , the *Artemia* bioassay method is still one of the most promising and simple techniques for determining the toxicity of environmental pollutants. Consequently much attention has been devoted to determining the sensitivity of different developmental stages to pollutants as well as the influence of environmental factors on pollution-related mortality (e.g. Centero *et al.*, 1993a,b. Brine shrimp have recently been used, as part of a battery of ecotoxicological tests, to predict human acute toxicity to the first 50 MEIC chemicals (chemicals that are known to be harmful and are released into the environment) (Calleja *et al.*, 1994).Testing of effluents that are to be dumped at sea using mortality and reproduction of *Artemia* as benchmarks is central to the legal requirements of the Oslo Convention on Sea Dumping (Hayward, 1984).

2.5 Concluding remarks

We can see from what has gone before that, both in terms of aquaculture and basic research, we have benefited much from this strange but interesting animal, the brine shrimp. And yet despite all of the, at times detailed, attention paid to this animal we still know very little about its basic ecology and how the natural environment affects the its biology - what makes this surprising is that much of this information is required for the successful and efficient culture of brine shrimp (Persoone & Sorgeloos, 1980).

While we are beginning to find out about the effects of salinity and temperature, singly or in combination, on brineshrimp function (see Sections 2.3.2.1 and 2.3.2.2), we still know very little about the effect of oxygen. As highlighted above oxygen lack, or hypoxia, is likely to be a periodic problem in water bodies (natural or artificial) that support brine shrimp and so it is vital that we know how this hypoxia, chronic and periodic will affect the culture and physiological function of brine shrimp. For this reason the rest of this thesis is dedicated to examining aspects of just these features.

Chapter 3 Effect of Hypoxia on Growth, Development and Reproduction

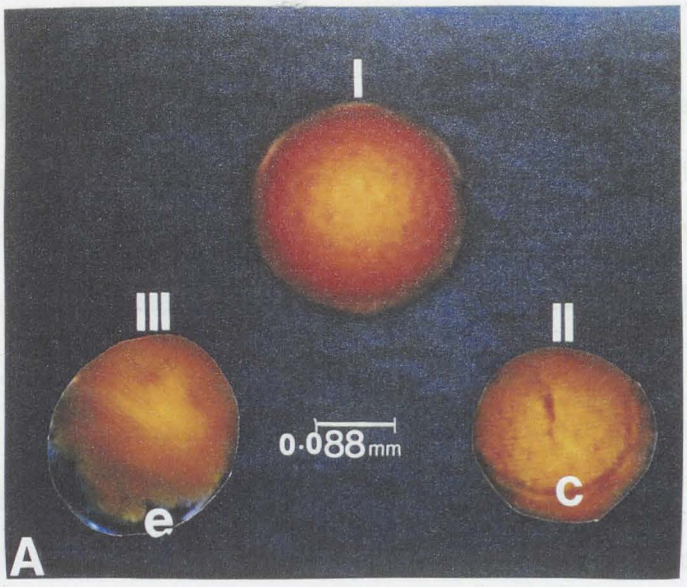
3.1 Introduction

3.1.1 Hatching, growth and development of brine shrimp

Technically speaking, the production of newly-hatched *Artemia* nauplii by incubation of cysts in sea water under continuous illumination, is a relatively simple and straight-forward process (Sorgeloos, 1980). Sorgeloos (1980) has given a good account of the process and also the effect of a number of physico-chemical factors (namely temperature, salinity, pH, oxygen, cyst density, and illumination) in the culture process (cf. Section 2.4.2). The technical details of the hatching and culture technique used in my studies are given below (Section 3.2.1).

The pattern of growth and development is very similar for most of the brine shrimp species studied. The brief account given here is for *Artemia franciscana* and so will be based primarily on my own personal observations, recorded both in the text and in the accompanying figures, supplemented where appropriate, by the works of Heath (1924), Anderson (1967), Benesch (1969) and Criel (1991a,b).

A few hours after cysts are rehydrated, the outer membranes break open and the embryo emerges. Each embryo is surrounded by a thin membrane (termed the hatching membrane) and the only obvious features are the progressive beating of the differentiating antennae (soon to be used in swimming) and the presence of a nauplius eye (Figure 3.1). A short time later the hatching membrane is ruptured and the nauplius becomes free swimming. Most of the cells of the newly emerged nauplius are packed with yolk, giving the overall body an orangish-brown colour (Figure 3.5A). Within a day or two, the nauplius starts to grow and differentiate, first thoracic, and then abdominal segments. The gut, which is blocked until now, becomes functional and food particles are filtered from the water column by the setae on the swimming appendages. It is about this time that the yolk is exhausted and the whole body becomes more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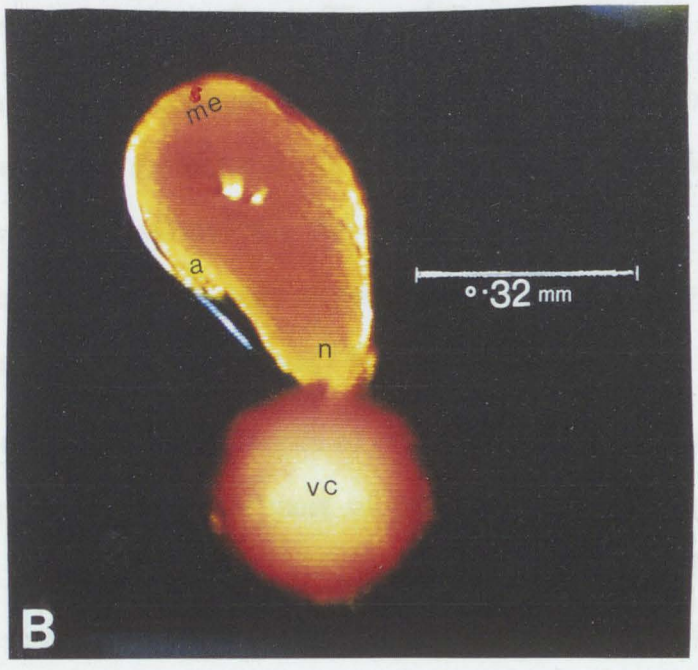
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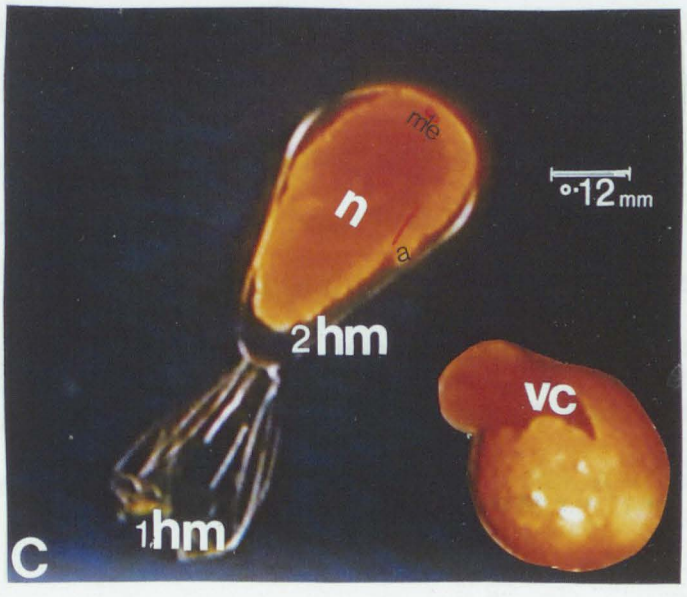
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Figure 3.1 Cyst and cyst hatching in *A. franciscana*.

A) Activation of dormant cyst, I dormant cyst immediately after hydration-related activation, II & III cyst 16 - 18 h after activation (T = 28°C, S = 35 ‰), note the cyst is beginning to 'crack open' (c) and the embryo (e) is just visible through this opening.

B) Newly-emerged nauplii (n), within second hatching membrane and still attached to the vacated cyst (vc). me = medial (nauplius) eye; a = antenna.

C) Nauplii, 2 h after emergence (n), still within second hatching membrane (hm) but completely separate from the vacated cyst (vc). Note the first hatching membrane (1hm) that in B) was within the vacated cyst is still attached. a = antenna.

Scale is indicated on each photograph.

or less transparent (Figure 3.5B&C). Clearly visible at this time is the larval salt gland on the head which accomplishes the processes of osmotic and ionic regulation until the gills form on the thoracopods (Figure 3.2). With the addition of each thoracic segment, there is the initial budding of paired appendages. These will eventually become the thoracopods, which will be used for both swimming, food gathering and gas exchange (Figure 3.2). The heart also forms and begins to beat early in this thoracic stage and lateral complex eyes begin to form. In the abdominal stage of development individuals begin to become sexually mature. It is around this time that the antennae loose their locomotory function (which is taken over by the thoracopods) and undergo sexual differentiation; in males they develop into grasping appendages, while they degenerate in the female (Figure 3.3).

Unfortunately such a qualitative account, while it gives a general idea of the developmental changes that take place during the ontogeny of brine shrimp is not sufficient for use in experimental studies where we would wish for more quantitative ways of categorising development. Consequently there have been a number of different (and often conflicting) attempts at how to determine developmental stage in these animals.

For example Heath (1924) described the rate of growth of *Artemia salina* and included in his paper measurements and drawings of most of the developmental stages he assigned. However, his staging is unsatisfactory as it is difficult to use; one cannot easily assign what is observed in life, to his stages with confidence as each of his stages is 'too wide' and covers quite marked changes in development. The next scheme proposed was that of Weisz (1946) in which developmental stage was quantitatively assessed by the number of body segments present. He also gave a detailed account of the changes in structure and internal organs of *Artemia*. There was a more detailed study of early larval development by Anderson (1967), who examined more closely the early initial 'Stage I' of Heath (1924) and differentiated that stage into a number of different and more helpful categories. This was followed by the study of Benesch (1969) who described the complete development from the egg to the adult. Such an attempt was also made by Criel (1991b) but the scheme she devised is probably far too complicated and cumbersome for normal use. Part of this is due to the fact that it is very

Figure 3.2 Scanning electron micrograph, dorsal view of free swimming nauplii [Weisz's (1946), Stage 9] towards the end of thoracic development (about 8 d after emergence). The salt larval gland (sg) is very conspicuous. Gills (g) are beginning to differentiate on the first three thoracic appendages (th). The segmented tubular heart (h) is clearly visible immediately beneath the dorsal surface (magnification x 160).

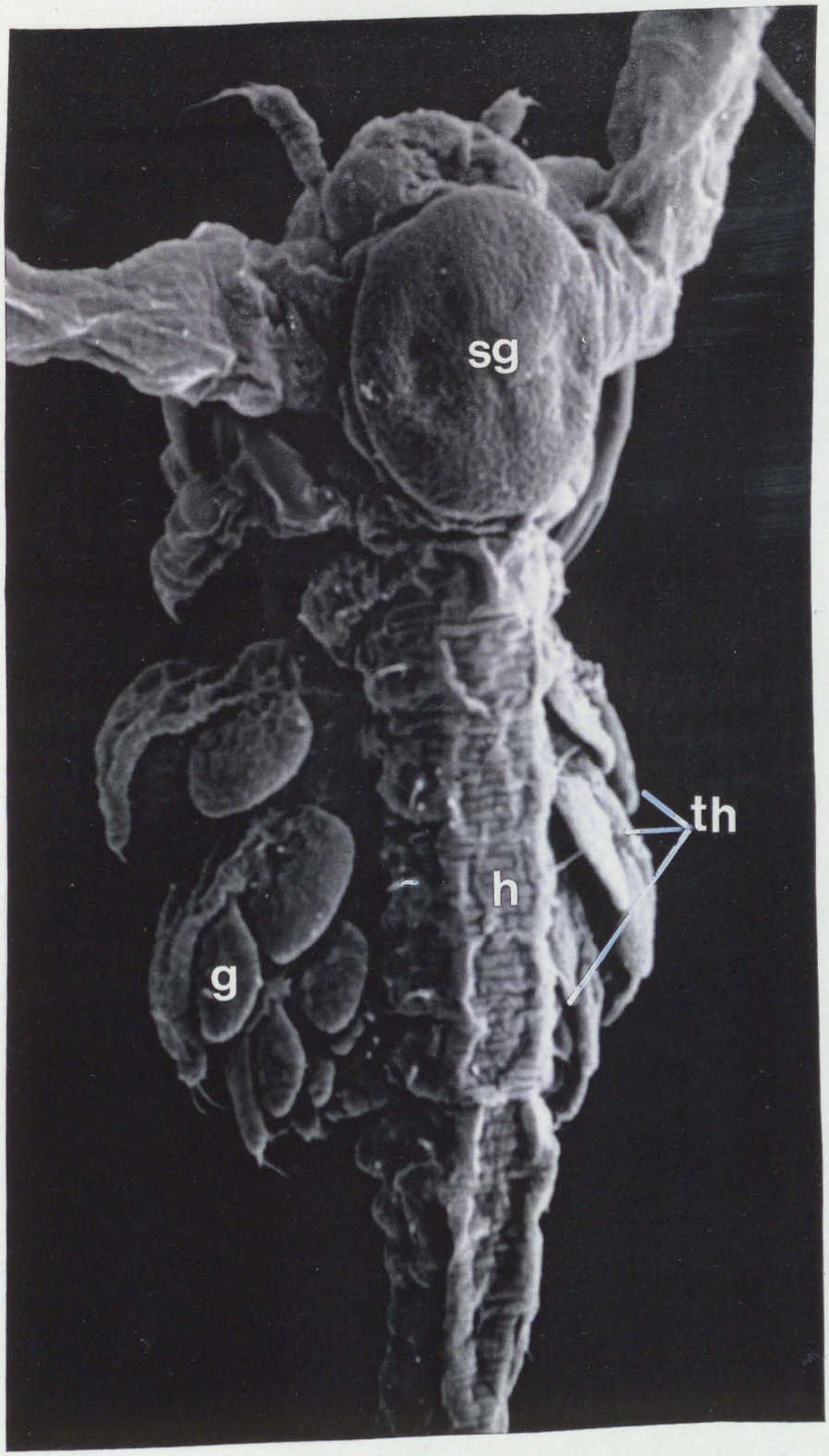
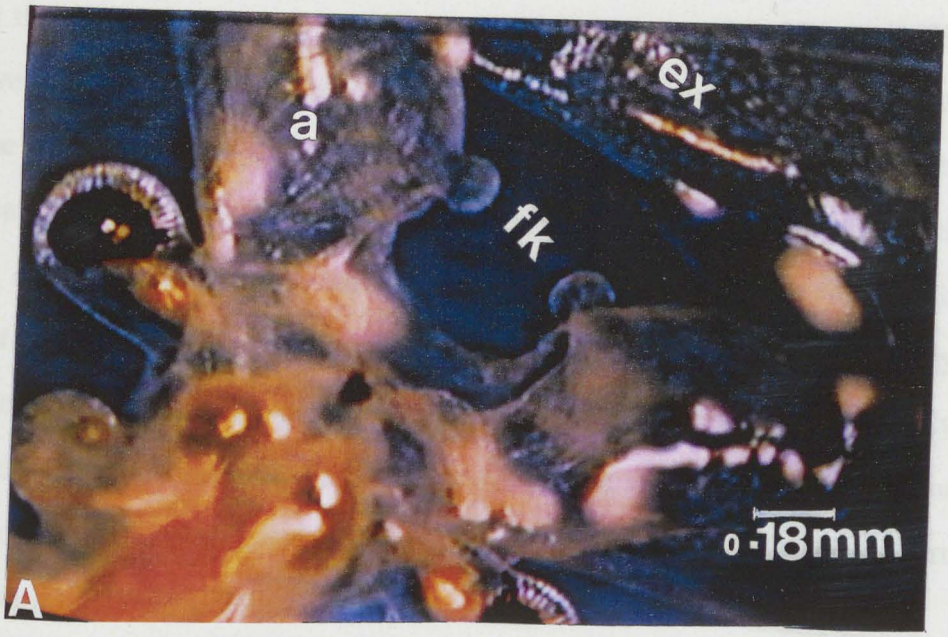

sg
th
h
g

Figure 3.3 Sexual differentiation in brine shrimp

A. The head of adult male *A. franciscana* showing the positions of the frontal knob (fk) located on the antenna (a) . The expodite (ex) is curled along the lateral edge.

B. The head of adult female *A. franciscana* showing the relatively simple antenna (a) compared with the male. al = antennule; ce = compound eye; me = medial eye. Scale is indicated on the photographs.


a
ex
fk
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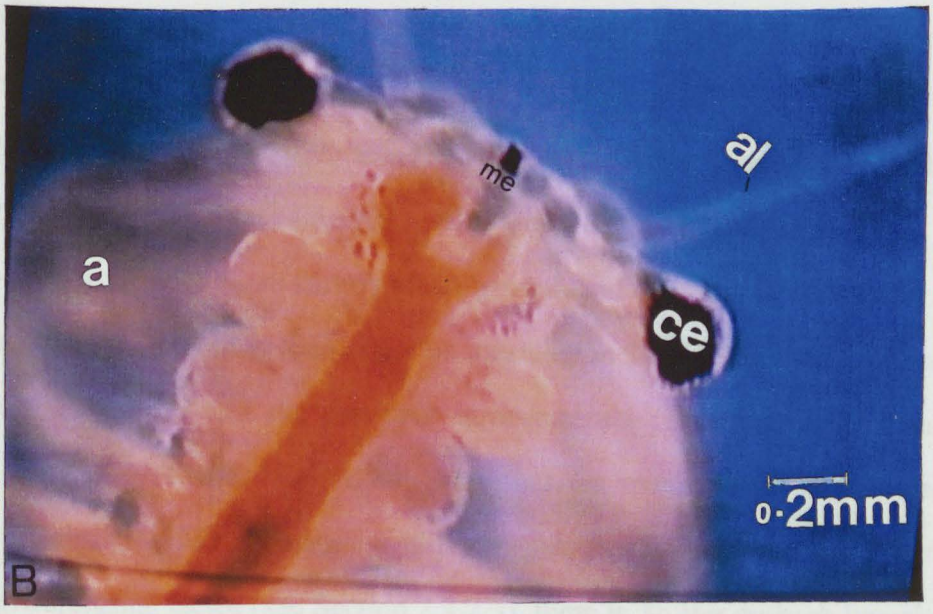
A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difficult to determine instars (the number would appear to be variable even within the same species) for these animals. Also we should mention the precise study of Olson (1979), which while it gave detailed comments concerning the time of development from hydration of the cysts and the larvae, neglected the later stages and so was not much of an improvement on the work of Heath (1924) and Anderson (1967). Blake (1979) made measurements of the width and length of the body, antenna and antennules for the first eight 'instars' of larval development but again his work is open to the same criticism as Olson's (1979) study. By far the easiest scheme to use, in my opinion, is that of Weisz (1946, 1947) - it is relatively easy to determine the developmental stage and each stage can be assigned a meaningful numerical value. Therefore in the experimental chapters that follow the Stages or staging referred to is that put forward by Weisz (1946, 1947).

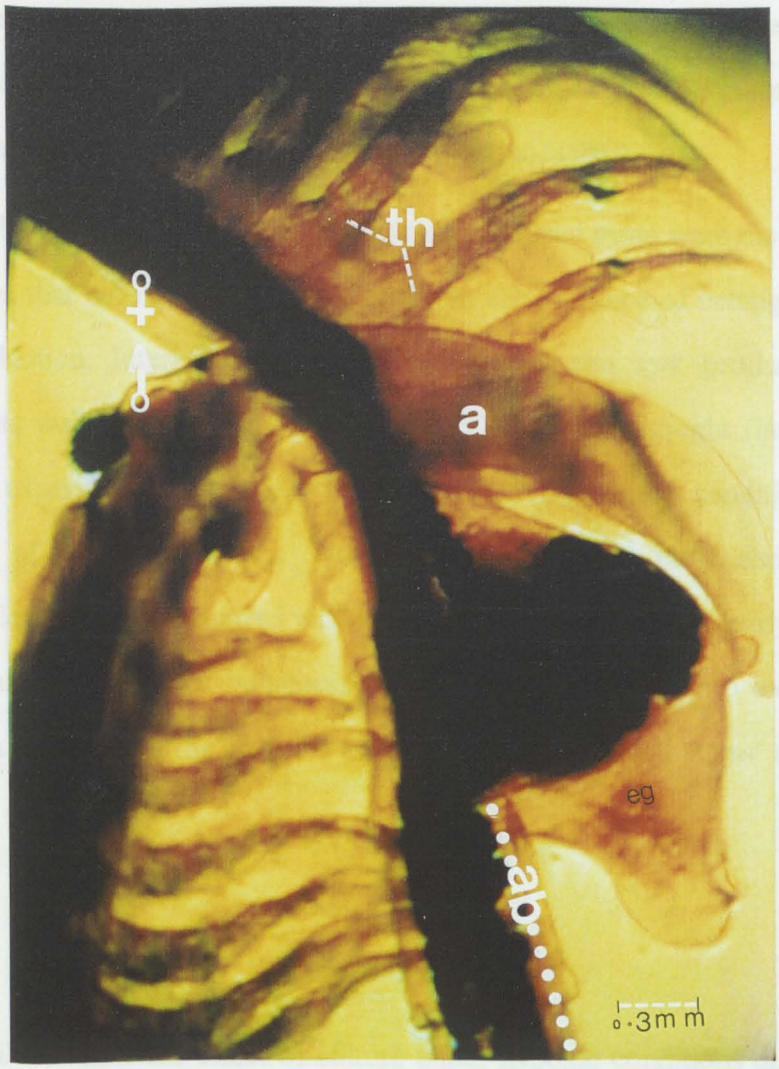
There are a number of accounts of the effect of intrinsic and extrinsic factors on growth and development on brine shrimp. The effect of food quality and quantity has been studied in some detail (Gibor, 1956, 1957; Mason, 1963; Reeve, 1963, D'Agostino & Provasoli, 1968; Dwivedi *et al.*, 1980; De Walsche *et al.*, 1991; Mitchell, 1991; Rosowski & Efting, 1992; Dierckens *et al.*, 1995). In general, and not too surprisingly, both growth and development were retarded if either food quality or quantity was reduced. Also studied has been the effect of environmental factors, such as acidity (Doyle & McMahon, 1995), temperature (Anderson & Hsu, 1990), salinity (Heath, 1924; Bond, 1932, 1933; Weisz, 1946; Gilchrist, 1959) both individually and in combination (Gilchrist, 1960; Von Hentig, 1971; Sorgeloos & Persoone, 1975). The pattern of how these factors affect growth and development was very similar to what we saw when we looked at their effect on mortality in the Ecology section (Section 2.3). Furthermore it is claimed that the prevalent environmental conditions can influence actual body form (Bond, 1933; Stella, 1933) although this has, for specific instances or stresses, been questioned (Gilchrist, 1959) Surprisingly,although oxygen is often mentioned as the next important environmental factor (see Section 2.3.2.3), there is, to this author's knowledge, only one study of the effect of oxygen on growth and development in brine shrimp. This is the work of Gilchrist (1959), in which, working only with late stage males and females, found that

growth rate of females (but not males) was retarded due to the low oxygen content of the surrounding sea water. And yet such studies are vital if we are to (a) disentangle the relative effects of salinity, temperature and oxygen on survival, growth and reproduction and so come to a closer understanding of what happens in the natural environment, (b) carry out studies on the effects of environmental factors on the physiology of brine shrimp. Hypoxia may act indirectly on a physiological function, by slowing down, or uncoupling growth and development, so making it much more difficult to say what the effects of hypoxia on physiology are *per se*.

3.1.2 Brine shrimp reproduction and effect of environmental factors

Based on personal observations made during the course of my experimental work, and supplemented by the literature (see references given in first paragraph of Section 3.1.1), reproduction in the brine shrimp *Artemia franciscana* takes place as follows. To initiate copulation, male brine shrimp clasp, using their specially modified second antennae, the dorsal surface of the female's abdomen, between the uterus and the last pair of thoracopods (Wolfe, 1973; Figure 3.4). He then twists the female's abdomen around in order to insert his copulatory processes (they are eversible and each contains an opening to the vas deferens) into the female gonopore (Wiman, 1981). Maternal tissues give rise to a special sac into which she lays the eggs, the eggs issuing from the glandular uterine chamber (Figure 2.2B). The eggs laid can be one of two types. They can be thin-shelled eggs, which are characterised by rapid development, and upon hatching within the female uterus are released as free-swimming nauplii (ovoviviparous reproduction). However, under certain adverse environmental conditions thick-shelled dormant eggs are produced. The fertilized egg reaches the gastrula stage before development is arrested and the eggs (or cysts as they are now) become dormant (oviparous reproduction) (Lochhead & Lochhead, 1940; Bowen, 1962, 1964; Ballardin & Metalii, 1963). These cysts remain in this dormant condition, until environmental cues, indicative of the return of more 'favourable' environmental conditions, result in a resumption in development and rapid hatching.

Figure 3.4 Amplexus in *A. franciscana*: Posterior lateral surface. Note the male (♂) clasps the dorsal surface of the female (♀) abdomen using the modified second antenna (a). ab = abdomen, th = thoracic appendage. Scale is indicated on the photograph.


th
♀
a
eg
ab
0.3mm

However, the mechanisms behind this change in reproductive strategy, from ovoviviparity to oviparity, are still not fully understood. Both alteration of the quality and/ or quantity of the food (Dutrieu, 1962; D`Agostino & Provasoli, 1968) and increases in environmental salinity (Abonyi, 1915; Barigozzi, 1939; Gilchrist, 1959), have been implicated to some extent. We should also note that Versichele & Sorgeloos (1980) based on their experiments were forced to conclude that neither the food source nor the water salinity controlled the mode of reproduction in *Artemia*. It has even been suggested that cyst production may be an uncontrolled hypo-illumination alternate to oviparity (D`Agostino, 1980).

It was first noticed by Dutrieu (1960) that females that produced cysts were more 'red' in colouration than females that did not and he postulated that the presence of hemoglobin was required for cyst production. Interestingly this correlation between cyst production and hemoglobin synthesis fits well with the view of Sorgeloos (1975) who thought (but without any basis of rigorous experimental evidence) that when environmental conditions were 'favourable' the mode of reproduction was ovoviviparous, but when brine shrimp were exposed to low oxygen levels this co-occurred with the induction of oviparity. Clearly we need to know more specifically exactly what influences this shift from oviviparity to oviparity in brine shrimp rather than just refer to the stimulus as 'general environmental stress' (Ballardin & Metalli, 1963).

3.1.3 Aims of study

In this chapter we will examine the effect of culture under hypoxic conditions on growth, development and reproduction in the brine shrimp *Artemia franciscana*. Although this is interesting in its own right, it is essential 'background' for understanding, and perhaps interpreting, the respiratory responses to hypoxia of individual brine shrimp as examined in Chapters 4 and 5. As the respiratory pigment hemoglobin is potentially important in respiratory performance (Chapter 4), and has also been implicated in cyst production (Section 3.1.2), the hemoglobin content of adults and eggs/cysts cultured under hypoxia will also be

examined. Taking into account the data summarised in Sections 2.1.1 and 2.1.2 the following hypotheses were tested.

1) Exposure to environmental stress often results in decreased growth and development. Therefore, it was predicted that culture under chronic hypoxia would result in a decrease in growth (measured as body length and weight) and development (measured as developmental stage) in the brine shrimp *Artemia franciscana*. Furthermore culture under periodic hypoxic conditions was predicted to have an effect intermediate to that of the control and the chronic hypoxia treatment.

2) Exposure to chronic hypoxia can result in an increase in the surface area of gas exchange surfaces of many animals (e.g. larval amphibian gills and surface area (Drastich, 1927; Bond, 1960)). Therefore it was predicted that the earlier nauplii stages of *Artemia franciscana*, lacking specific gas exchange surfaces at this stage in development (Section 3.1.1) would be characterised by a greater overall surface area when cultured under hypoxia, when compared with normoxic controls.

3) Crustaceans exposed to chronic hypoxia often show an increase in the concentration of the respiratory pigment present be it hemoglobin (Fox, 1949, 1954; Fox & Phear, 1953; Chandler, 1954; Gilchrist, 1954; Hoshi & Kobayashi, 1972; Calvalho, 1984) or hemocyanin (Mangum, 1990). Therefore it was predicted that brine shrimp cultured under hypoxia would have greater concentrations of hemoglobin than normoxic controls. Not only was total hemoglobin assessed for *Artemia franciscana* but exactly where the hemoglobin was localised was examined too, as this may potentially be informative when we come to consider the role of hemoglobin in gas exchange (Chapter 4).

4. If hemoglobin is involved in the determination of the reproductive method then it is not unreasonable to assume that if there is a hypoxia-related increase in hemoglobin this will be accompanied by a shift from ovoviparity to oviparity and so implicate low oxygen as a potential trigger for cyst production as suggested previously.

Consequently as well as assessing changes in hemoglobin content of female brine shrimp and eggs, the effect of hypoxia on reproductive mode will also be assessed.

3.2 Material & methods

3.2.1 Origin and Culture of Animal Material

Individuals of the brine shrimp *Artemia franciscana* were obtained from dried cysts purchased from King British Aquarium Accessories Co Ltd, Bradford, U.K.. These cysts were collected from the South Arm of the Great Salt Lake, Utah (personal communication to Dr. J.I. Spicer from the manager, King British Aquarium Supplies, UK). Unless stated otherwise all cysts were hatched in artificial sea water (Tropic Marine, S = 35 ‰) under conditions of continuous illumination (60 - 100 MicroEinsteins. $m^{-2}.sec^{-1}$) and aeration (using a free standing Aquarium air pump). All of the artificial sea water solutions were autoclaved and filtered before use in any culture. Culture vessels and aeration equipment were also autoclaved. A large number of culture vessels (Vol. = 1.5 l) were maintained in water baths thermostatically controlled at a temperature of 28°C.

After hatching, the nauplii were separated from their shells and the remaining unhatched cysts discarded. Newly hatched nauplii were then washed thoroughly using filtered (0.2 μm, Whatman WCN1 filter) artificial sea water.

All culturing took place at 28°C. Newly hatched individuals (2000 - 3500 individuals per flask) were transferred to culture flasks (Vol. = 2 l) containing artificial sea water. The exact number of introduced into each culture flask (approximately 2 500) was determined from some preliminary experiments on the effect of density on growth and development where different numbers of individuals were cultured in the same vessel. There were always at least three replicate flasks for each set of culture conditions. The different culture conditions were defined as follows.

1. Normoxia - the water was vigorously aerated with air supplied from a bench air line.

2. Periodic hypoxia - the water was vigorously aerated with air for 16 h each day. For the remaining 8 h the oxygen content of the water was reduced to 50 ± 4 % of normoxia.

3. Chronic hypoxia - the oxygen content of the water was maintained continually at 50 % of normoxia.

The gas mixture used to induce and maintain hypoxic conditions was produced using mixtures of nitrogen (oxygen free) and air using precision gas mixing apparatus (Wöstoff, Bochum, Germany). The oxygen saturation of the water was checked periodically using an oxygen electrode (E5046, Radiometer, Copenhagen) linked to an oxygen meter (Strathkelvin Instruments, Glasgow). In Chapters 4, 5 and 6 the phrase 'chronic hypoxia' will refer to culture under the conditions referred to above by the same name (3, above). This also applies to the phrase 'periodic hypoxia' (2, above).

Individuals in each of the culture flasks were fed every 2 d on Liquifry (0.4 ml) until Stage 6. After this time the volume of Liquifry was increased to 0.8 ml, until around Stage 10. From Stage 10 through the late Stage cultures, each culture consisting of approx. 100 individuals, brine shrimp were supplied with 0.6 ml of Liquifry every 2 d. This feeding regime, worked out in detail in initial experiments, ensured that growth was not resource limited, but also ensured that the water quality was not compromised. The culture medium was replaced every 4 d.

3.2.2 Measurement of animal length, weight, surface area, development and mortality

Individuals were removed from culture vessels at both a large number of different time intervals and each of the different developmental stages encountered. The total body length was measured (± 5 μm), under low power magnification, using an ocular micrometer. Body length was taken as the distance from the front of the median eye to the posterior margin of the telson (excluding cercopods) (Figure 3.5).

Dry body weight was measured as follows. Individuals were counted and briefly washed in distilled water, to remove salt deposits, before they were gently blotted dry on filter paper.

After being placed on small pre-weighed aluminium foil squares animals were dried to constant weight at 60 °C for 24 h. Then individual animals were weighed to an accuracy of ± 1 μg on a micro balance (Mettler ME30).

Developmental stage was determined using the scheme devised by Weisz (1946, 1947) in which stages are numbered 0 - 19, depending on when the last thoracic (0 - 13) or abdominal (14 - 19) segment rudiment first appears. Stage 19 is taken as the final stage and covers all of the remaining life cycle of the sexually mature adult.

Total surface area of individual nauplii (Stages 0 - 4) was estimated as follows, based on key measurements (Figure 3.5) made under low power light microscopy, using a calibrated eyepiece micrometer.

Total surface area of stage 0 individuals was calculated by assuming that the head was approximately spherical and that the trunk approximated to a cone. So the total surface area = using the formula of two sections ,the head (sphere) and the(trunk) cone . So the total surface area $4\Pi r_s^2$ (area of sphere) + $\Pi r_c^{\ 2}\sqrt{r_c^2+h^2}$ (area of cone) where h= the altitude of the cone, and r_s and r_c the radius of the head and the cone (Figure 5A).

The surface area of individuals at either Stage 1,2,3 or 4 (Figure 5B & C) was calculated assuming that the head approximated to a sphere, the trunk approximated to a cylinder and each bud approximated to a half sphere. Therefore the total surface area for an individual animal was calculated using the equation $4\Pi r_s^2 + 2\Pi r_c h + ½(4\Pi r_b^2$ for each of the buds present) (See Figures 5B & C for details).

Mortality of brine shrimp under each of the different experimental culture conditions was assessed by counting all of the individuals present in each of the culture flasks when the culture medium was replaced.

3.2.3 Effect of hypoxia on reproduction

Paired brine shrimp (i.e. when males had clasped females) were separated from mass cultures as soon as they were noted. Individual pairs were then transferred to a culture flask

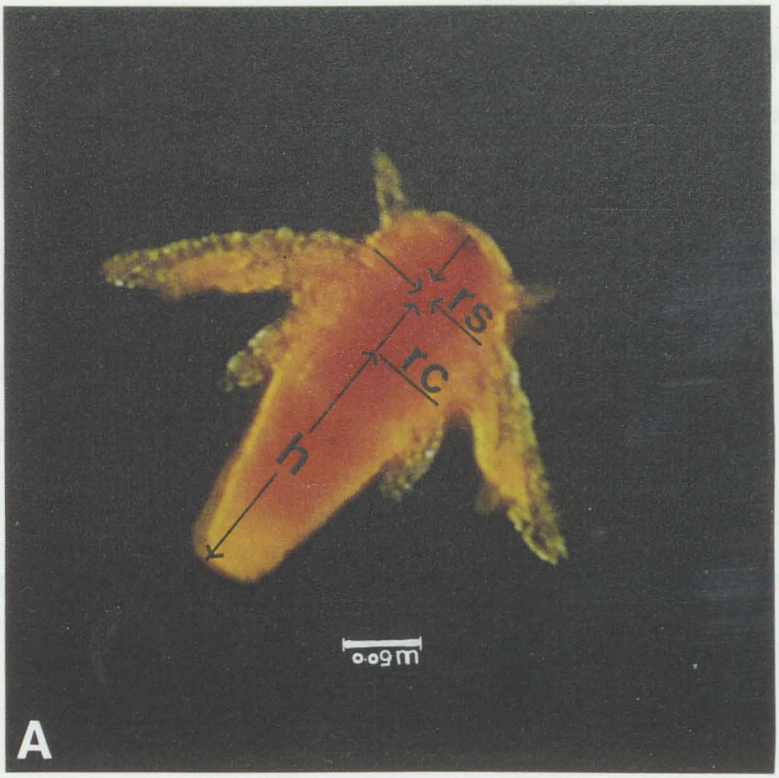
Figure 3.5 Measurements taken to enable the surface area of nauplii to be calculated (see text for details)

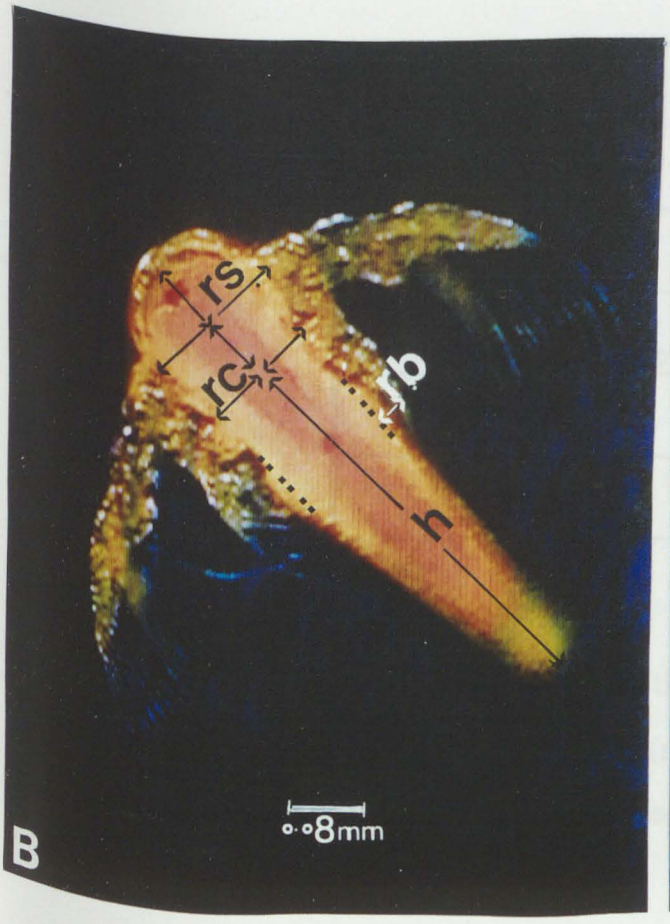
A. One hour old nauplii (Weisz's Stage 0) (dorsal view). The intense orange body colour is due to intracellular yolk stores.

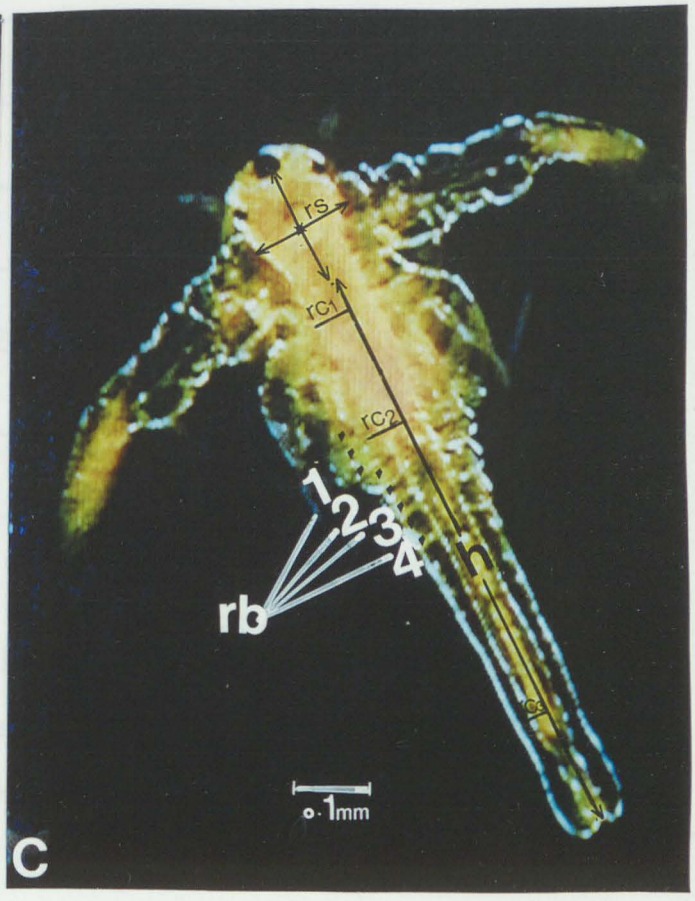
B. Nauplii at the beginning of the thoracic stage of development (Weisz's Stage 1) about 4 h after hatching (dorsal view).

C. Nauplii in the thoracic stage of development, about 48 h after hatching (ventral view). The first four segments are clearly visible (Weisz's Stage 4). The yolk is exhausted and the body becomes transparent.

Measurements made were as follows: Nauplii were divided into two; a head section (approximated to a sphere) and the rest of the body (in Stage 0 approximated to a 'cone', in Stages 1- 4 approximated to a cylinder). h = length of 'cone'; rb = radius of limb bud (different limb buds are numbered according to segment of origin); rc = radius of 'cone' (Stage 0) or cylinder (Stages 1-4); rs = radius of head section .Scale is indicated on each photograph.


rs
rc
h
0·05m
A


rs
rc
rb
h
0·08mm
B


rs
rc1
rc2
1
2
3
4
rb
h
0·1mm
C

(Volume = 100 ml), containing artificial sea water (S = 35 ‰, T = 28°C) maintained under normoxic or hypoxic conditions as described above (Section 3.2.1) Brine shrimp were supplied with 0.2 ml of diluted Liquifry (1 Liquifry: 3 culture medium) every 2 d and the water was changed after each brood. Each day offspring (as nauplii or cysts) were removed from each of the experimental flasks and counted. Cysts were removed by filtering culture water through filter paper, some of them were stored in dry air (R.H. = < 10%, T = 28 - 30°C) and the remaining eggs were directly rehydrated without desiccation.

The length of the female producing the eggs or cysts was measured as outlined in Section 3.2.2 and her hemoglobin content was estimated, semi-quantitatively, using the method of Chow (1968), which assigns a score to individual brine shrimp on the basis of how red they are (Table 3.1)

Table 3.1 The 'hemoglobin index' of Chow (1968)

SCORE	COLOUR
0	no colour
+	light pink
++	light pink, few spots red
+++	red
++++	dark red

To examine the effect of hypoxic culture on the reproduction of isolated females. Females from normoxic and hypoxic culture conditions (n = 10 in each case) were isolated from the time they were 14 d old. They were then reared in isolation, under normoxic and hypoxic

culture conditions for two months. Egg and/or cyst production was monitored as was the colour of (and its localisation on) the mother.

3.2.4 Localisation of hemoglobin

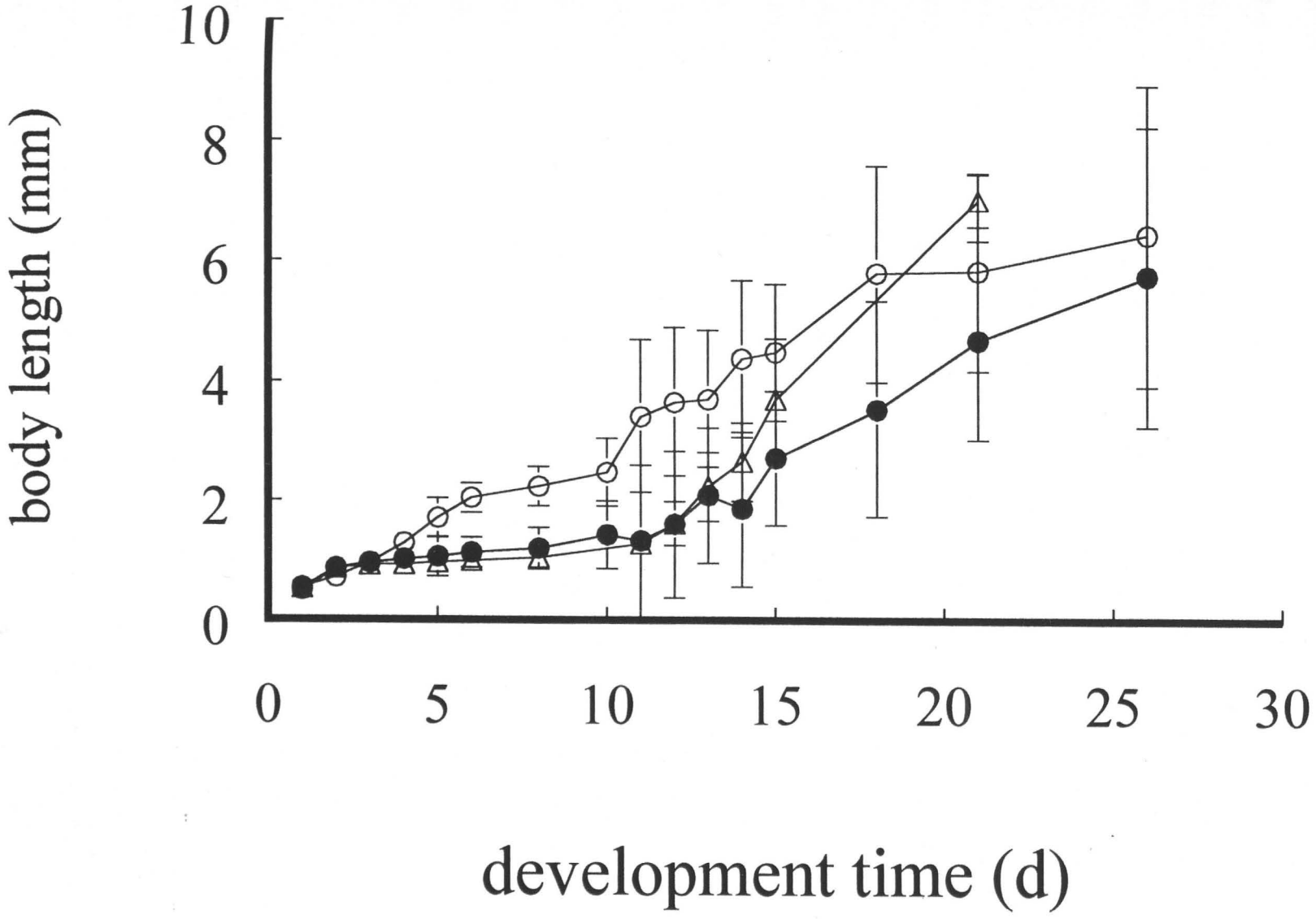
Specific staining for hemoglobin was carried out on prepared tissues of late stage *Artemia franciscana*, cultured under different oxygenation regimes. Whole animals were first fixed in buffered formalin (formaldehyde 4%) for 24 h before being embedded in paraffin wax. Transverse, longitudinal and sagittal sections (for different individuals) were then cut (5 - 6 µm thick) using (Anglia Scientific Rotary microtome). Thin sections were briefly rinsed in distilled water before being stained for 5 min in leuco patent blue V reagent. The leuco patent blue V technique (the method of Lison- Dunn as modified by Kiernan, 1990) is highly specific for hemoglobin. The reagent, which is reduced to the leuco form by nascent hydrogen, recolourises (stains blue) in the presence of hemoglobin peroxidase. After staining with leuco patent blue, the nuclei were counter stained using 1% Aqueas safranin for 1 min, before being rehydrated, cleared and finally mounted using D.P.X. All sections were examined visually using high and low powered microscopy (Leica Biomed) and photomicrographs were taken using a dedicated microscope with camera attachment (Nicon Apophot).

3. 3 Results

3.3.1 Effect of hypoxic culture on growth and development

For the control culture and both experimental cultures, there were significant relationships demonstrated between body length and developmental time for *Artemia franciscana* from each culture (Figure 3.6). Subsequent covariance analysis showed that there was a significant difference between the control and the chronic hypoxia cultures ($F_{1,477} = 4104$; $P < 0.05$) but not between the control and the periodic hypoxia cultures ($F_{1,366} = 2.7$; $P > 0.05$). However

Figure 3.6 Relationship between body length (mm) and developmental time for *A. franciscana* cultured under different oxygenation regimes. ● = normoxia; O = chronic hypoxia; Δ = periodic hypoxia. Each value represents mean ± 1 standard deviation of 15 - 45 determinations.


body length (mm)
10
8
6
4
2
0
0
5
10
15
20
25
30
development time (d)

closer observation of the graphs, followed by the use of Student's 't' test, indicated that there was only a significant difference between normoxic and chronic hypoxic culture for each interval between 6 and 15 days of culture ($P < 0.05$ in each case), and not before or after that time. In other words culture under chronic hypoxia resulted in individuals being of a greater body length than the controls although this difference was not detectable by day 15 of culture.

There were, however, no significant effects of culture under periodic or chronic hypoxia on either the relationship between body length and developmental stage ($F_{2,660} = 0.97$; $P > 0.05$) (Figure 3.7) or body weight and developmental stage ($F_{2,660} = 1.31$; $P > 0.05$) (Figure 3.8). This means that the increase in length of hypoxic cultured animals, noted above is solely due to an increase in developmental rate. This can be seen clearly in Figure 3.9 where the proportions of each of the developmental stages present at any one time are plotted against 'real' time. Although the resultant plots are complicated some general trends are evident. In normoxia culture within 24 h of hatching brine shrimp have developed to Stages 3 and 4 and these remain the dominant stages until 6 - 7 days after hatching (Figure 3.9A. Within 11 days of hatching the first sexually mature individuals (Stage 19) have developed and within 24 h all of the individuals present are Stage 19. Culture under periodic hypoxia resulted, at least initially, in a similar overall pattern except that Stage 4 became the more dominant stage more quickly (Figure 3.9B). However, the first sexually mature individuals were noted until day 12 and after this it was a further 4 d before all of the individuals present in the culture were sexually mature.

Culture under chronic hypoxia resulted in individuals apparently 'rushing through' the first 4 developmental stages and beyond and while the first sexually mature brine shrimp were noted earlier than in any of the other culture conditions (9 d) it was not until day 15 that all of the individuals in the culture were sexually mature (Figure 3.9C). Total surface areas were calculated only for Stages 0 - 4 from normoxic and chronic hypoxic culture conditions. Student's 't' test indicated that there was only a significant difference at Stage 2 with hypoxic cultured individuals having a greater surface area than normoxic controls (Figure 3.10).

Figure 3.7 Relationship between body length (mm) and developmental stage for *A. franciscana* cultured under different oxygenation regimes. ● = normoxia; O = chronic hypoxia; Δ = periodic hypoxia. Each value represents mean ± 1 standard deviation of 15 - 45 determinations.

body length (mm)
4
3
2
1
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
developmental stage

Figure 3.8 Relationship between body weight (μg) and developmental stage for *A. franciscana* cultured under different oxygenation regimes. ● = normoxia; O = chronic hypoxia; Δ = periodic hypoxia. Each value represents mean ± 1 standard deviation of 5 - 10 determinations.

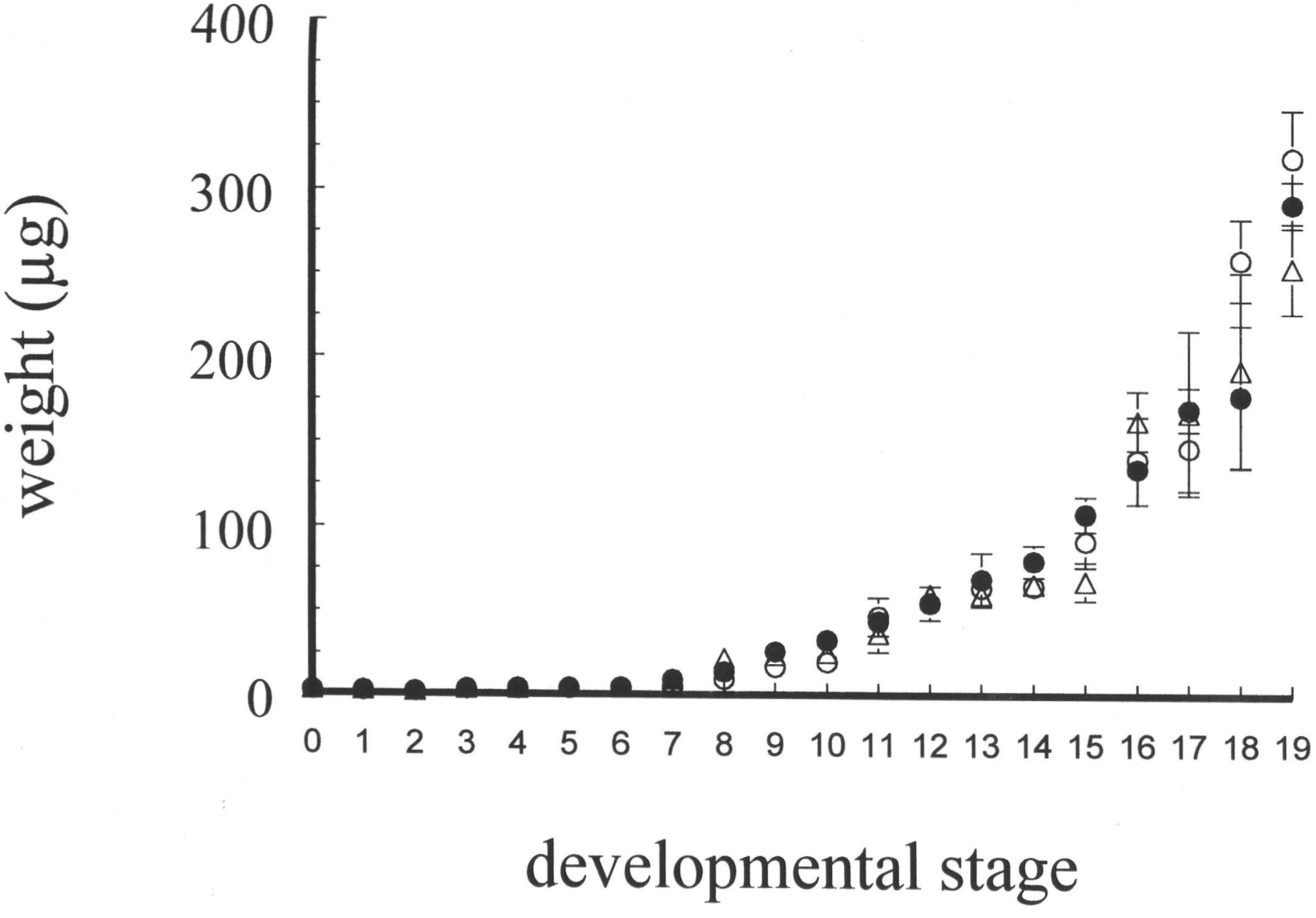
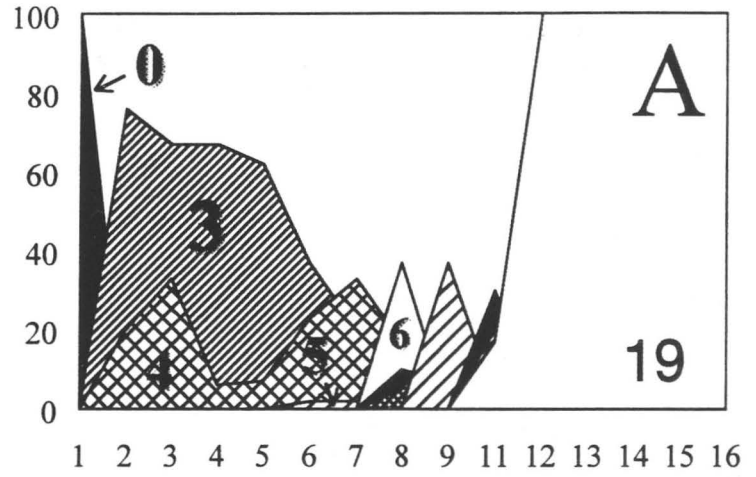
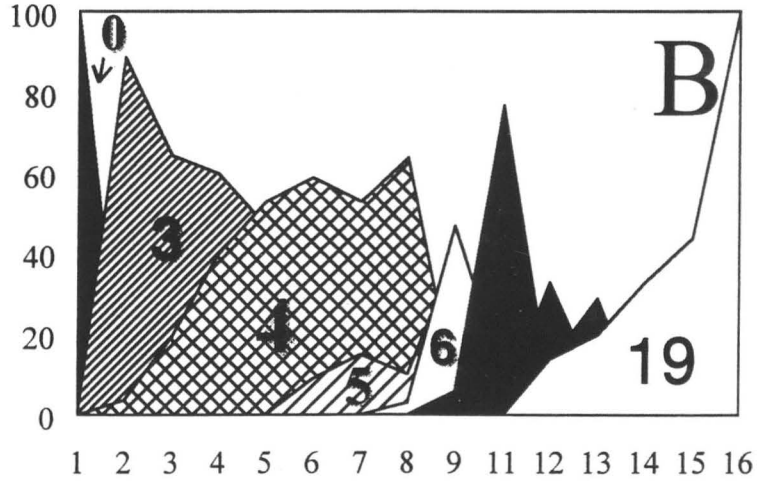
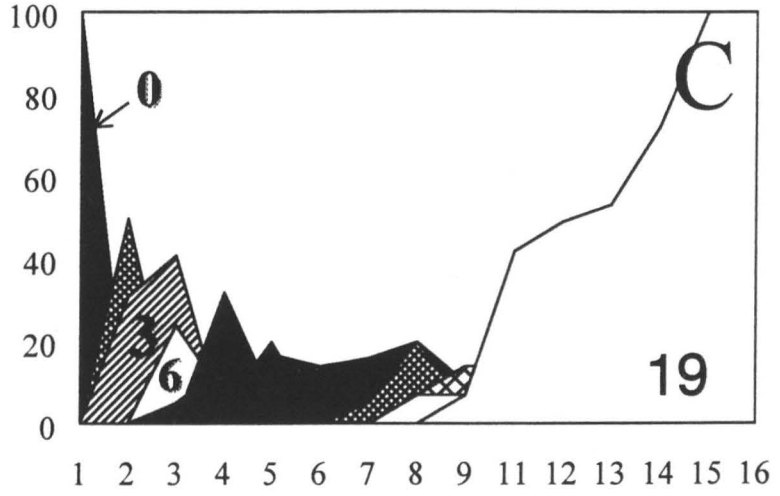

400
300
200
100
0
weight (μg)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
developmental stage

Figure 3.9 Graphic representation of how the proportions of different developmental stages of *A. franciscana*, cultured under different oxygenation regimes, changes with time. A = normoxia, C = chronic hypoxia, B = periodic hypoxia. Key Stage values are given in bold and are equivalent to those of Weisz (1946).



Stage present (%)





developmental time (d)

Figure 3.10 Relationship between total surface area (μm^2) and developmental stage for early developmental stages of *A. franciscana*. ● = normoxia; O = chronic hypoxia. Each value represents mean ± 1 standard deviation of 10 - 20 determinations.

total surface area
600
400
200
0
0
1
2
3
4
developmental stage

However, as we have seen above Stage 2 does not last very long and rarely comprises a significant proportion of the culture at any particular time.

3.3.2 Mortality under hypoxia culture

It is difficult to disentangle the two distinct processes responsible for the patterns in the data presented above, namely growth/development and mortality. To help us do this we will now examine the effect of the experimental cultures on mortality. Presented in Figure 3.11 is the effect of hypoxic culture on total mortality, from immediately after hatching to reaching late Stage (19+). There was no significant difference in total mortality (25 - 30 %) between hypoxic culture and control (normoxic) culture ($P > 0.05$). Perhaps surprisingly mortality was significantly lower in *A. franciscana* cultured under periodic hypoxia than those cultured under either normoxia or periodic hypoxia (about 14 %) ($P < 0.05$). Mortality was stage dependent (Figure 3.12), with greatest mortality sustained between Stages 5 - 9 (> 50 % in each case). This figure seemed to be independent of the oxygenation of the culture media. Just more than one fifth died before reaching Stage 4. Mortality from Stage 10 onwards accounted for about one twentieth of the total observed mortality.

3.3.3 Induction of hemoglobin by hypoxia

Presented in Figure 3.13A is a photograph of three representative brine shrimp, each cultured under a different oxygenation regime. This is presented semi-quantitatively for female *A. franciscana* cultured under normoxic (Figure 3.13B) and chronic hypoxic (Figure 3.13C) conditions. *Artemia franciscana* , of both sexes, cultured under chronic hypoxia were a pronounced red colour when compared with individuals cultured under either periodic hypoxia or normoxia. However, it is true to say that females cultured under chronic hypoxia were invariably more strongly pigmented than males of equivalent size. Normoxic individuals showed little red pigmentation .Brine shrimp cultured under periodic hypoxia were

Figure 3.11 Total mortality (% of hatchlings) of *A. franciscana* cultured under different oxygenation regimes. Values are expressed as means ± 1 standard deviation, n = 3 in each case.

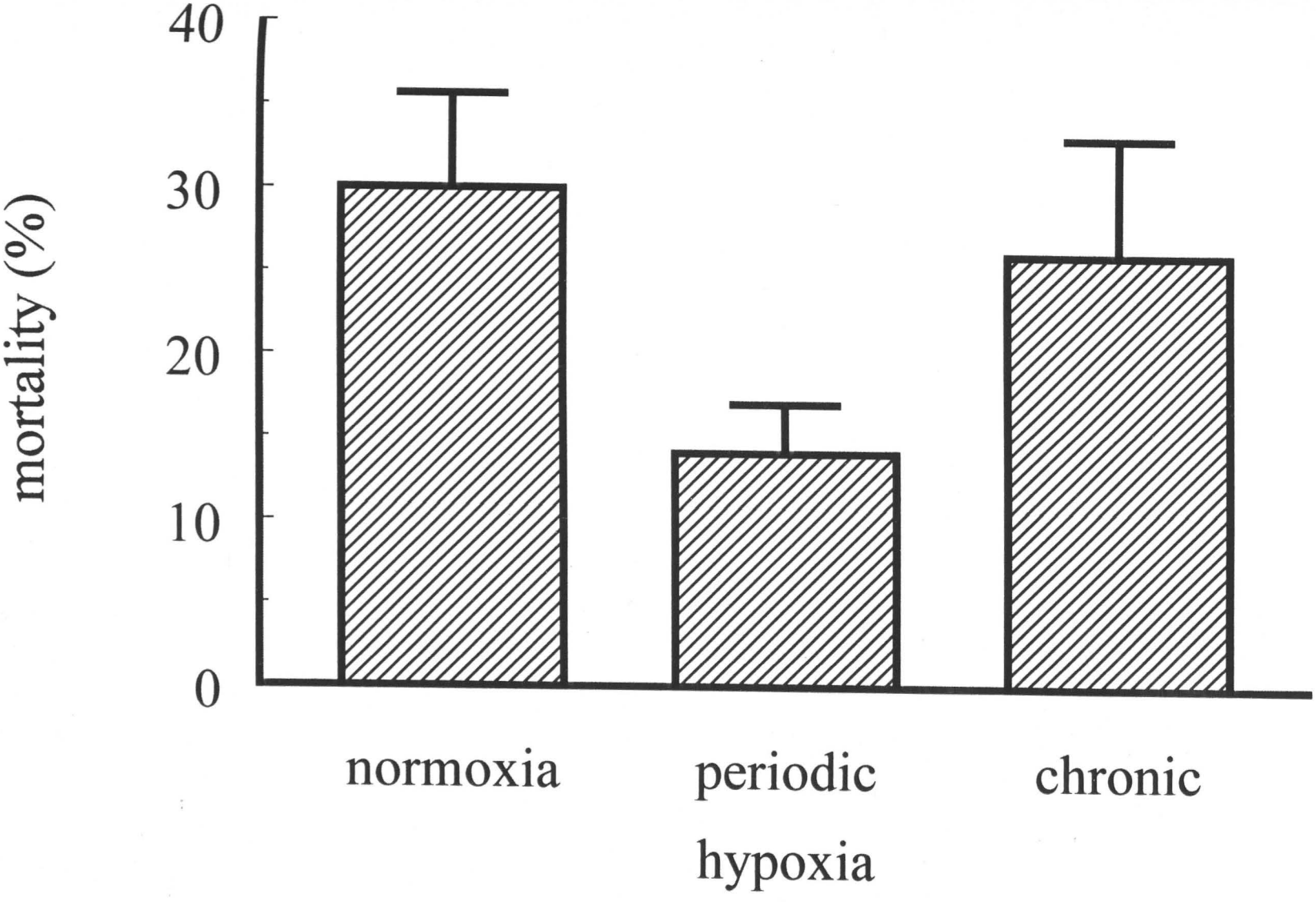

40
30
20
10
0
mortality (%)
normoxia
periodic hypoxia
chronic

Figure 3.12 Relationship between Stage-related mortality, expressed as a percentage of total mortality, and developmental stage in *A. franciscana*. Clear = normoxia; filled = chronic hypoxia; hatched = periodic hypoxia. Values are given as means ± 1 standard deviation (n = 2 - 3).

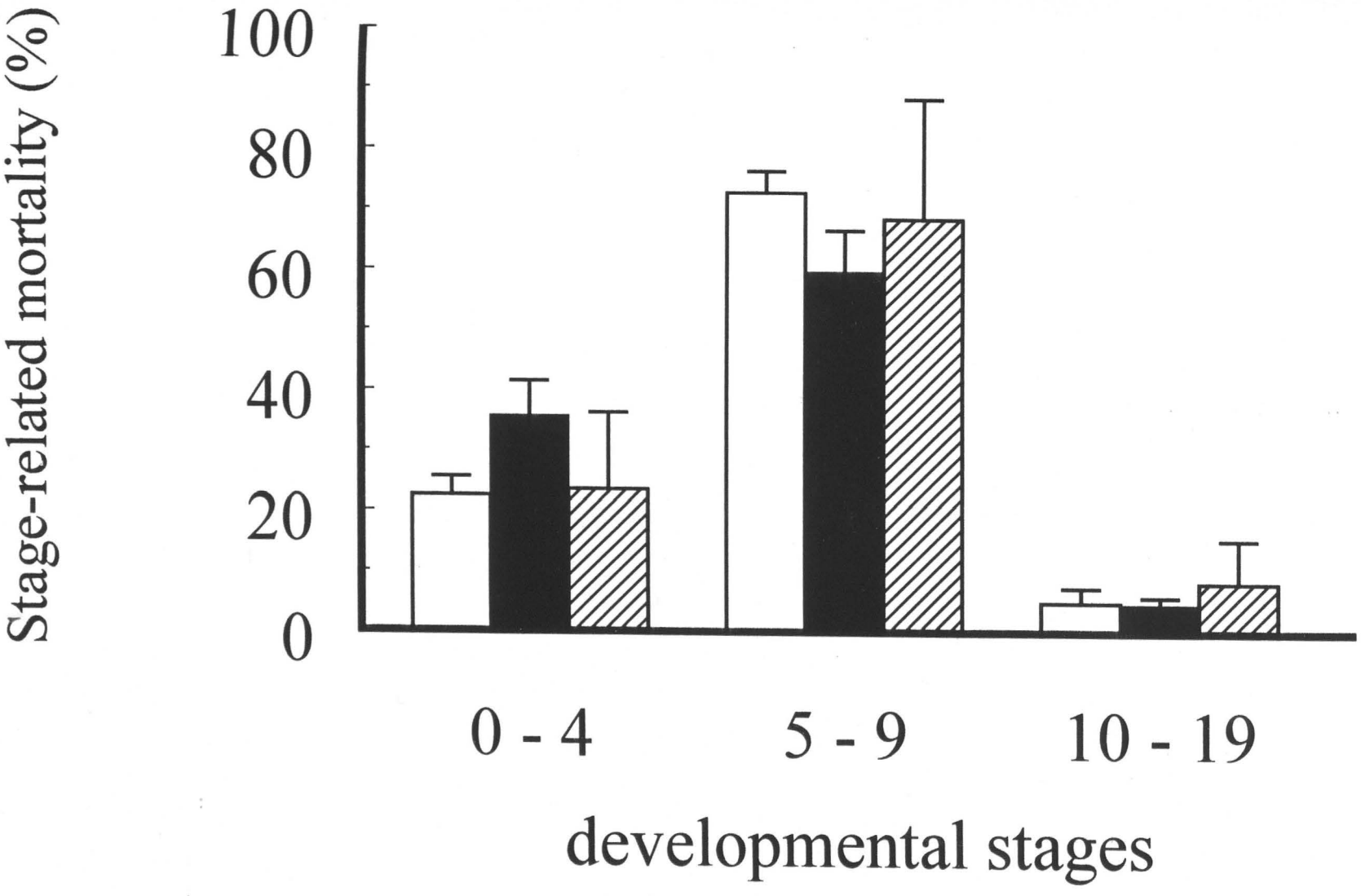
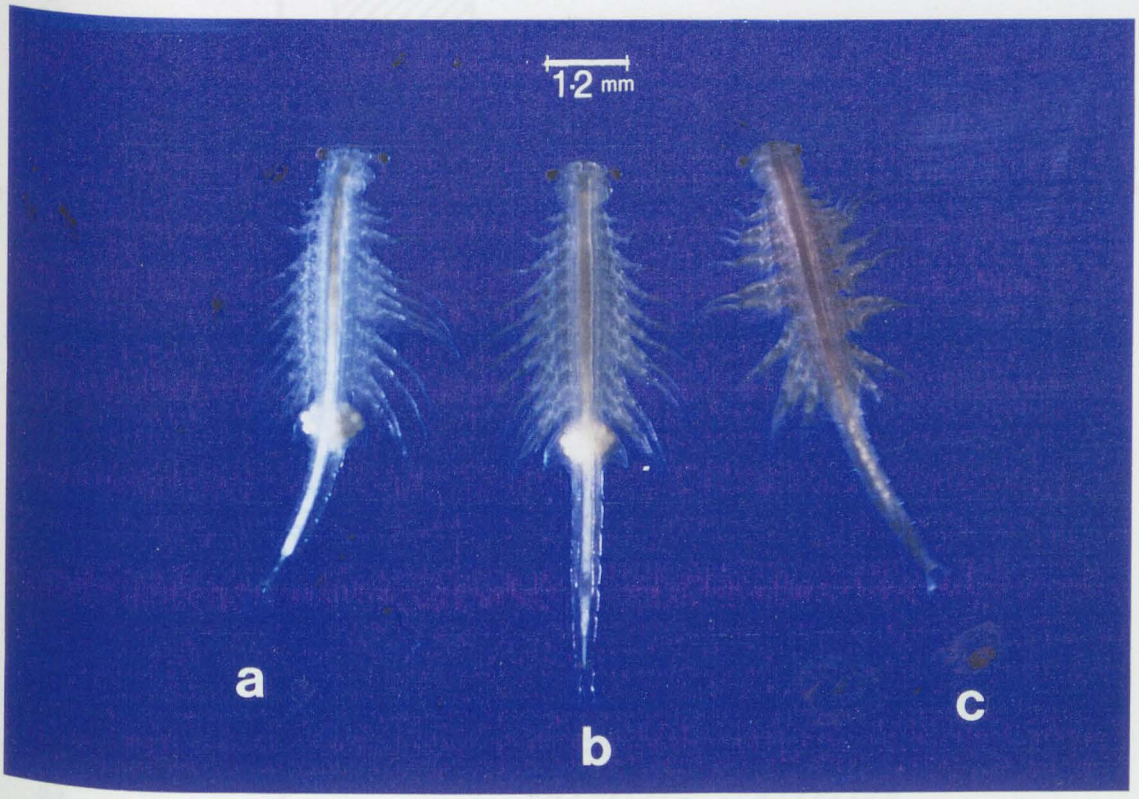
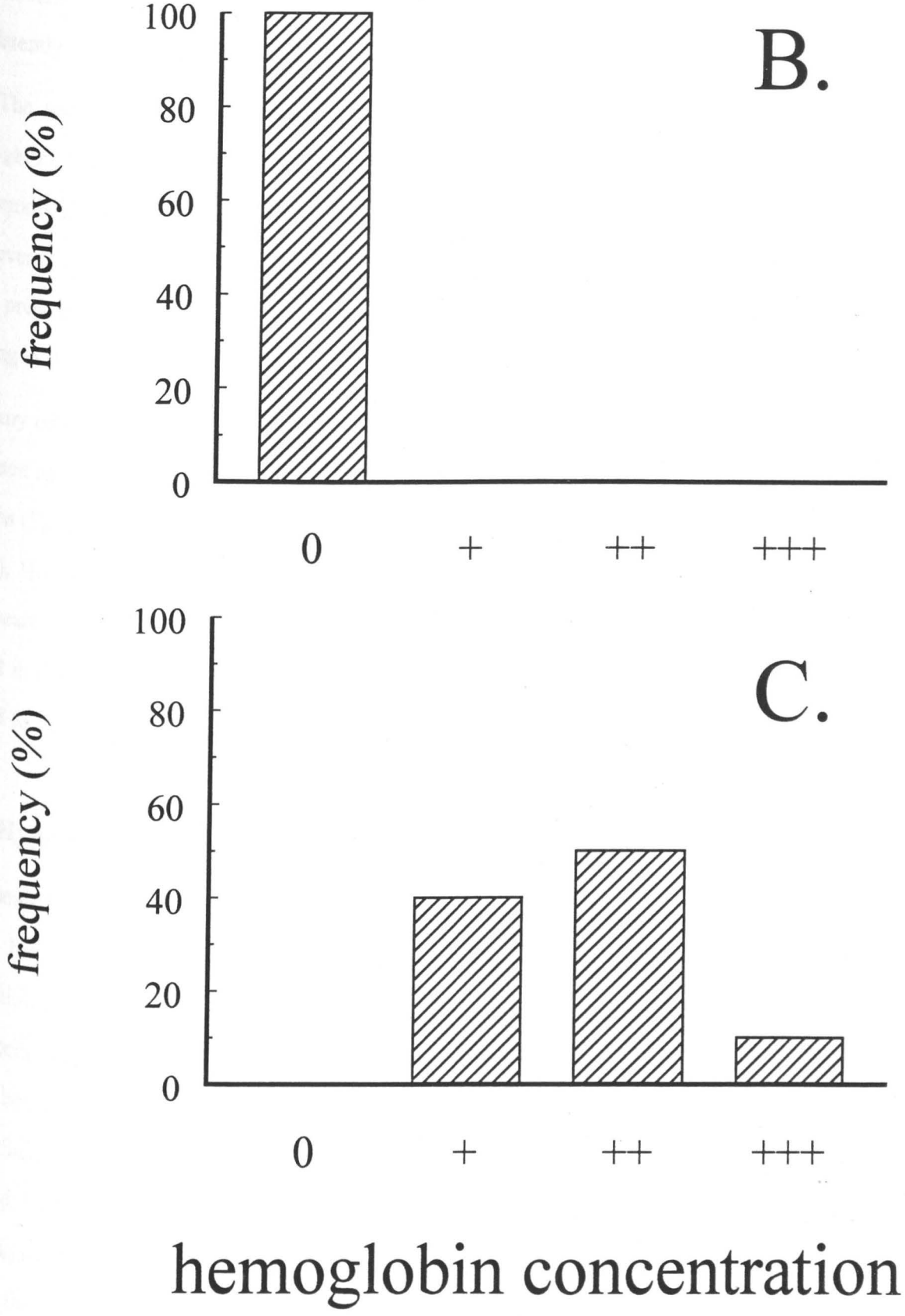

Stage-related mortality (%)
100
80
60
40
20
0
0 - 4
5 - 9
10 - 19
developmental stages

Figure 3.13 Effect of hypoxic culture on hemoglobin concentration in adult female *A. franciscana*.

A Representative individuals cultured under different oxygenation regimes. a = normoxia, b = periodic hypoxia and c = chronic hypoxia. Scale is indicated on the photograph.

B & C Semi-quantitative assessment of hemoglobin concentration (after Chow, 1986, see Section 3.2.3 for method) for animals cultured under normoxia (B) and chronic hypoxia (C).


1·2 mm
a
b
c


B.
frequency (%)
100
80
60
40
20
0
0
+
++
+++
C.
frequency (%)
100
80
60
40
20
0
0
+
++
+++
hemoglobin concentration

intermediate in colour to normoxic and hypoxic cultured individuals. These differences were consistently observed through-out the experimental work.

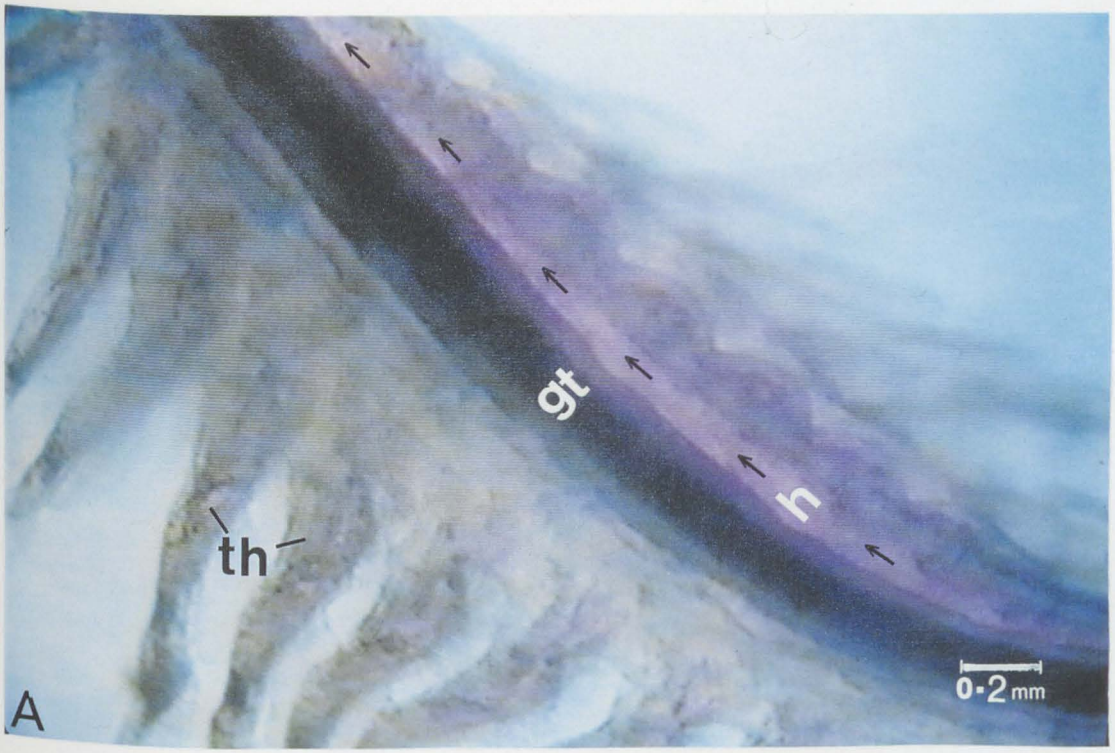
The series of photomicrographs presented below allow us to see the localisation of hemoglobin in the tissues of *Artemia franciscana*. Hemoglobin is normally found dissolved in the hemolymph of invertebrates and this is also the case for *A. franciscana* (Figure 3.14A). However, culture under hypoxic conditions resulted in a greater concentration of hemoglobin being present in the hemolymph, compared with the control, as indicated by the more intense staining in the former (Figure 3.13A)

Many of the body tissues also stained positive for hemoglobin and the concentration also increased as a result of culture under hypoxia. This was most pronounced in the thoracic limb muscles (Figure 3.15) and particularly in females (Figure 3.15B) compared with males (Figure 3.15C). However, similar results were also found for other tissues; abdominal muscles, gut wall, heart wall (Figure 3.14B&C) and hemolymph (see Figure 3.16). Hemoglobin was even present in the nervous system but, unlike any of the other tissues examined, the concentration seemed to be unaffected by culture under hypoxia (Figure 3.17).

3.3.4 Hypoxia, reproduction and hemoglobin provisioning

Presented in Figure 3.18A is a graph of the total number of offspring produced by a female against her body length for individuals cultured under normoxia and chronic hypoxic. Correlation analysis indicated that there was no significant relationship between these two parameters in either case ($r^2 \leq 0.26$, $n = 9$, $P > 0.05$ in each case) despite such a relationship having been demonstrated previously for the closely-related fairy shrimp *Siphonophanes grubii* (Saiah & Perring, 1990). Under normoxic conditions 369.8 ± 103.2 offspring were produced by each female with about two thirds of those free-swimming nauplii, and the remaining third, dormant cysts (Figure 3.18B). However for individuals cultured under chronic hypoxia the number of offspring produced by each female had fallen to 146.0 ± 61.1. This difference was statistically significant (Students 't' test, $t = -5.48$, d.f. $= 11$, $P < 0.05$). Furthermore over 90 % of the offspring were in the form of dormant cysts (Figure 3.18B).

Figure 3.14 Heart of adult female *A. franciscana* cultured under chronic hypoxia. A = lateral view, B = dorsal view and C = transverse section of myocardium stained for hemoglobin. The hemoglobin present in the hemolymph passing through the heart is clearly visible. ← = direction of hemolymph flow, h = heart, he = hemoglobin, gt = gut, pg = 'red spot' pigments, th = thoracic appendages. Scale for A & B is indicated on the photograph. For C (Magnification x 750).


gt
h
th
0·2 mm
A

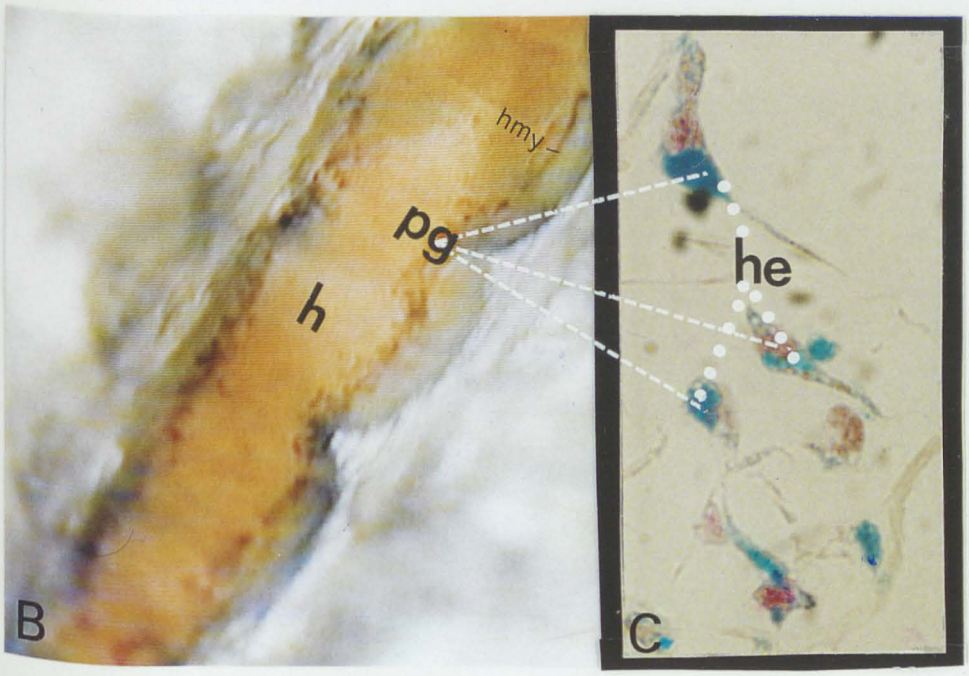

hmy
pg
h
he
B
C

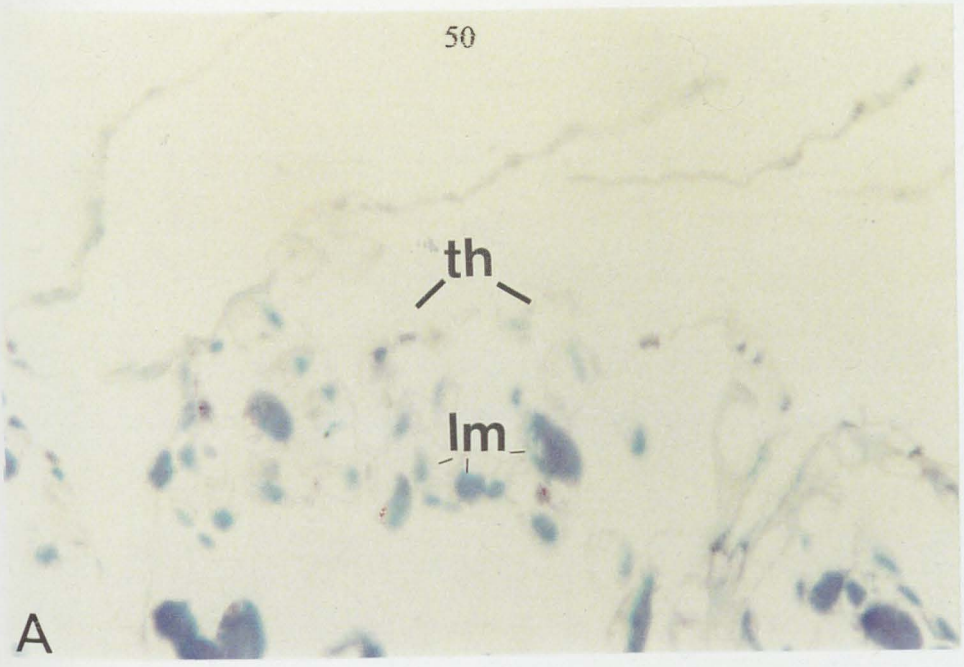
Figure 3.15 Representative light micrographs of sections through thoracic limbs staining for hemoglobin using Leuco patent blue.

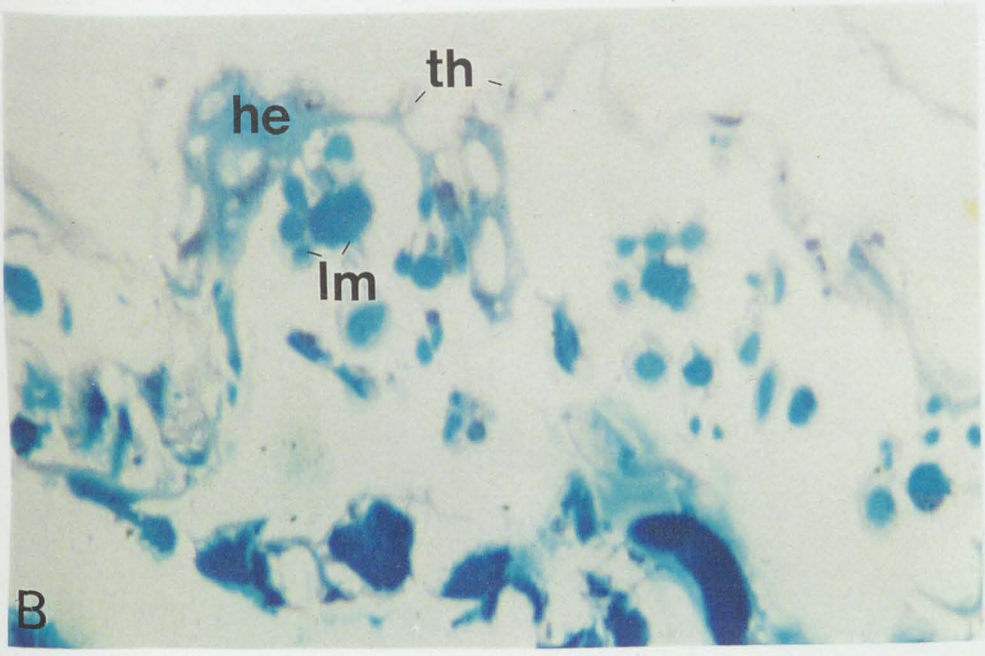
A Longitudinal section through adult female cultured under normoxia.

B Longitudinal section of adult female cultured under chronic hypoxia.

C Sagittal section of adult male cultured under hypoxia.

(magnification X 160). bc = blood forming cells, he = hemoglobin, lm = limb muscles, th = thoracic limbs.


th
lm
A


th
he
lm
B

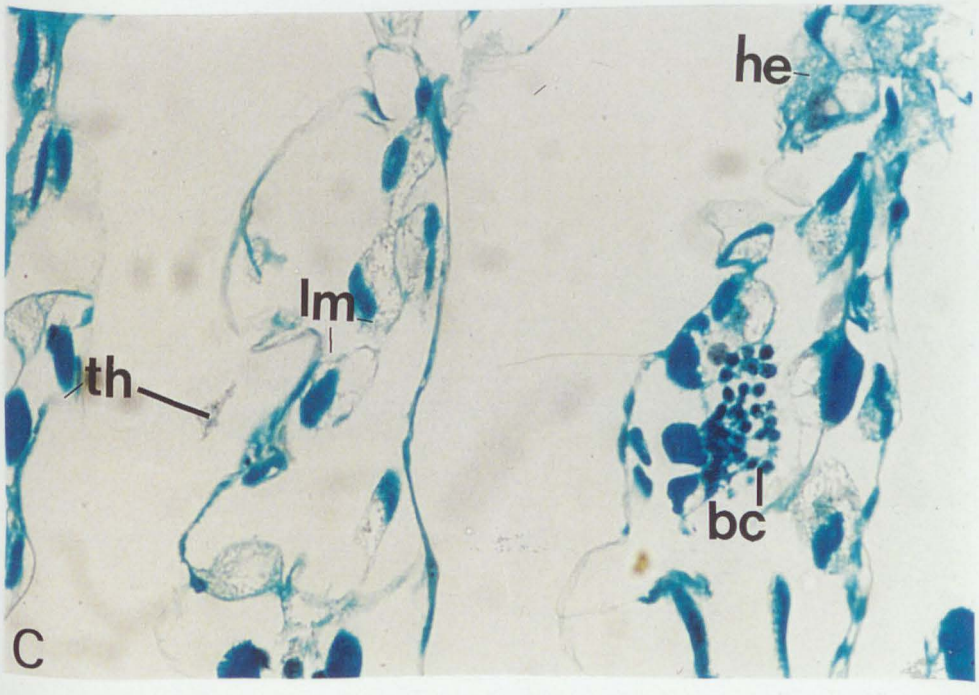
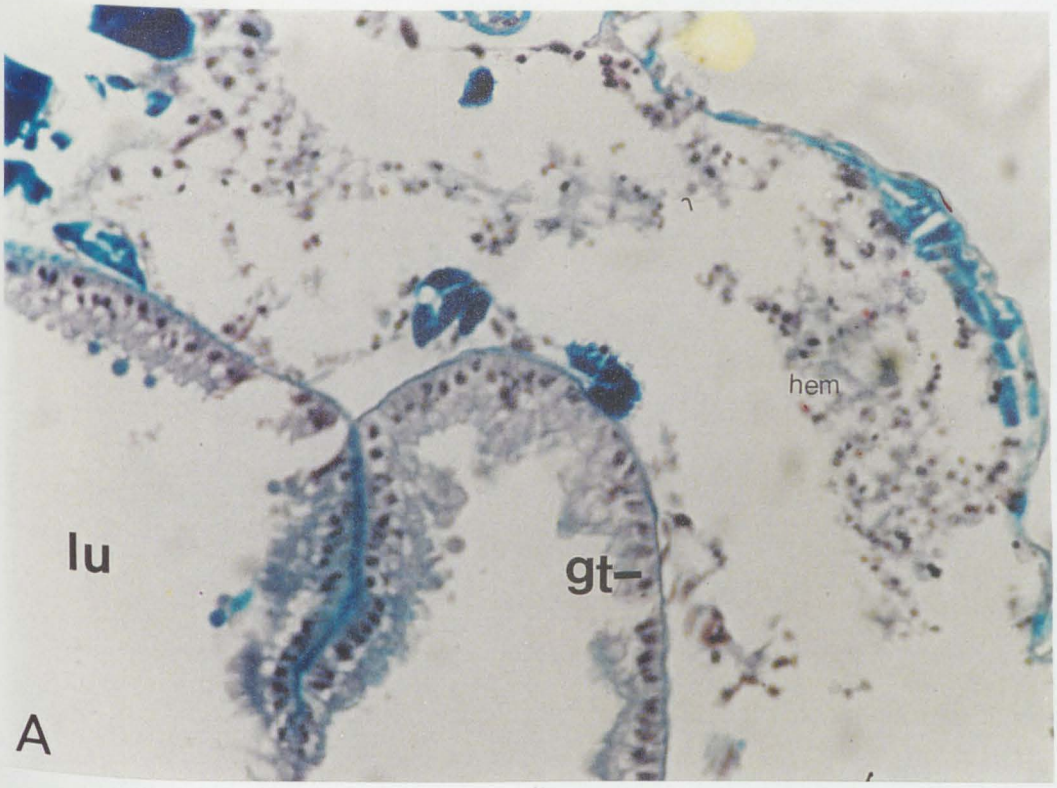

he
lm
th
bc
C

Figure 3.16 Representative light micrograph of transverse section through the female abdomen of individuals cultured under normoxia (A) and chronic hypoxia (B). Stained with Leuco patent blue V. (Magnification X 160). gt = gut epithelium, hem = hemolymph, lu = lumen.


hem
lu
gt
A

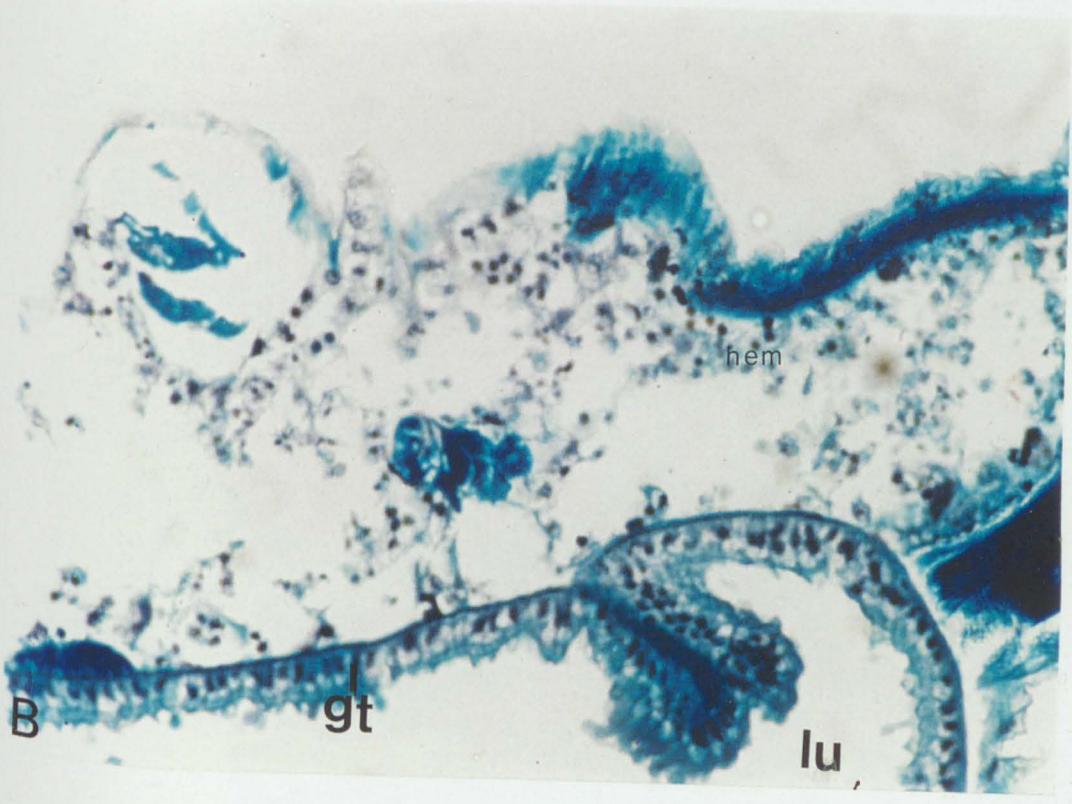
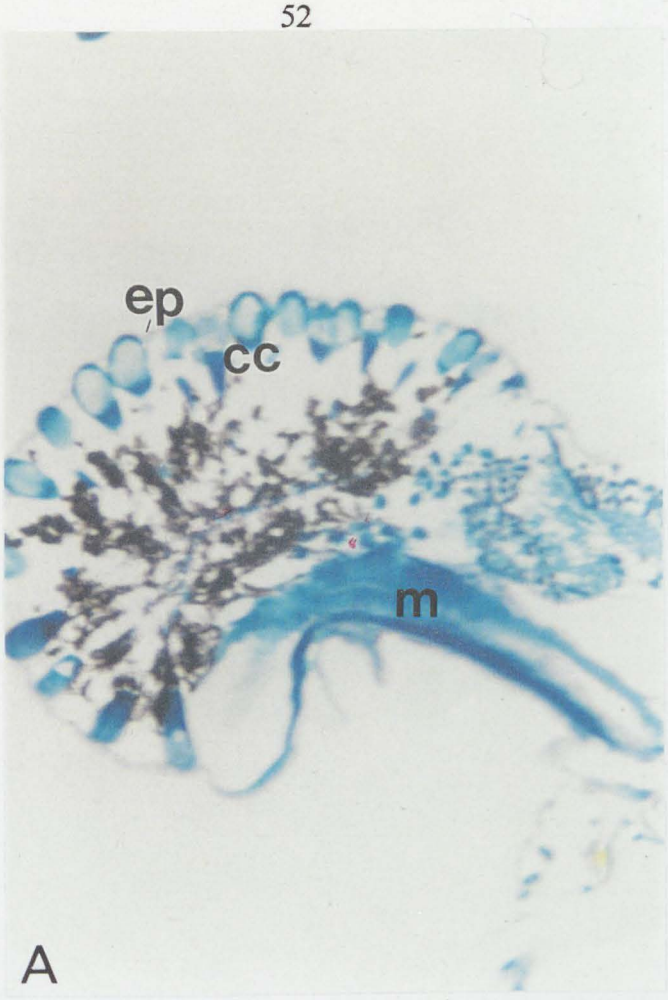

hem
B
gt
lu

Figure 3.17 Representative light micrograph of a sagittal section through compound eye stalk in individuals cultured under (A) normoxia and (B) chronic hypoxia . Stained with Leuco patent blue V. (Magnification X 160). cc = cristal cone, ep = epidermis and cuticle, m = medulla of the optic lobe.


ep
cc
m
A

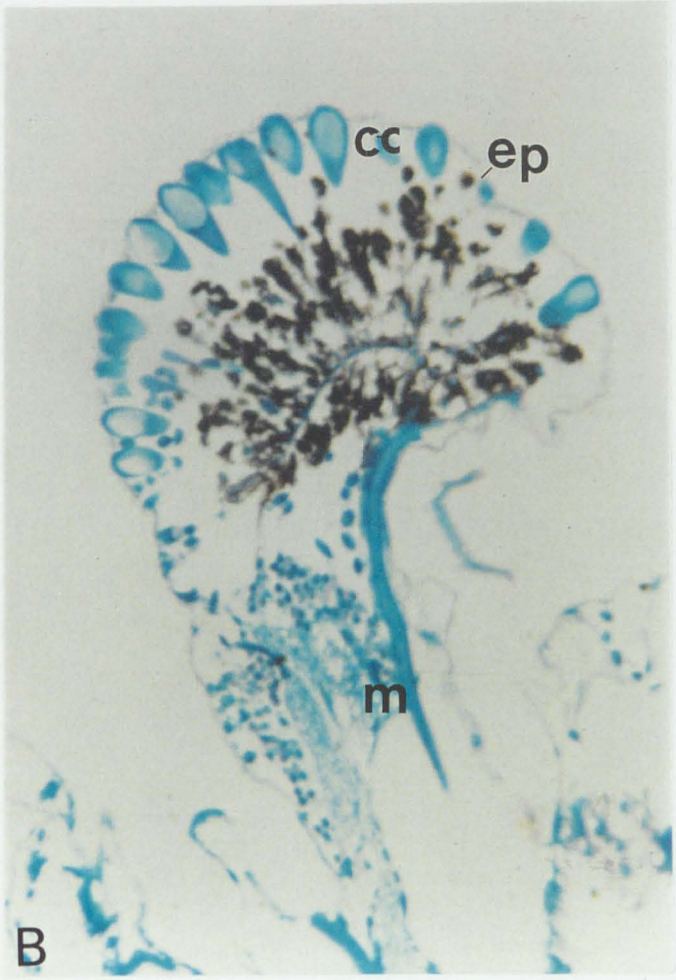
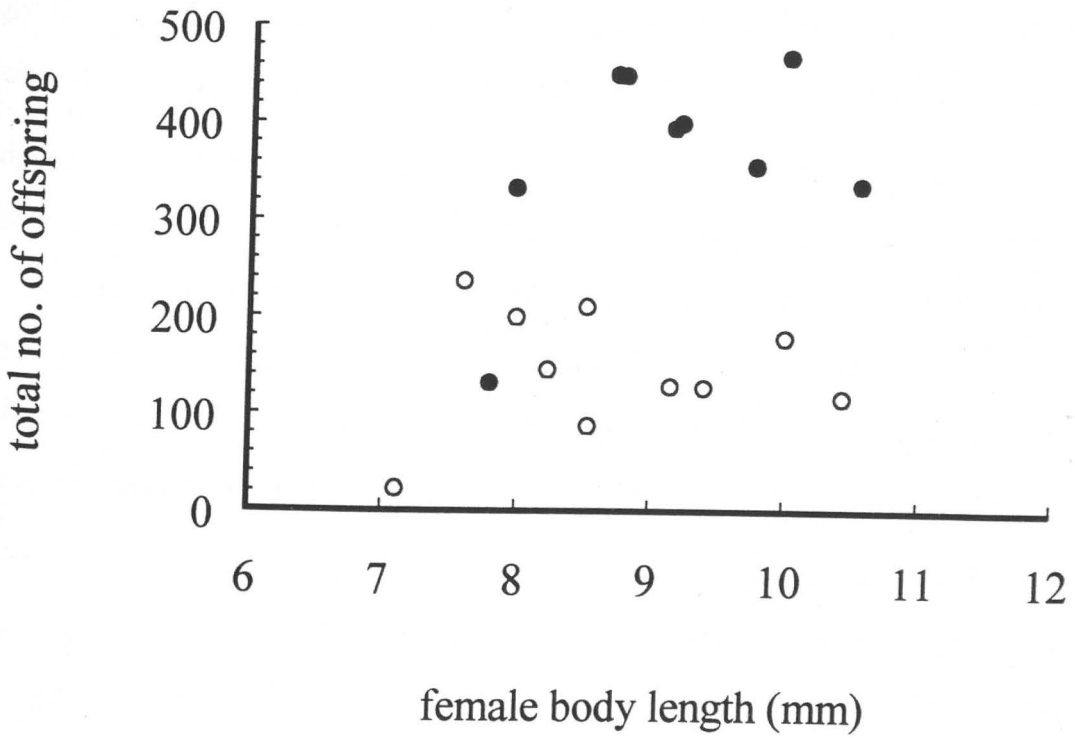

cc
ep
m
B

Figure 3.18 Effect of hypoxia on reproduction in brine shrimp

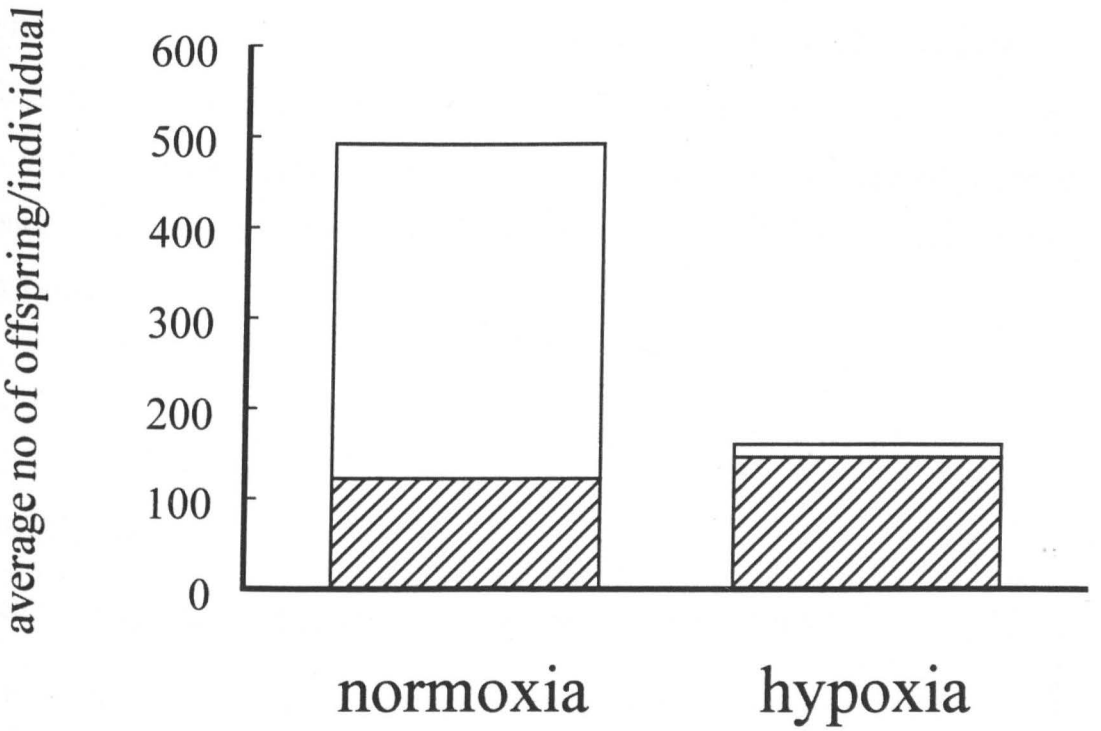
A The relationship between total reproductive output of individual female *A. franciscana* and female body length for individuals cultured under different oxygenation regimes. ● = normoxia, O = chronic hypoxia.

B The effect of hypoxic culture on the average number of cysts and live young produced by each female. Clear = live young, hatched, filled = dormant cysts.

A


total no. of offspring
500
400
300
200
100
0
6
7
8
9
10
11
12
female body length (mm)

B


average no of offspring/individual
600
500
400
300
200
100
0
normoxia
hypoxia

Interestingly there was no significant difference between the number of cysts produced by mothers cultured under chronic hypoxia and those from the normoxic control treatment.

Ovigerous females cultured under normoxia were pale in colour. This was markedly different from females cultured under hypoxic which varied from light pink to red in colour indicating, at least in part, that these individuals contained a greater concentration of hemoglobin than controls (Figure 3.13B,C). The 'redness' of the cyst or nauplii was strongly related to the redness of the mother; "colourless" normoxic mothers produced pale coloured eggs whereas 'red' mothers produced red-coloured eggs (Figure 2.1, 2.2.B)

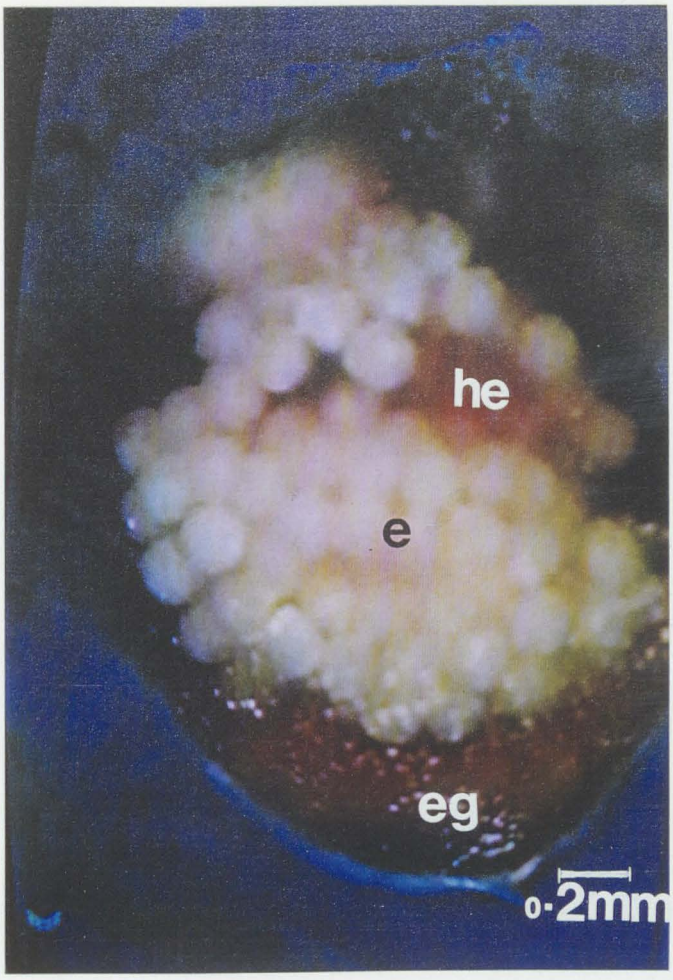
In the case of isolated females there were no viable eggs or cysts production for both normoxic and hypoxic cultured animals. In all cases the ovaries were seen to produce pale non-viable eggs (Figure 3.19). In the case of females cultured under hypoxia, the concentration of hemoglobin increased in the mother and in the ovaries in particular. However, in the case of the latter the hemoglobin did not cover/envelop the eggs. After 2 months the eggs carried by the hypoxia cultured female had increased in both size and number and the concentration of hemoglobin both in the mother and the ovary was very great (+++) (Figure 3.20). By this time the ovary and uterus were greatly distended and differed considerably in size and shape from normoxic controls. This is markedly different from what happens if a male is present. In this case the reproductive cycle, from ovulation to release of eggs or nauplii, is usually from 3 - 5 d, and the time between broods in eggs deposition is less than half than that when nauplii are produced.

3.4 Discussion

3.4.1 Effect of hypoxia on growth, development and mortality

Culture under chronic hypoxia did affect growth and development but not in the way that was predicted. Instead of compromising growth and development, early in ontogeny the rate of development of *Artemia franciscana* actually increased. This had the result of 'hurrying

Figure 3.19 Representative photomicrographs of eggs produced by (A) an isolated female (ventral view of dissected egg sac) and (B) a sexually reproducing female (ventral view, egg sac intact) of *A. franciscana* cultured under hypoxia. Scale is shown on photograph. e = egg, eg = egg sac, he = hemoglobin.


he
e
eg
0·2mm

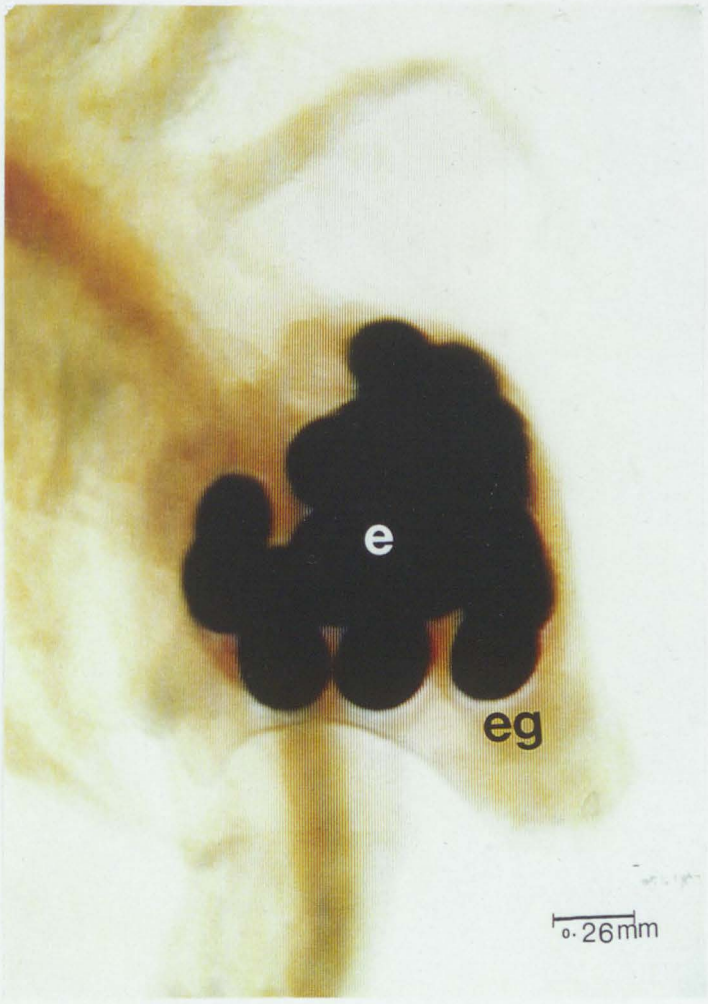
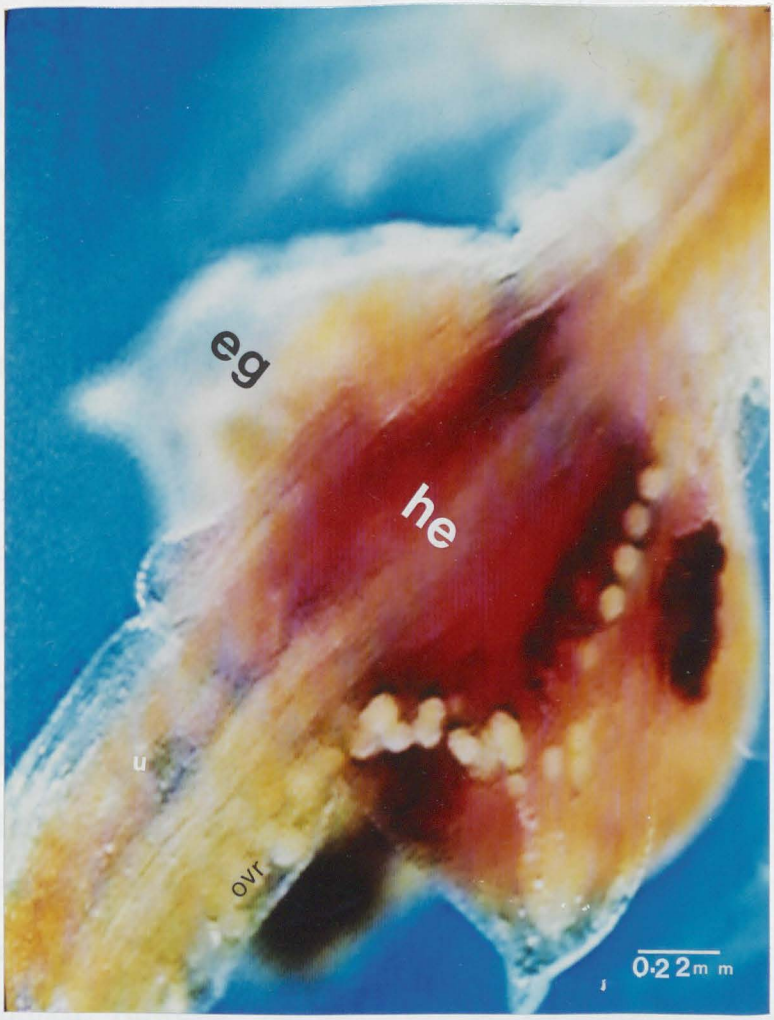
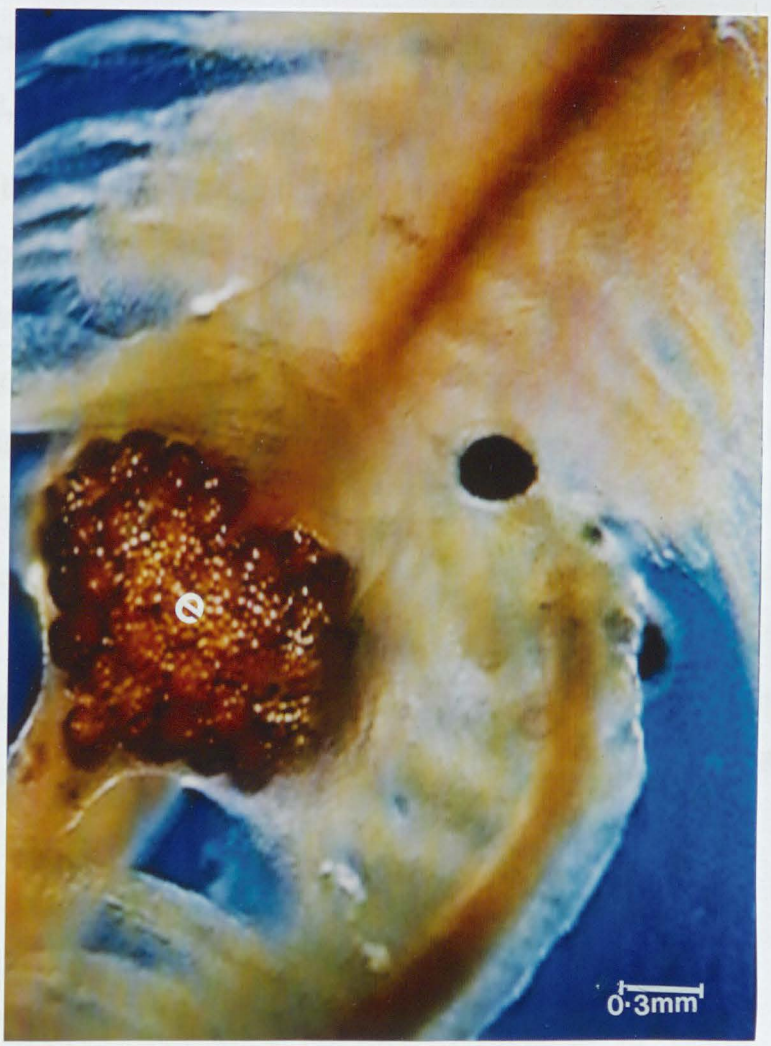

e
eg
0·26mm

Figure 3.20 Representative photomicrographs of ovaries (ovr) and egg sac (eg) of isolated female (A - dorsal view) and sexually reproducing females (B - ventral view) of *A. franciscana* cultured under hypoxia. e = egg, he = hemoglobin, Scale is shown on photograph.


eg
he
ovr
0·22m m


e
0·3mm

through' the potentially hypoxia-sensitive stages 5 - 9. These stages contain many of the key events of organogenesis as described above (Section 3.1.1) including heart and gill formation. Accelerated growth through early critical developmental stages was also found by Weisz (1946) when he studied the effect of the salinity on the growth rate of *Artemia*. He found that, during the early stages of development, the nauplii grew more rapidly in more concentrated medium (which in passing also possessed a lower concentration than more dilute media) but, as found in this present study growth 'slowed down' just before the individuals became sexually mature. Furthermore, also as found here, the initial increase in developmental rate noted by Weisz (1946) was achieved without compromising growth (as measured by total body length and total weight), e.g. in this study hypoxia cultured stage 4 individuals still had the same length and weight as control stage 4 individuals but they developed in a much shorter time from hatching than the controls. It should be noted, however, that 'rushing through' these sensitive developmental stages in *A. franciscana* was also accompanied by substantial mortality. The greatest mortality was associated with the period in which a functional heart and gills were formed. However, the fact that mortality was unaffected by hypoxic acclimation, indicated that the stages themselves are sensitive to the developmental process 'going wrong' and, interestingly, this was not exacerbated by hypoxia.

Effectively the early developmental iternary was telescoped into a much shorter time period. Better growth in hypoxia, compared with normoxia, has been demonstrated before but mainly for animals that inhabit (near) chronically hypoxic environments such as the freshwater oligochaete *Tubifex*, the freshwater snail *Planorbis* and larval chironomids and others (Huss, 1913; Juday, 1908; Leitch, 1916; Collip, 1920; Berkeley, 1921; Ewer, 1931; Brundin, 1951; Fox, 1945, 1954; Fox & Taylor, 1954). Also these studies have mainly considered growth rather than development. In general, for most other, primarily aerobic, marine and freshwater animals, exposure to chronic hypoxia resulted in a decrease in development rate or growth rate or even both (Green, 1956; Gilchrist, 1959). This is quite different from what is described here for *A. franciscana*. However hypoxia-related increased developmental rate in these brine shrimp is not the whole story.

Before *A. franciscana* reached sexual maturity, the developmental rate had slowed down and now was not significantly different from normoxic controls. Furthermore, while the proportion of hypoxia cultured *A. franciscana* reaching sexual maturity was the same as normoxic controls, a greater proportion of normoxic animals reached sexual maturity earlier. Thus it could be suggested that the 'cost' of accelerated development early in ontogeny is paid for by a slowing down in development immediately prior to sexual maturity; such life history trade-offs are not uncommon (Sibly & Calow, 1986).

3.4.2 Hemoglobin induction

Induction of invertebrate hemoglobins by exposure to low oxygen is well known and extensively documented (Fox, 1949, 1954 ; Fox & Phear, 1953; Chandler, 1954; Hoshi & Kobayashi, 1972; Weber, 1980; Kobayashi & Hoshi, 1982; Calvalho, 1984). Such is, usually, taken as being of respiratory significance. Possessing a greater concentration of a respiratory pigment by an animal acclimated to hypoxic conditions may result in it possessing a greater ability to sustain oxygen uptake at low oxygen tensions (Weber, 1980; Mangum, 1990; but see Chapter 4). However, it is also often the case that modification of the oxygen affinity, or the manner in which the pigment binds oxygen is equally important (Mangum, 1990). In the case of *Artemia* we know from the literature that acclimation to low oxygen produces a higher affinity pigment than is found in control animals (Heip *et al.*, 1978, 1980; Vos *et al.*, 1979). There is no reason why this should not also be the case for *Artemia franciscana* studied here (cf. Chapter 6).

What was interesting in this present study was that not only did the hemolymph of individuals cultured under hypoxia stain for hemoglobin but so too did many of their tissues (discussed more fully in Chapter 6). This could indicate that these animals generate an intracellular respiratory pigment that would aid in oxygen diffusion during exposure to, or culture under, hypoxic conditions. The fact that the nervous tissue examined stained for hemoglobin in both normoxic and hypoxic cultured individuals, and that hypoxic culture did not seem to result in an increase in the concentrations in this tissue, is interesting. This may

reflect the fact that nervous tissue in this animal is highly aerobic and even under normoxic conditions requires respiratory pigment to ensure adequate oxygenation of these tissues.

3.4.3 Hypoxia, reproduction and hemoglobin provisioning

Female brine shrimp have paired ovaries from which the egg passes into a pair of lateral pouches (ovisacs), where fertilization takes place, and then onto the median brood pouch (uterus). Finally either eggs or nauplii are liberated from the maternal brood pouch. Under the normoxic culture conditions used in this study both oviparity and ovoviviparity were recorded for the brine shrimp *Artemia franciscana*. In fact sometimes both nauplii and eggs were produced in the same brood. This was similar to what was found by Gilchrist (1959) who when rearing *Artemia* in sea water noted that while, in the main, females reproduced ovoviviparously, occasionally females produced shelled eggs, which she referred to as 'resting eggs'. Production of both types of offspring seems to be a reasonably common feature of both temperate (Saiah & Perrin, 1990) and tropical brine shrimp (Hildrew, 1985) and has been referred to as 'bet-hedging' in the face of an unpredictable and variable environment. Certainly there was no fixed pattern for the type of deposition noted during this study as documented by Ballardin and Metalli (1963). They observed that parthenogenetic diploid *Artemia* from S. Gilla, when subjected to hypo-illumination, reproduced ovoviviparously on the first deposition and oviparously on the subsequent depositions.

When cultured under hypoxic conditions oviparity became the dominant reproductive pattern accounting for about 80 % of the total reproductive output although the total output of offspring itself was depressed when compared with normoxic controls. Interestingly the hypoxia-related decrease in reproductive output was not achieved by merely producing fewer cysts and live young; the number of cysts produced was independent of the oxygenation status of the environment, the lower total reproductive output being accounted for by the hypoxic cultured female greatly reducing (or ceasing) the production of free swimming nauplii. In other words the response to hypoxia is not a shift from ovoviviparity to oviparity but instead a cessation of ovoviparity, in an animal which under 'normal conditions' is both oviparous and

ovoviviparous. Whereas production of both cysts and live young by brine shrimp can be seen as 'bet-hedging' when faced with an unpredictable environment (Saiah & Perrin, 1990) presumably the hypoxia-generated shift to oviparity means that, the animal no longer considers that bet-hedging is worthwhile in what has become a 'predictable' environment.

Mothers cultured under hypoxia had a greater concentration of hemoglobin present both in their hemolymph and in the tissues generally. This increase in hemoglobin concentration was most marked in the ovaries and the uterus where the eggs were 'immersed' in hemoglobin. There could be two (non-exclusive) explanations for this observation. Firstly the hemoglobin could be producing a localised high oxygen environment that may be required for successful egg development, in a generally hypoxic environment. This is likely to be the case even if the primary stimulus for hemoglobin production lies elsewhere. Secondly, the female may be 'provisioning' the young with hemoglobin as a respiratory pigment or perhaps even as a source of material for egg construction. This idea is strengthened by the fact that the uterine hemoglobin 'disappeared' during egg development and the eggs and/or cysts became more red coloured with increasing development. From this it may be inferred that there was a transfer of hemoglobin (or some product of hemoglobin) from the mother to the offspring: The 'reddest' mothers produced the 'reddest' eggs. This may also lie behind the fact that adult females cultured under hypoxia seem to have a greater concentration of hemoglobin than adult males from the same culture. the mother requires more not for her own respiratory needs but to provision the offspring.

Furthermore, based on the results of the experiments with isolated females, it is suggested that fertilisation is, in some way, critical to the provisioning by the mother of eggs, produced under hypoxia, with hemoglobin; in the absence of fertilisation the hemoglobin in the ovaries and uterus continued to increase in concentration, and did not decrease, during egg/cyst development and the non-viable offspring remained pale in colour. The respiratory significance of this provisioning is examined experimentally in Section 4.4.3 and discussed in detail in the concluding chapter (Section 6.2).

3.4.4 Conclusions

Culture under chronic hypoxia did not result in impairment of growth and development, at least initially. In fact the principle effect of hypoxia was to accelerate development through the early more vulnerable developmental stages, while still producing sexually mature adults of the same size and weight. There was evidence, however, that there were 'costs' associated with bringing the developmental internary forward in time; in general it took longer, overall, for the majority of the individuals, cultured under hypoxic conditions, to come to sexual maturity and the total number of offspring was dramatically reduced. Culture under chronic hypoxia did not result in any significant deviations from the controls, contrary to our predictions.

Hypoxic culture resulted in brine shrimp being better provisioned with respiratory pigment both within their hemolymph and within their tissues. This was more noticeable in females when compared with males.

Hypoxic culture resulted in a shift from a mixed reproductive strategy(ovoviviparity and oviparity) to oviparity alone, with the number of cysts produced by any female, on average, being independent of the oxygenation status of the environment. Furthermore mothers cultured under hypoxic conditions provisioned their offspring (and the local enclosed environment of the offspring) with hemoglobin. The provisioning appears to be linked with post-fertilization events within the egg.

Chapter 4. Effect of Hypoxia on Whole Animal Oxygen Uptake

4.1 Introduction

4.1.1 Hypoxia and oxygen uptake by aquatic crustaceans

The effect of acute exposure to environmental hypoxia on the oxygen uptake of crustaceans has been well studied, at least in adult stages. All crustacean species examined to date can be roughly divided into one of two groups (two ends of a sliding scale) with respect to their ability to maintain oxygen uptake under conditions of declining oxygen tensions. They are either conformers, that is animals in which oxygen uptake decreases as environmental oxygen tensions decreases, or they are regulators. Regulators are able, at least to some degree (and this varies from species to species as well as depending on the environmental history of the group) to maintain a constant oxygen uptake down to a critical oxygen tension (Bridges & Brand, 1980a,b cf. annelids, Mangum, 1970 and molluscs, Bayne, 1971; Taylor & Brand, 1975). This critical oxygen tension is referred to as the P_c point. After this point is reached oxygen uptake decreases with decreasing oxygen tensions. The effect of intrinsic and extrinsic factors on P_c have been discussed at great length (Mangum & van Winkle, 1973; Herreid, 1980; Aldrich, 1986; Aldrich & Regnault, 1990; Spicer, 1995b) as have been the methods proposed for calculating P_c (e.g. Tang, 1933; Bridges & Brand, 1980a). However many of the problems associated with the calculation and value of P_c are still unresolved.

How crustacean regulators manage to maintain oxygen uptake during acute exposure to declining oxygen tensions is relatively well understood and well documented. The most common responses of regulators are that there is an increase in ventilation rate, and also an increase in heart stroke volume (although not necessarily heart rate) in response to

acute hypoxia (Arudpragasam & Naylor, 1964; Johansen *et al.*, 1970; Taylor *et al.*, 1973; McMahon & Wilkens, 1975, 1977, 1983; Taylor, 1976; Butler *et al.*, 1978; Jouve & Truchot, 1978; Wheatly & Taylor, 1981; Taylor, 1982; Bradford & Taylor, 1982; Hagerman & Uglow, 1985; Taylor & Spicer, 1989; Airriess & McMahon, 1994). There is also some evidence that there may be changes in the hemolymph micro environment that influence oxygen binding by the respiratory pigment (when present) and result in more efficient oxygen transport during acute hypoxia (Mangum, 1983, 1990).

During exposure to more prolonged hypoxic stress the role of ventilatory and perfusion responses, so prominent under conditions of acute exposure, are replaced by, at least in some cases, more efficient oxygen transport (Butler *et al.*, 1978, Mangum, 1983, 1990). This is brought about by both by changes intrinsic to the respiratory pigment itself as well as (to a lesser extent) by modifications of hemolymph constituents. Very little attention has been given to other possible respiratory responses to hypoxia (rather than anoxia), e.g. the possibility of reducing metabolic demand (hypometabolism), supplementing aerobic with anaerobic metabolism.

If the effect of prolonged exposure to hypoxia on oxygen uptake has received comparatively little attention, when compared with acute exposure, then the effect of periodic hypoxia has remained completely unexamined, despite the fact that, if brine shrimp encounter hypoxia in their natural environment it will most likely be periodic in nature (Section 2.3.2.3).

4.1.2 Changes in oxygen uptake, and its responses to hypoxia, in *A. franciscana* during development

Most studies of oxygen uptake by brine shrimp, and their response to hypoxia have been for adult individuals, using single factor experiments, where only oxygen was

manipulated (Kuenen, 1939; Eliassen, 1952; Gilchrist, 1956, 1959; Conover, 1960; Dutrieu, 1960; Mitchell & Geddes, 1977; Vos *et al.*, 1979; Decleir *et al.*, 1980, 1989; Herbst & Dana, 1980). However, some other, comparatively recent, studies take a multifactorial approach, investigating the possibility of describing interrelations between environmental factors. For example, Lange *et al.* (1972) showed that the rate of oxygen uptake in saline waters varied proportionally to the solubility of oxygen and De Wachter & Den Abbeele (1990) alleged that the oxygen concentration was a more important factor than the oxygen tension in determining the rate of oxygen uptake in different strength saline waters. In all of these studies brine shrimp have been shown to possess very well developed powers to regulate their oxygen uptake under conditions of declining oxygen tensions.

Despite our good knowledge of adult physiology, we have no *a priori* reason to believe that by studying the effect of hypoxia on a late developmental stage we have an entire picture for the species as a whole. It would not be surprising if early developmental stages respond to an environmental challenge differently from adults due to both qualitative (e.g. organogenesis) and quantitative (size difference) differences and resultant physiological demands (Burggren, 1992). Unfortunately while we have a lot of information on the effects of environmental factors on the oxygen uptake of adult brine shrimp we have only a handful of studies of the respiration of nauplii and the physiological strategies employed by individuals at different stages of their life cycle (Eliassen, 1952; Bertalanffy & Krywienczyk, 1953; Zeuthen, 1953; Dutrieu, 1960; Engel & Angelovic, 1968; Bernaerts *et al.*, 1981; Varo *et al.*, 1991, 1993; Hemamalini & Munuswamy, 1994; Spicer, 1995c).

Eliassen (1952) was the first to investigate variations in metabolic rate (oxygen uptake) of *Artemia salina* with the body size over the whole size-range (different developmental stages?). From his results he emphasised the great influence of seasonal variation upon the

smaller nauplii and that the effect of this decreased with increasing size and age. Bernaerts *et al.* (1981) measured the oxygen uptake *Artemia* during the whole ontogeny (life cycle lasted approximately 38 d). They found that maximum oxygen uptake occurred during a moulting phase 26 h after hatching. Thereafter oxygen uptake decreased slowly until the adult rate was attained. The ability to regulate oxygen uptake during exposure to declining oxygen tensions seems to appear very early in the ontogeny of *Artemia* (Varo *et al.*, 1991, 1993; Spicer, 1995c) although, due to difficulties in comparing studies, it is difficult to say categorically if this is as well developed as found in the adult.

Even with this handful of studies, both the scarcity and incompleteness of our information about these early life stages can lead to misunderstanding and consequently misinterpretation of what is actually happening during ontogeny. For example, Eliassen (1952), who was one of the first to study metabolism during ontogeny in brine shrimp, neglected the first 3 days of development. Engel & Angelovic (1968) and Varo *et al.* (1991, 1993) have studied only 'one day old' nauplii and did not appear to consider the possibility that these nauplii may have been at different developmental stages. Consequently, we still require a complete description of how oxygen uptake in brine shrimp changes during ontogeny. Furthermore we still need to know when the ability to regulate oxygen uptake during declining oxygen tensions appears and how this ability changes during ontogeny, never mind an examination of the effect of acclimation to different oxygen tensions on these processes.

4.1.3 Brine shrimp hemoglobins

The fluid circulated in the hemolymph vessels and spaces of anostracan brine shrimp is known to contain the respiratory pigment hemoglobin (Fox, 1945; Bowen *et al.*, 1966, 1969; Moens *et al.*, 1991). This is also the case for conchostracans such as the water flea *Daphnia* (Fox, 1945, Hildemann & Keighley, 1955; Mangum, 1983, 1990) The other

crustaceans that possess a respiratory pigment are primarily malacostracans (lobsters, crabs, shrimps) and they possess a different type of pigment, hemocyanin, which has copper as its base, rather than iron as in the case of hemoglobin (Mangum, 1983, Truchot, 1993b). Respiratory pigments, such as hemoglobin are known to bind oxygen reversibly, and co-operatively, in a fashion that is presumed to enhance oxygen uptake, transport and delivery to the tissues. The structural and functional properties of brine shrimp hemoglobins have been extensively studied, as has the effect of environmental factors on these properties, and they have been reviewed, if not very critically, by Moens *et al.* (1991). Fox (1947) was the first to notice that hemoglobin synthesis in invertebrates could be stimulated by exposure to low oxygen. Gilchrist (1954) some years later found that adult *Artemia salina*, cultured for 2 - 3 weeks at 18 - 20 °C in water only 10 - 20 % saturated with air, developed 'pink blood' which showed strong oxyhemoglobin absorption bands.

In adult brine shrimp the latest view is that there at least three types of hemoglobin subunit, which in turn give rise to at least 10 phenotypes on the basis of the presence and mixture of these subunits (Vinogradov *et al.*, 1993). Furthermore these phenotypes have been shown to change during ontogeny (Moens *et al.*, 1991) although many more focused studies are required to substantiate this finding. There appears to be a transition from a high to a low oxygen affinity pigment some time during development. It is known that, at least in adults, acclimation to hypoxia increases the production of one hemoglobin subunit, Type III. This particular subunit possesses a greater affinity for oxygen than the remaining two subunits (D'Hondt *et al.*, 1978; Heip *et al.*, 1978; Weber, 1980). This is very similar to the recent work on the waterflea *Daphnia* which, as mentioned above, also possesses extracellular hemoglobins. This work has shown that individual waterfleas develop, during ontogeny, the ability to respond to hypoxia by both increasing the content and oxygen affinity of the hemoglobins present (Kobayashi, 1982; Kobayashi *et al.*, 1987, 1990).

The extent to which hemoglobins function in gas transport in various invertebrates remains the subject of continuing debate due to the fact that 1) hemoglobin-containing species show tremendous variation in pigment concentration even between individuals in the same culture and at different times during ontogeny. 2) this pigment occurs sporadically amongst invertebrates that are exposed to low oxygen tension in nature. On the other hand we cannot neglect the fact that the spectacular diversity of hemoglobin structure in invertebrates may reflect adaptive divergence to a wide range of environmental conditions (Vinogradov *et al.*, 1993). Also there are a number of studies showing the function of invertebrate hemoglobins *in vivo* and, using carbon monoxide (CO) to block the oxygen binding function of hemoglobins, the importance of this molecule in maintaining oxygen uptake (Gilchrist, 1954; Weber, 1980).

Given that we know that the structure, function and concentration (Sections 3.4.2, 3.4.3) of brine shrimp hemoglobins can change during ontogeny, the question remains - does it make any difference? In other words to what extent does brine shrimp hemoglobin contribute to the maintenance of oxygen uptake during ontogeny, both under normoxic and hypoxic conditions. Gilchrist (1954), using CO to inhibit hemoglobin function, showed that proper functioning of this respiratory pigment was essential for maintaining adult oxygen uptake even under normoxic conditions. However, that changes in hemoglobin structure and function have any influence on the development of oxygen uptake in brine shrimp has yet to be demonstrated experimentally.

4.1.4 Aims of study

On the basis of what has gone before the aims of this chapter are as follows.

1. Changes in oxygen uptake (and particular its response to declining oxygen tensions) during ontogeny in *Artemia franciscana* will be followed and recorded in

detail through-out its entire life cycle, from hatching to sexual maturity. Exactly how these changes are related to developmental stage, animal size, sex and the environment of the parent will be given special consideration. The hypotheses to be tested are that a) oxygen uptake can be predicted on an allometric basis, and on the basis of size alone, i.e. developmental stage *per se* does not affect oxygen uptake and b) as newly hatched nauplii and adults co-occur in the same ephemeral pool both should possess equally well developed powers to regulate oxygen uptake when exposed to declining oxygen tensions, i.e. the null hypothesis, P_c will not change during ontogeny.

2. The effect of culture under hypoxic conditions on the changes in oxygen uptake (and response to declining oxygen tensions) during ontogeny will be examined and compared. Both chronic and periodic hypoxia will be examined, the former because it is easy to maintain, the latter because, arguably, it most closely resembles what these animals would experience in their natural environment. The hypotheses to be tested are a) that animals cultured under hypoxia should show a reduced rate of oxygen uptake when compared with animals cultured under normoxia as a way of resorting to hypometabolism and so 'saving energy'. b) that animals cultured under hypoxia should show an improvement in their ability to maintain oxygen uptake during exposure to declining oxygen tensions, i.e. P_c should decrease, when compared with animals cultured under normoxia thereby increasing the chances of survival in a hypoxic environment. c) that the respiratory performance (oxygen uptake, P_c) of individuals exposed to periodic hypoxia should be intermediate to those exposed to normoxia and chronic hypoxia, indicating that hypoxia-related changes in respiratory performance are related to the duration, not the pattern (i.e. periodic) of exposure to hypoxia.

3. The effect of CO (which irreversibly binds to hemoglobin and prevents it from loading oxygen) on individuals, at different developmental stages and from different culture (normoxia, periodic and chronic hypoxia) conditions will be examined to ascertain the importance of this respiratory pigment in maintaining oxygen uptake during exposure to declining oxygen tensions. The hypothesis to be tested is that if hemoglobin is essential to maintaining rates of oxygen uptake, of different developmental stages under different experimental conditions, this maintenance will be compromised after treatment with CO. Furthermore if newly-hatched individuals receive hemoglobin from their parent, there should be a difference in the effect of CO on the respiratory response of individuals from hemoglobin 'rich' and individuals from hemoglobin 'poor' parents.

4. The capacity to survive anoxia (i.e. no oxygen) of individuals cultured under normoxia and hypoxia will be examine. The hypothesis to be tested is that culture under hypoxia will result in an increase in tolerance to anoxia. This may either be because individuals cultured under hypoxia a) have greater powers of anaerobiosis or b) contain more hemoglobin which acts as a store for oxygen.

4.2 Material and methods

4.2.1 Measurement of oxygen uptake

The oxygen uptake during declining oxygen tensions, of individuals of different developmental stages and from different experimental treatments was examined using a closed respirometer technique. The closed respirometer used was the RC 300 microrespirometer (Strathkelvin Instruments, Glasgow) which had a modifiable chamber volume (200 - 1000 µl). This technique and apparatus employed is now well established

has been used previously for examining the oxygen uptake of *Artemia* individually and in groups of 100 or more (Varo *et al.*, 1991; Spicer, 1995c).

Either groups of individuals (of equivalent weight and developmental stage) or individual specimens (in the case of adults) were placed into the respirometer chamber and left to acclimate to experimental conditions for 5 min in each case. The water in the chamber was sterile, being identical to that used in the appropriate culture technique, constructed from autoclaved distilled water and sea salts (see Section 3.2.1 for details). The chamber was sealed and the individual(s) allowed to deplete the available oxygen. The rate of oxygen depletion was followed on a chart recorder down to about 5 % of normoxic saturation. After this time the experiment was terminated and the dry weight of the animal(s) determined as mentioned previously (Section 3.2.2). Oxygen uptake under conditions of declining oxygen tensions was then calculated and expressed as either $\mu l\ 0_2.h^{-1}$ or $\mu l\ 0_2.mg^{-1}.h^{-1}$. Each run lasted < 1.5 h, the exact time depending on the chamber volume used. After the completion of each run the respirometer chamber was cleaned using absolute alcohol and then rinsed with distilled water. This reduced gradual contamination of the respirometer chamber by micro-organisms. The critical oxygen tension, or P_c, was defined as the point (expressed in kPa) at which the ability to maintain a constant rate of oxygen uptake ceased. This was estimated from a visual examination of the chart recorder trace.

4.2.2 Use of CO to block hemoglobin function

In order to assess the importance of the respiratory pigment hemoglobin in maintaining the oxygen uptake of brine shrimp during exposure to declining oxygen tensions the following experiment was carried out. *Artemia franciscana* were pre-exposed to carbon monoxide (CO), a gas which binds irreversibly to hemoglobin and prevents the molecule

functioning in gas exchange, following very closely the method used previously on *Artemia salina* (Gilchrist, 1954).

Brine shrimp (developmental stages 0 (from hypoxic and normoxic parents), 3, 6 and late (cultured under either normoxia or chronic hypoxia) were kept in total darkness (for 40 min in the case of nauplii and 60 min in adults) in flasks of well-aerated artificial sea water (vol. = 22 ml) to which sufficient CO-saturated artificial sea water (1 ml) had been added to make the partial pressure of carbon monoxide approximately one-sixth that of the dissolved oxygen. This method was sufficient to eliminate the ability of hemoglobin to reversibly bind oxygen but avoid any potential inhibition of cytochrome *c* oxidase activity. Controls were also run where the experimental protocol was followed as outlined above but no CO was added. After being treated with CO, the oxygen uptake of brine shrimp during conditions of declining oxygen tension was measured as described above (Section 4.2.1). At the end of each set of experiments the presence of carboxyhemoglobin in the blood was verified visually, under low power magnification (x 10) by placing individuals on an indented glass slide supporting a few drops of a solution of sodium dithionite ($Na_2S_2O_4$, 100 $mmol.l^{-1}$). This solution contains no dissolved oxygen, and any oxyhemoglobin present in the blood would be quickly deoxygenated.

4.2.3 Tolerance to severe hypoxia

Tolerance to anoxia of individuals (males and females) cultured under normoxia and hypoxia was compared as follows. To allow comparability with published data, the experimental protocol employed was as close as possible to the method used to examine anoxic tolerance of the water flea *Daphnia magna* (Fox *et al.*, 1951) and *Artemia salina* (Gilchrist, 1954). Individuals (n = 5, six replicates) from each experimental treatment were transferred to a) a glass stoppered vessel (volume = 500 ml) completely filled with artificial sea water with a low dissolved oxygen content or b) an open dish of fully aerated

artificial sea water. For each of the experimental bottles the oxygen tension of the artificial sea water was lowered by bubbling nitrogen through it. The oxygen tension was reduced to a level just above that at which the hemoglobin of *A. franciscana* became deoxygenated. This level, 4 to 4.9 kPa, was estimated by recording when the hemoglobin became deoxygenated using a hand-held spectroscope as described by Fox *et al.* (1951). All experiments were carried out at room temperature (T = 24 - 26 °C) and no food was given. The mortality in both control and experimental treatments was then assessed visually. Individuals were termed dead when both locomotory and cardiac movements ceased. The time at which 50 % of the individuals in any particular treatment died (if reached) was then determined graphically. The oxygen tension of the water in each of the vessels was measured at the beginning and at the end of the experiment using a microelectrode, thermostatted at the experimental temperature.

4.2.4 Effect of density on oxygen uptake determinations

During preliminary attempts at measuring the oxygen uptake of different developmental stages, it was noticed that animal density could have an important influence on the value calculated for oxygen uptake by individuals. This was only really a potential concern for the earliest developmental stages. Consequently the densities employed when early developmental stages were examined were set by the least number of individuals required for any density dependent effects to be counteracted. Estimates of P_c were independent of density.

4.3 Results

4.3.1 Effect of culture under chronic and periodic hypoxia on oxygen uptake during exposure to declining oxygen tensions.

4.3.1.1 Changes with development

Presented in Figure 4.1 are some representative figures, each from an individual experimental run, that illustrate the type of relationship that exists between oxygen uptake and environmental oxygen tension in *Artemia franciscana*, cultured at 28°C under normoxic conditions, at different stages of development. It can be seen that the oxygen uptake for each stage was regulated over a very wide range of oxygen tensions and only begins to break down below 9 kPa. Furthermore, the range over which regulation was possible seemed to become wider as the animals develop, i.e. the critical oxygen tension, or P_c point, decreased in value. That animals earlier in development possessed a rate of oxygen uptake greater than older animals, as observed here, was not unexpected given that smaller animals would be expected to have a greater weight-specific metabolism. However, the figure presented here tells us little of the nature of the relationship that exists between body weight and oxygen uptake.

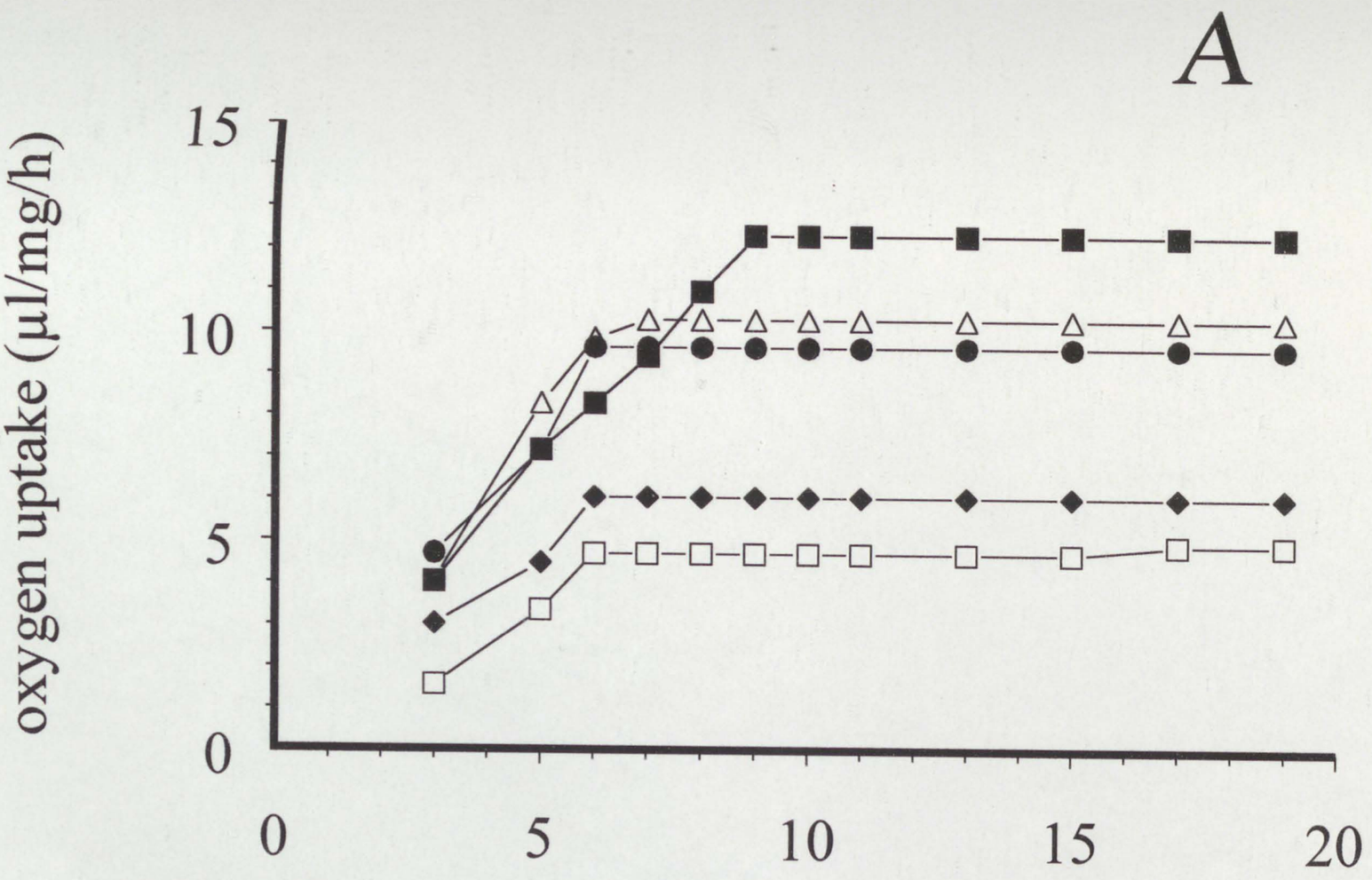
4.3.1.2 Effect of body weight on oxygen uptake of animals cultured under different hypoxic regimes

Presented in Figure 4.2 is the relationship between oxygen uptake and body weight, after double logarithmic transformation, for *A. franciscana* cultured under each of the experimental conditions; normoxia, chronic hypoxia and periodic hypoxia. In each case there was a significant relationship between oxygen uptake and body weight (r^2 = 0.971 (d.f. = 1,122), 0.988 (d.f. = 1,104) and 0.915 (d.f. = 1,113) respectively, all significant at

Figure 4.1 Oxygen uptake of representative individuals, at different developmental stages, of *A. franciscana*, under conditions of declining oxygen tensions (T°C = 28, S = 35 ‰).

A. Relationship between oxygen uptake and oxygen tension for different stages Developmental stages: 0 = ■; 3 = Δ; 6 = ●; 10 = ◆; late = □.

B. Chart recordings for each of the developmental stages (illustrated) referred to above showing the critical oxygen tensions (P_c points).


A
oxygen uptake (µl/mg/h)
15
10
5
0
0
5
10
15
20
oxygen tension (kPa)

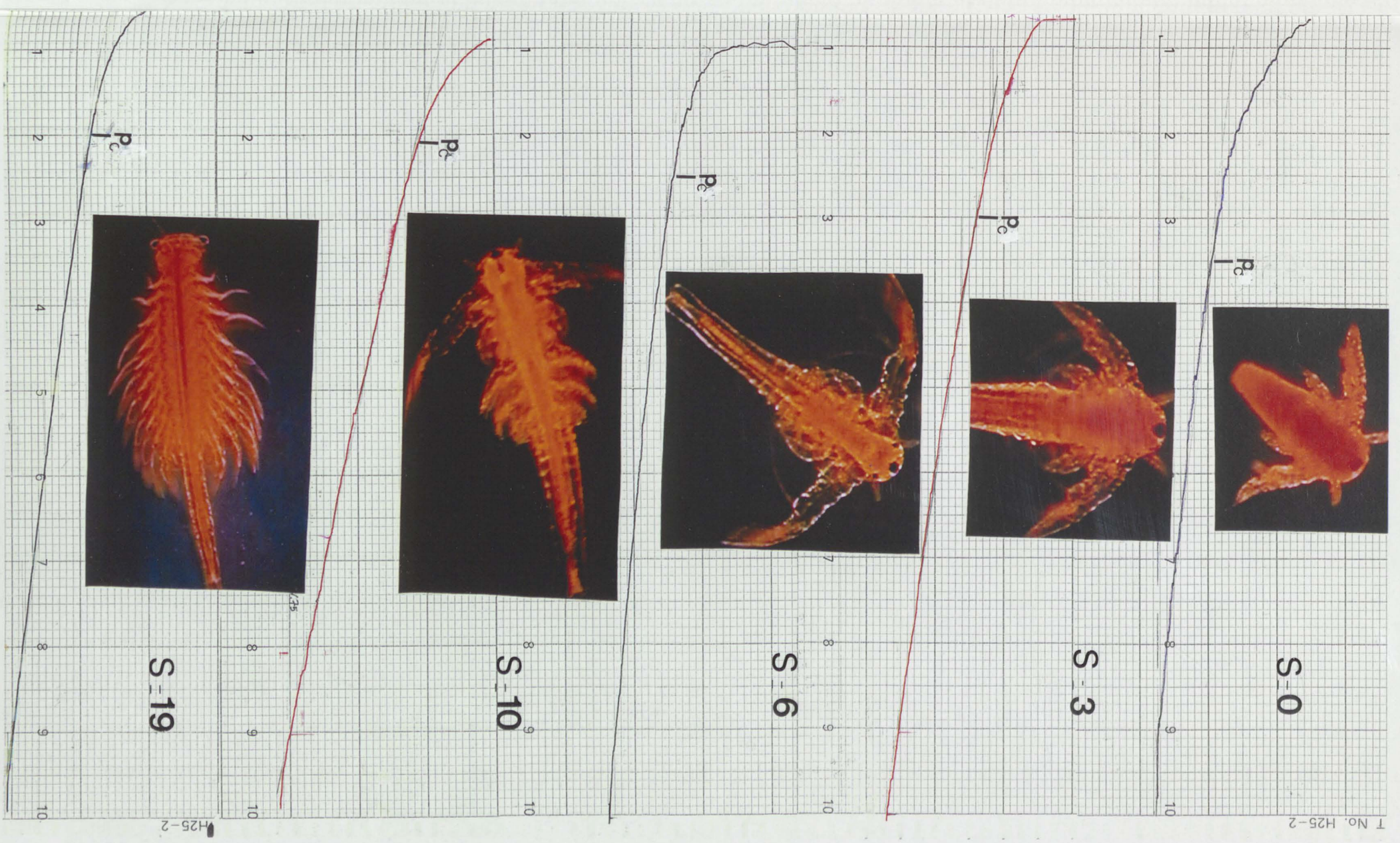
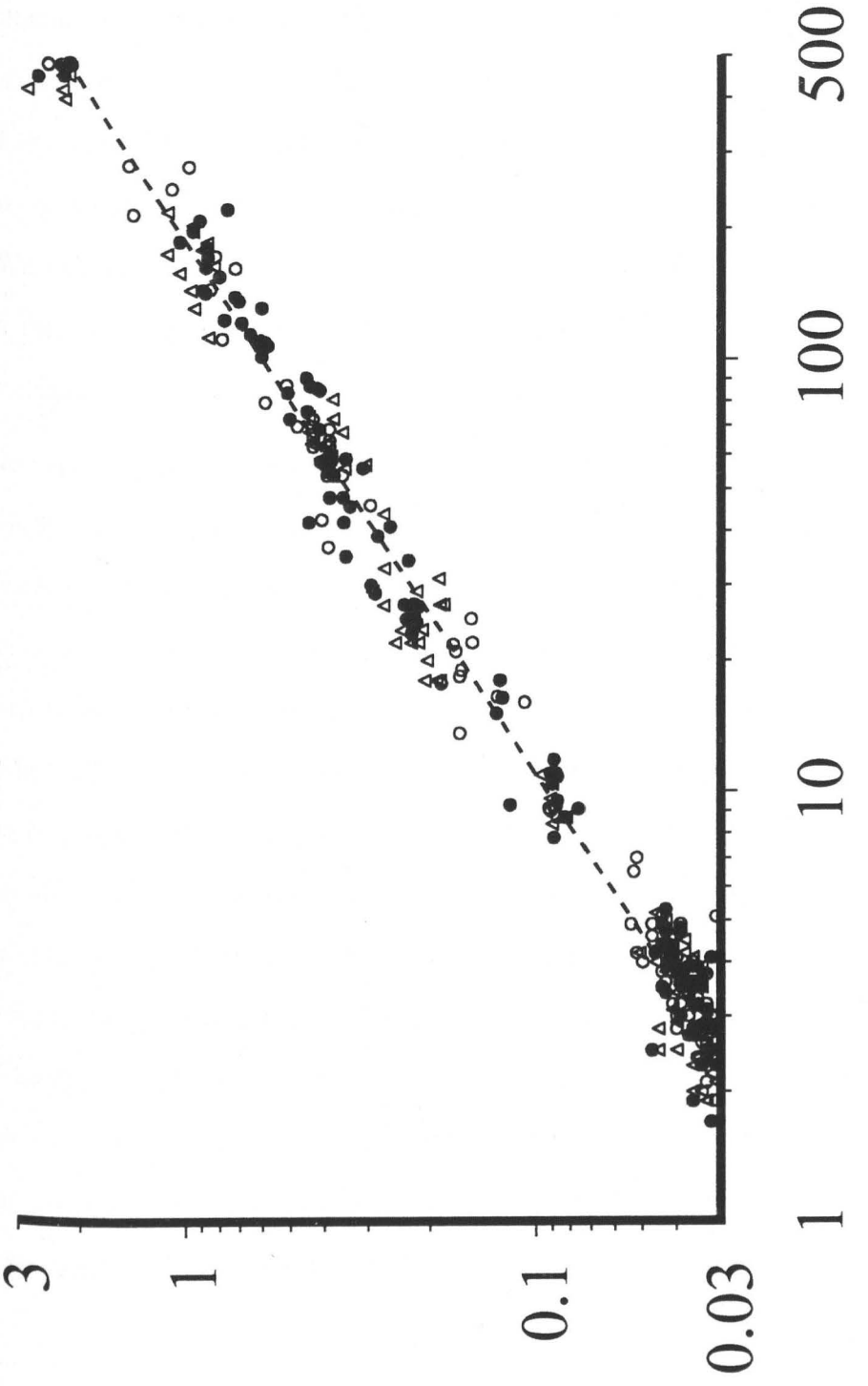

S_0
S_3
S_6
S_10
S_19
Pc

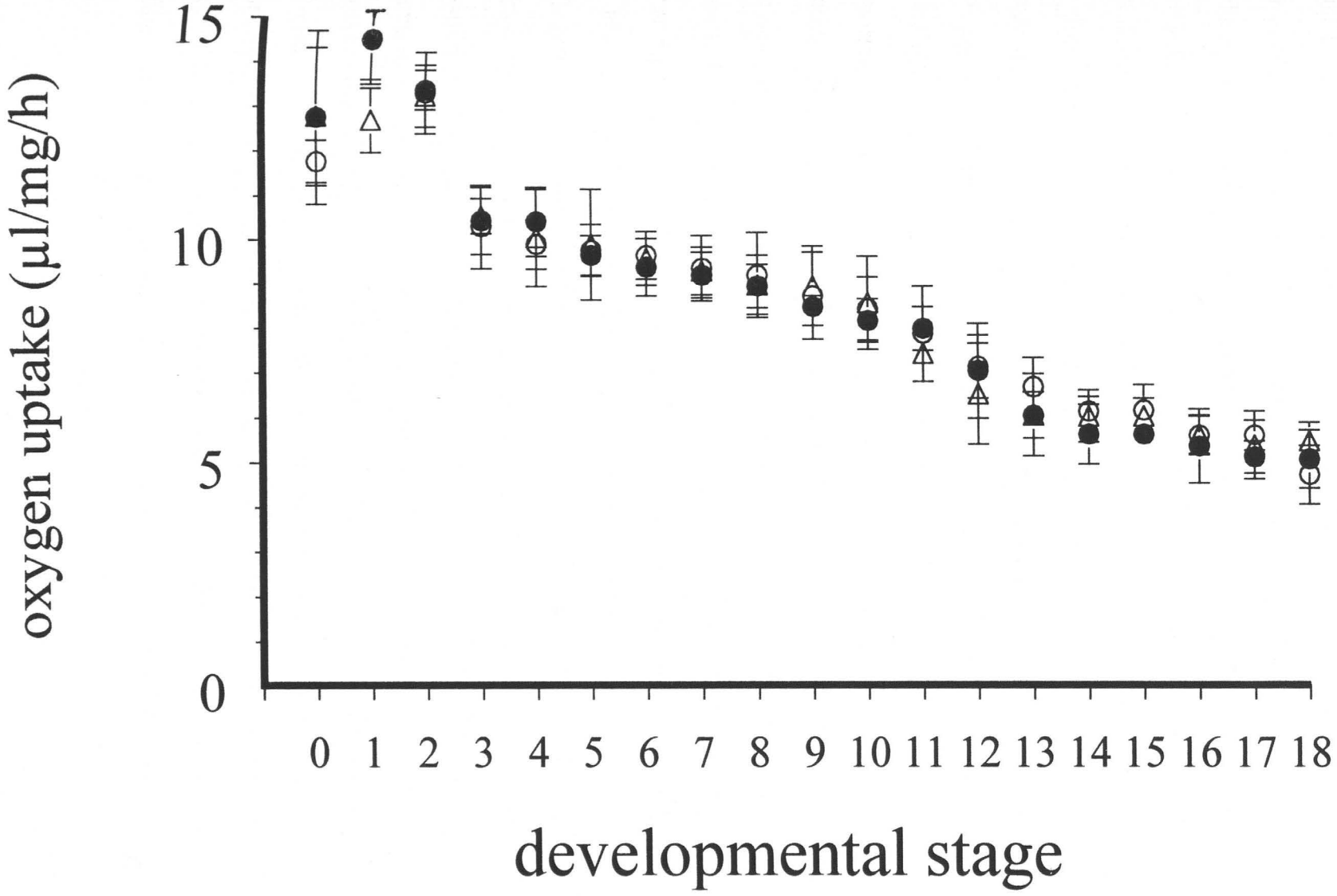
Figure 4.2 The relationship between oxygen uptake and body weight in individuals of *A. franciscana* (T°C = 28, S = 35 ‰) cultured under different oxygenation regimes: normoxia = ●; chronic hypoxia = ○; periodic hypoxia = Δ.


oxygen uptake (µl/h)
3
1
0.1
0.03
1
10
100
500
body weight (µg)

$P > 0.001$). Covariance analysis indicated that there was no significant difference in these relationships that could be attributed to culture conditions ($F_{(2,341)} = 2.04$ $P > 0.05$). The overall relationship between body weight (x - expressed as μg) and oxygen uptake (y - expressed as μl/h) can be described by the equation $y = 0.80\ x - 1.83$. As the weight exponent (b) was + 0.80 we can see that there was an inverse relationship between weight specific oxygen uptake and body weight and that the exponent (b') of that relationship was - 0.20. We conclude that the hypoxic regimes imposed in this study had no detectable effect on rate of oxygen uptake, at either the oxygen tension of normoxic or hypoxic culture conditions.

If we now examine oxygen uptake related to developmental stage, the picture that emerges is not so straight forward (Figure 4.3). While there was a decrease in weight-specific oxygen uptake with each successive developmental stage from Stage 1 onwards, there was an increase between Stages 0 and 1 and oxygen uptake values for Stages 1 and 2 appear to be much greater than we might have expected. The solution to this puzzle may be found in the fact that Stages 1 and 2 were not as heavy as Stage 0. Consequently over these first two stages (0 and 1) weight actually decreased with development, meaning that our graph looks 'out of synchrony' as development and growth (in terms of weight gain) were not synonymous. This is why when we plot oxygen uptake against weight (Figure 4.2) this 'irregularity' does not arise. There was, however, a significant difference between rates of oxygen uptake of Stage 1 nauplii cultured under different oxygen regimes (ANOVA $F_{(2,18)} = 20.9$, $P < 0.001$) that could not be explained by differences in weight: there was no significant difference in the weights of Stage 1 individuals cultured under each of the experimental conditions (ANOVA $F_{(2,18)} = 1.68$, $P > 0.05$).

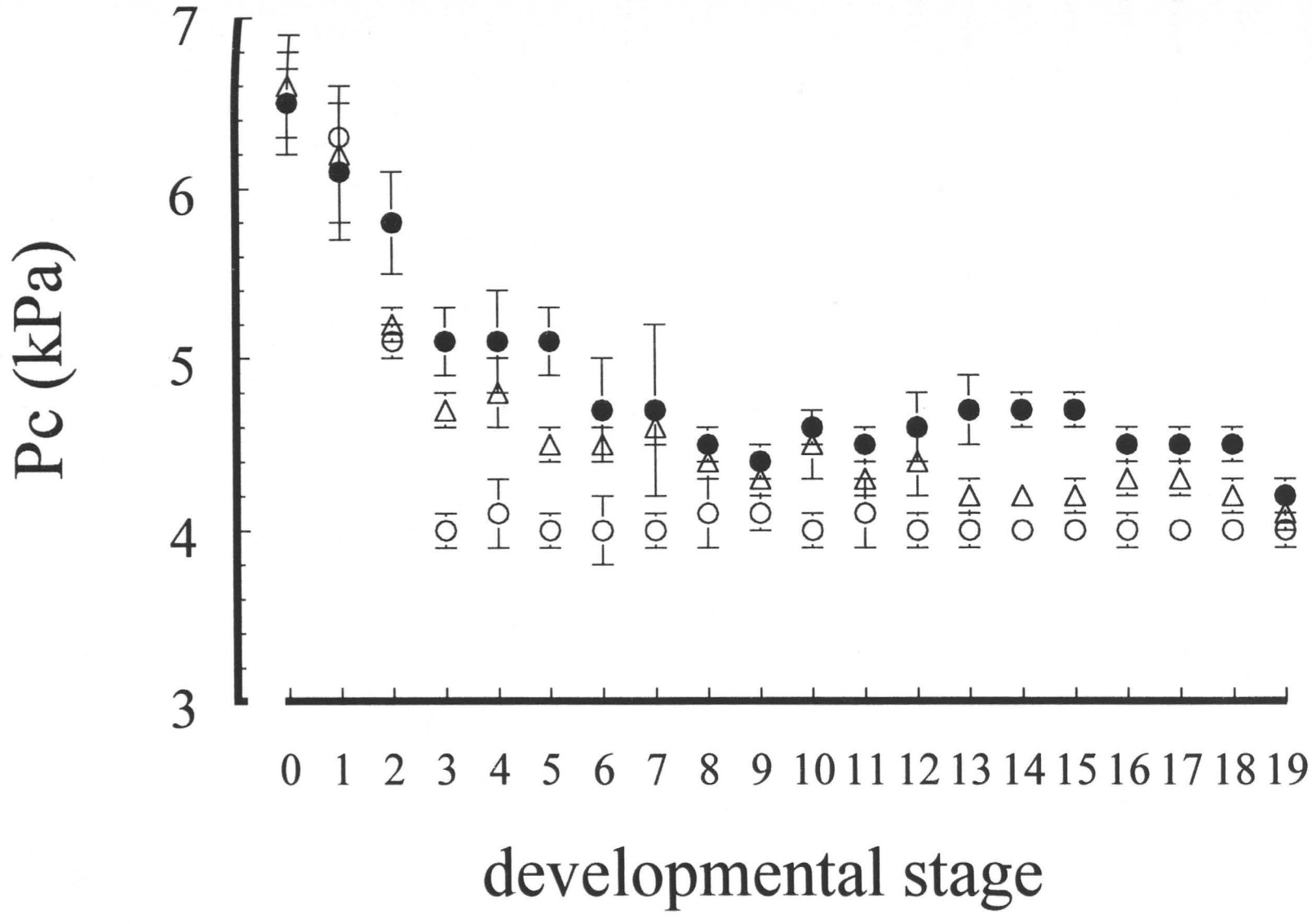
Figure 4.3 Weight-specific rates of oxygen uptake for *A. franciscana* (T°C = 28, S = 35 ‰) of different developmental stages, cultured under different oxygenation regimes: normoxia = ●; chronic hypoxia = ○; periodic hypoxia = Δ. Values are means ± 1 standard deviation of 5 - 16 determinations.


oxygen uptake (µl/mg/h)
15
10
5
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
developmental stage

4.3.1.3 Regulation of oxygen uptake and development

Presented in Figure 4.4 is the effect of culture under different hypoxia regimes on how the ability to regulate oxygen uptake over a wide range of environmental oxygen tensions changed during development. The overall pattern of change during development was very similar for individuals from each of the culture regimes. There was a steady increase in regulatory ability during early development (Stages 1 - 6), evidenced by a decrease in P_c. However from Stage 7 onwards, the established regulatory pattern varied little (with the possible exception of some stages cultured under normoxic conditions). Even though the general response was the same, there were also important and distinctive differences between individuals in the different culture regimes. Under normoxic culture conditions the P_c decreased from 6.5 ± 0.3 kPa for newly hatched individuals (Stage 0) to 4.6 ± 0.3 kPa in individuals at Stage 6. Thereafter there were no significant changes noted in P_c with development ($F_{(13, 91)} = 1.01$, $P > 0.05$, mean $P_c = 4.5 \pm 0.2$ kPa) except that the adult stage had a slightly improved P_c value of 4.2 ± 0.1 kPa. However in individuals cultured under chronic hypoxia the establishment of the adult pattern of regulation occurred much sooner: P_c decreased from 6.5 ± 0.2 kPa for newly hatched individuals (Stage 0) to 4.0 ± 0.1 kPa in individuals at Stage 3, and did not change significantly thereafter (ANOVA $F_{(15,84)} = 1.13$, $P > 0.05$) until the adult stage was reached when there was a significant improvement in respiratory performance ($P_c = 3.7 \pm 0.1$ kPa). Furthermore individuals cultured under chronic hypoxia possessed a greater regulatory ability (consistently lower P_c values) than controls once the 'adult' pattern of regulation had been established. Interestingly, individuals cultured under periodic hypoxia displayed a pattern of response (i.e. time to establish 'adult' pattern of regulation and the breadth of the ability to maintain oxygen uptake during exposure to declining oxygen tensions) intermediate to that of individuals cultured under chronic hypoxia and the normoxic controls.

Figure 4.4 Critical oxygen tensions (P_C) for *A. franciscana* (T°C = 28, S = 35 ‰) of different developmental stages, cultured under different oxygenation regimes: normoxia = ●; chronic hypoxia = ○; periodic hypoxia = Δ. Values are means ± 1 standard deviation of 5 - 16 determinations.


Pc (kPa)
7
6
5
4
3
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
developmental stage

4.3.2 Function of hemoglobin

4.3.2.1 Effect of CO on rate of oxygen uptake

Presented in Figure 4.5 is the effect of treatment with CO on the relationship between oxygen uptake (under normoxic conditions) and body weight for *A. franciscana* cultured under normoxic and hypoxic conditions. In each case there was a significant relationship between oxygen uptake and body weight (Table 4.1) allowing us to compare the lines using covariance analysis. There were no significant differences ($F_{(3,85)} = 2.14$, $P > 0.05$) as a result of treatment with CO.

Table 4.1 Correlation coefficients (r^2) for relationships of oxygen uptake and body weight under different experimental conditions

Treatment	r^2	d.f.	P
Normoxic culture, untreated	0.77	1,22	< 0.001
Normoxic culture, CO treated	0.70	1,23	< 0.001
Hypoxic culture, untreated	0.69	1,18	< 0.001
Hypoxic culture, CO treated	0.51	1,18	< 0.001

Figure 4.5 The effect of CO on the relationship between oxygen uptake (under normoxic conditions) and body weight in individuals of *A. franciscana* (T°C = 28, S = 35 ‰) cultured under different oxygenation regimes: normoxia, untreated = ●; normoxia, treated with CO = ■; chronic hypoxia, untreated = ○; chronic hypoxia, treated with CO = □.

oxygen uptake (µl/mg/h)
20
10
1
1
10
100
500
body weight (µg)

4.3.2.2 Effect of CO on respiratory response to declining oxygen tensions of different developmental stages

The effect of treatment with CO on the respiratory response to declining oxygen tensions was examined for *A. franciscana*, at different stages during development, cultured under different oxygenation regimes. Figures 4.6, 4.7 and 4.8 show that, for each of the developmental stages examined, while there was no effect of CO on oxygen uptake during exposure to declining oxygen tensions in individuals cultured under normoxic conditions, there was a marked effect on individuals cultured under hypoxic conditions. After treatment with CO individuals cultured under hypoxic conditions were unable to regulate their rate of oxygen uptake during exposure to declining oxygen tensions as efficiently as control individuals.

4.3.2.3 Effect of CO on respiratory response to declining oxygen tensions of newly hatched individuals from parents with different hemoglobin concentrations

There was no effect of either CO or parental origin of eggs (i.e. from hemoglobin 'rich' or from hemoglobin 'poor' parents) on oxygen uptake of newly hatched (Stage 0) individuals ($F_{(1,35)}$ = 3.92 and 2.45 respectively, $P > 0.05$ in each case) (Figure 4.9). Neither was there any significant effect of CO on rates of oxygen uptake during declining oxygen tensions in Stage 0 hatchlings from hemoglobin 'rich' or hemoglobin 'poor' parents (Figure 4.10).

4.3.2.4 Asphyxia resistance

Prior acclimation to hypoxia resulted in a decrease in sensitivity to anoxic exposure (Figure 4.11). After 54 h continuous anoxia less than 5% of hypoxia acclimated

Figure 4.6 The effect of CO on oxygen uptake during exposure of *A. franciscana* (Stage 3) to declining oxygen tensions (T°C = 28, S = 35 ‰). A = individuals cultured under normoxia. B = individuals cultured under chronic hypoxia. Closed symbols = untreated and open symbols = treated with CO. Values are means ± 1 standard deviation of 10 determinations. * = difference significant at $P < 0.05$ (t-test).

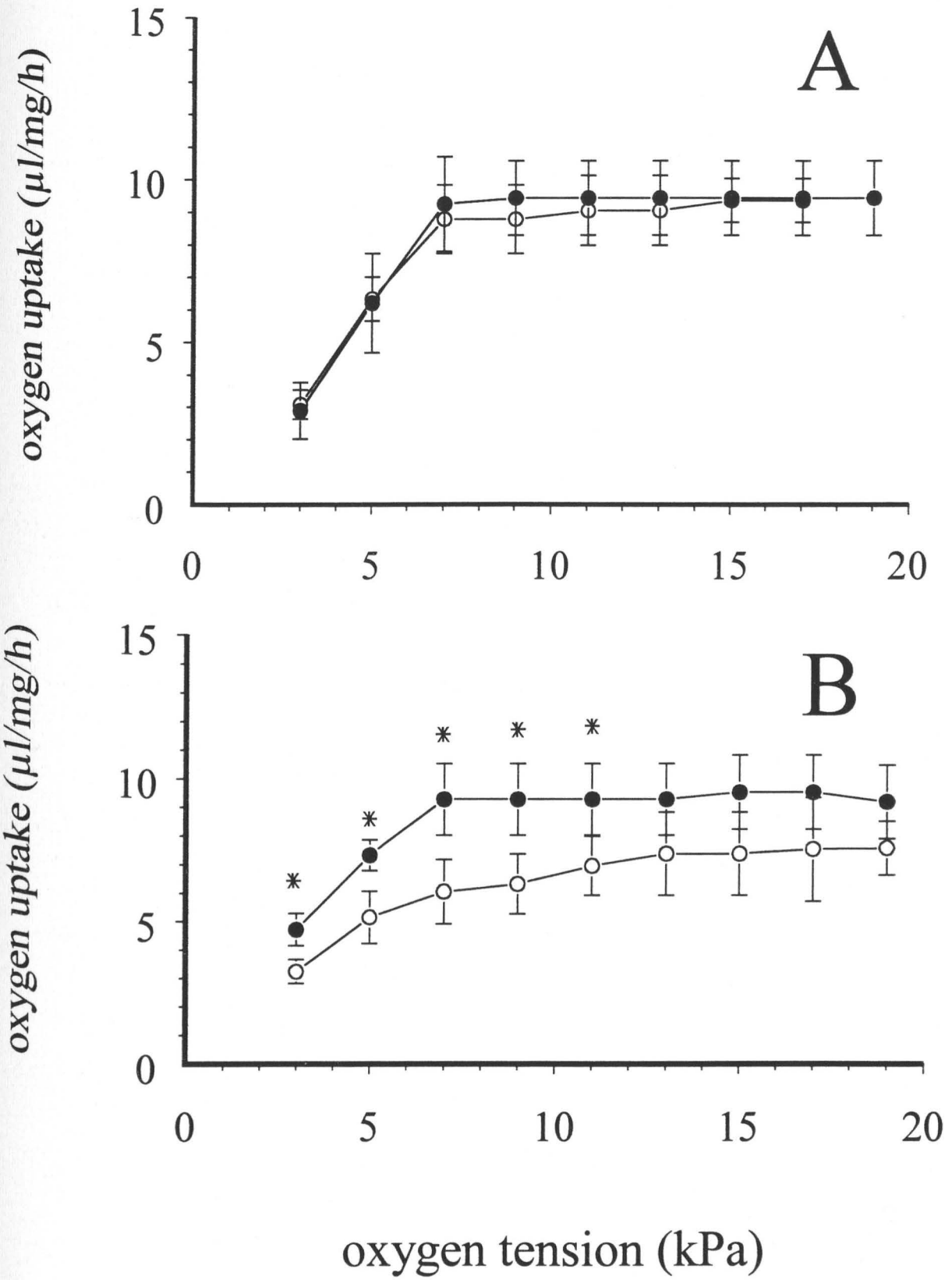

A
B
oxygen uptake (µl/mg/h)
oxygen uptake (µl/mg/h)
0
5
10
15
20
oxygen tension (kPa)

Figure 4.7 The effect of CO on oxygen uptake during exposure of *A. franciscana* (Stage 6) to declining oxygen tensions (T°C = 28, S = 35 ‰). A = individuals cultured under normoxia. B = individuals cultured under chronic hypoxia. Closed symbols = untreated and open symbols = treated with CO. Values are means ± 1 standard deviation of 8-10 determinations. * = difference significant at $P < 0.05$ (t-test).

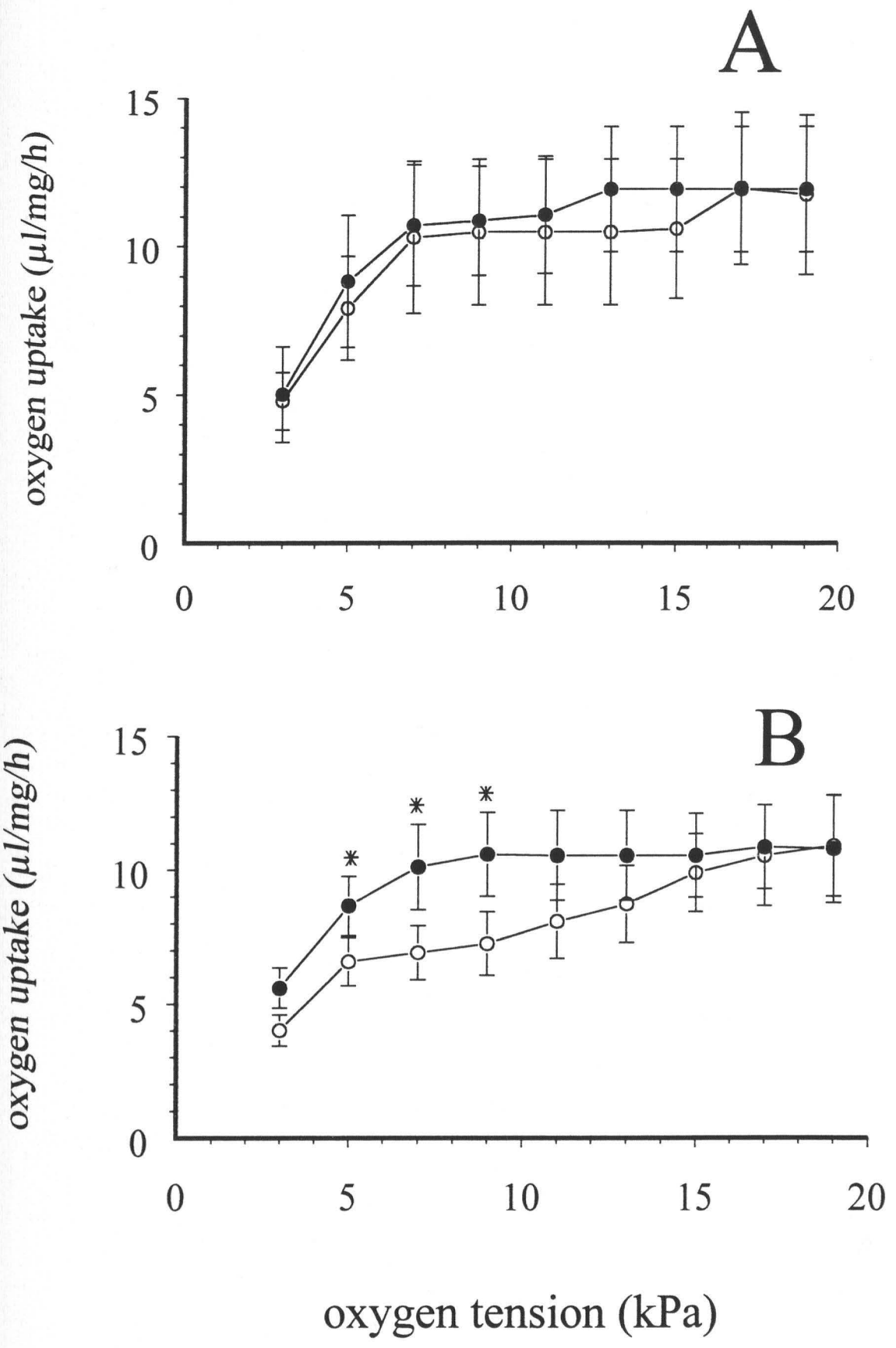

A
B
oxygen uptake (µl/mg/h)
oxygen uptake (µl/mg/h)
15
10
5
0
0
5
10
15
20
*
*
*
oxygen tension (kPa)

Figure 4.8 The effect of CO on oxygen uptake during exposure of *A. franciscana* (late Stage) to declining oxygen tensions (T°C = 28, S = 35 ‰). A = individuals cultured under normoxia. B = individuals cultured under chronic hypoxia. Closed symbols = untreated and open symbols = treated with CO. Values are means ± 1 standard deviation of 10 determinations. * = difference significant at $P < 0.05$ (t-test).

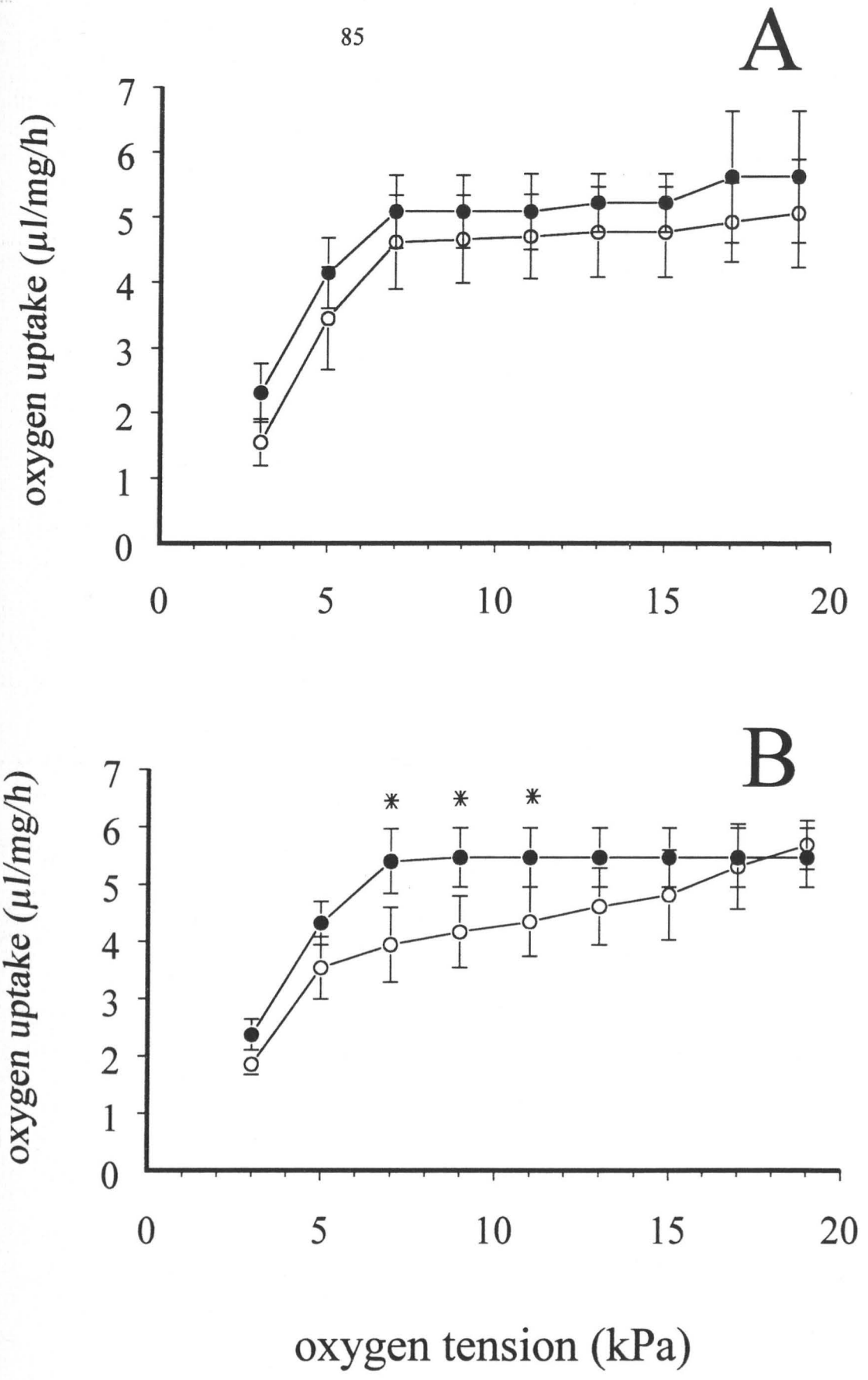

A
B
oxygen uptake (µl/mg/h)
oxygen uptake (µl/mg/h)
0
1
2
3
4
5
6
7
0
5
10
15
20
*
*
*
oxygen tension (kPa)

Figure 4.9 The effect of CO and origin of eggs (i.e. from hemoglobin 'rich' or 'poor' parents) on oxygen uptake by newly hatched (Stage 0) *A. franciscana* (T°C = 28, S = 35 ‰). Values are means ± 1 standard deviation of 10 determinations.

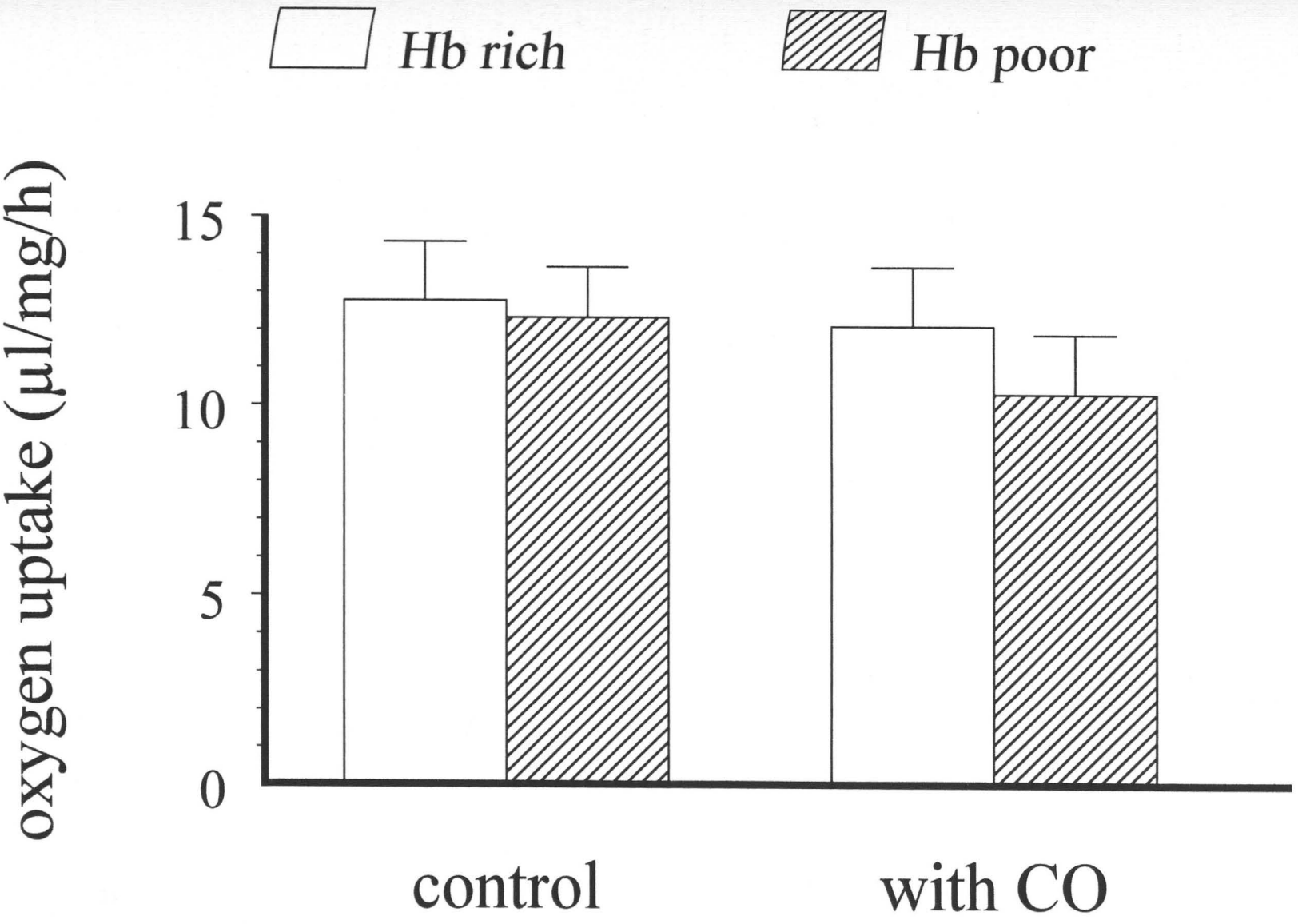

Hb rich
Hb poor
oxygen uptake (µl/mg/h)
15
10
5
0
control
with CO

Figure 4.10 The effect of CO and origin of eggs (i.e. from hemoglobin 'rich' or 'poor' parents) on oxygen uptake by newly hatched (Stage 0) *A. franciscana* during exposure to declining oxygen tensions (T°C = 28, S = 35 ‰). Squares = untreated and triangles = treated with CO. Values are means ± 1 standard deviation of 10 determinations.

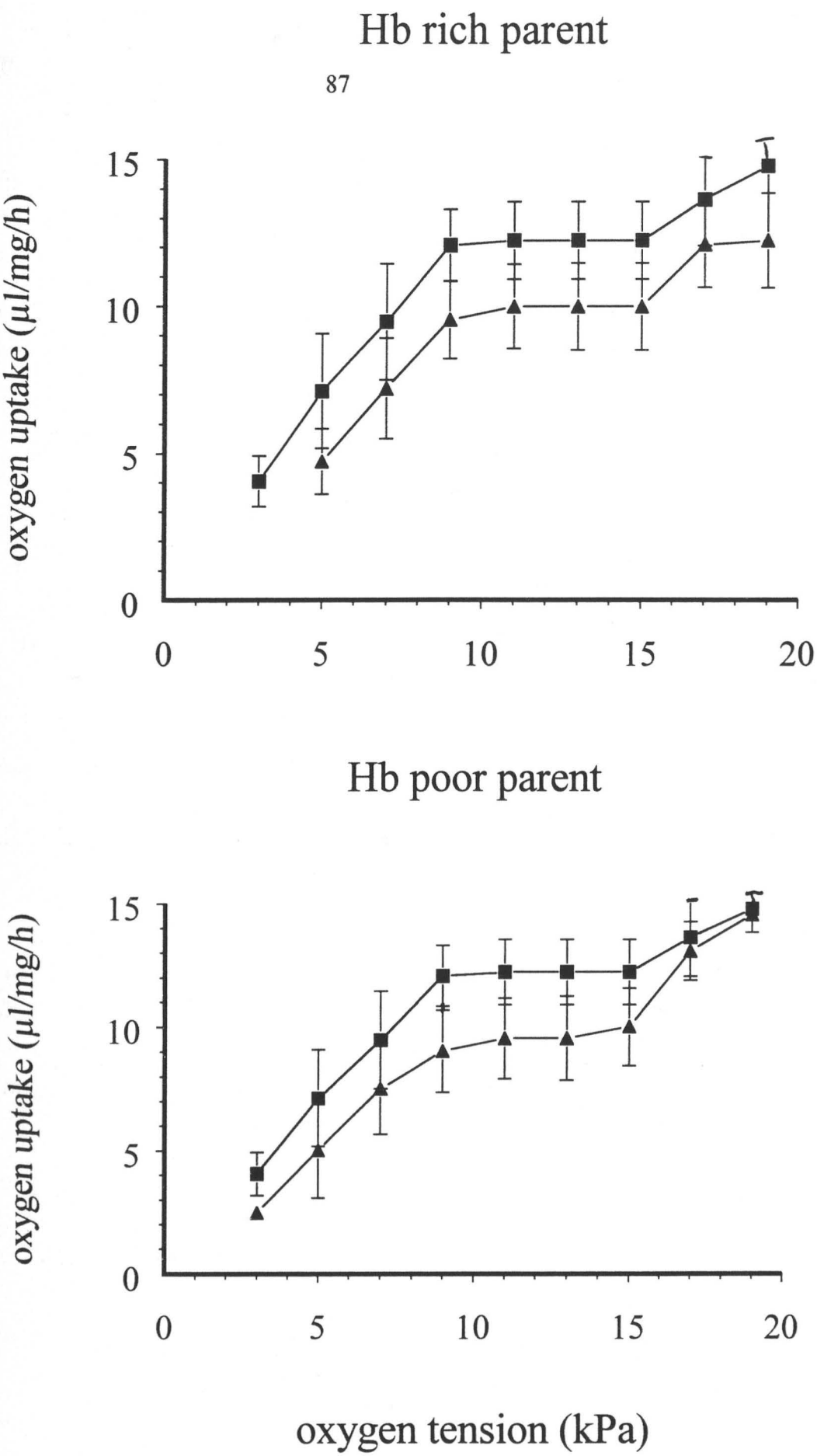
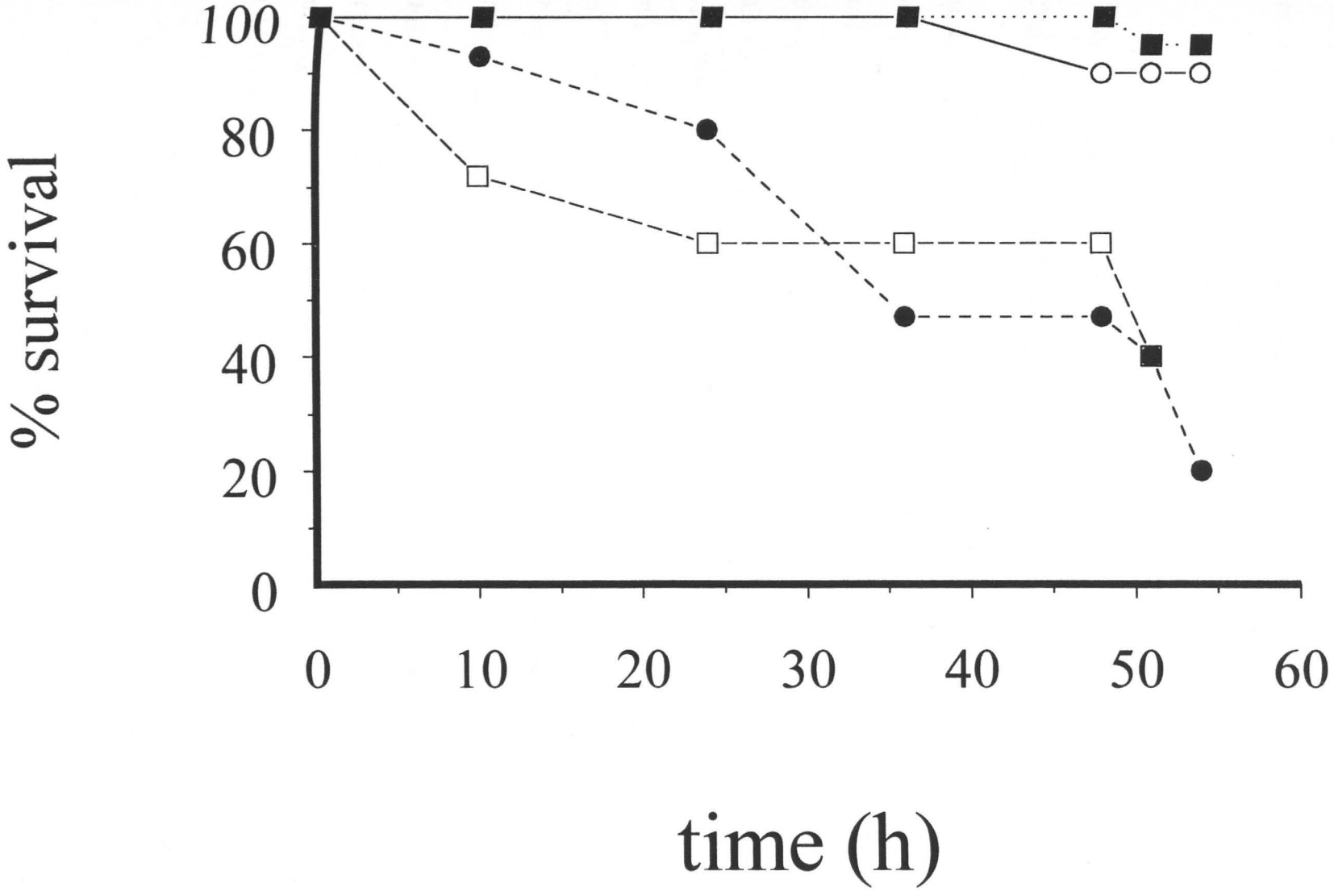

Hb rich parent
oxygen uptake (µl/mg/h)
15
10
5
0
0
5
10
15
20
Hb poor parent
oxygen uptake (µl/mg/h)
15
10
5
0
0
5
10
15
20
oxygen tension (kPa)

Figure 4.11 Anoxic survival of adult males and females of *A. franciscana* cultured under normoxic and hypoxic condition. ■ = females cultured under chronic hypoxia; □ = females cultured under normoxia; ○ males cultured under chronic hypoxia; ● = males cultured under normoxia.


% survival
100
80
60
40
20
0
0
10
20
30
40
50
60
time (h)

individuals were dead. This contrasted markedly with individuals culture under normoxic conditions with estimated LT_{50} values of approximately 36 h for males and 50 h for females. So even individuals cultured under hypoxia differed in their sensitivity to anoxia with males being more sensitive than females.

4.4 DISCUSSION

4.4.1 Changes in respiratory function during development

The oxygen uptake of individuals, encompassing the entire weight range encountered in this species (2.8 - 700 μg dry weight) could be predicted on an allometric basis. In other words total oxygen uptake increased with an increase in weight (slope of line, b = 0.80) and weight-specific oxygen uptake decreased with an increase in weight (slope of line = -0.20). This generally confirms many of the earlier studies on *Artemia* where, for example, b was calculated to be 0.82 (Gilchrist, 1959, cf. also Bernaerts *et al.*, 1981) and 0.76 (Bertalanffy & Krywienczyk, 1953). Eliassen (1952) claimed to detect three different values for b (0.75, 1.00 and 0.60), each value being characteristic of individuals of a particular age/size range. However, there seems very little reason for assuming that we have a similar pattern in the data presented here.

That there was a straight-forward relationship between oxygen uptake and body weight should not be surprising, as had been recognised about a century ago, such a relationship does seem to hold both within and between species generally, although the values for the slopes of the lines (as well as what they mean) are still disputed (Rubner, 1883, quoted in Drabkin, 1950); Bertalanaffy *et al.*, 1953; Zeuthen, 1953; Hemmingsen, 1960; John *et al.*, 1990).

The conclusion that there was a simple allometric relationship between oxygen uptake and body weight seems, at least initially, to be at variance with some of the data for *Artemia* (see Figure 18 on page 213 of Moens *et al.*, 1991, which depicts an increase in weight-specific oxygen uptake with weight in early development, followed by a decrease after approximately Stage 3), and some other crustaceans (e.g. barnacle nauplii (Lucas & Crisp, 1987) and lobster zoea (Capuzzo & Lancaster, 1979), where in very early development there was a decrease in oxygen uptake with increasing development, before the pattern observed above was established. However, on closer scrutiny of what actually happened during development we can see that in the case of both *Artemia* and barnacles, there was an initial decrease in weight with increasing development, and so at least some, if not all, of these examples still show a positive relationship between weight and oxygen uptake.

The ability of *A. franciscana* to maintain oxygen uptake in the face of declining oxygen tensions increased during development. Such a developmental transition has been observed before for crustaceans that (unlike brine shrimp) undergo a major metamorphosis. In both crab and lobster species planktonic larvae, which showed little ability to regulate oxygen uptake under conditions of progressive hypoxia, metamorphosed to benthic adults with a characteristically well-developed pattern of respiratory regulation (e.g. Belman & Childress, 1974; Spicer, 1995b). Interestingly in many bivalve mollusc species larger individuals showed a greater degree of respiratory independence during exposure to hypoxia than did smaller individuals (Bayne, 1967, 1971; Taylor & Brand, 1975) although in the cuttlefish *Sepia officinalis* respiratory independence decreased, with a shift from a benthic to a planktonic existence upon metamorphosis (De Wachter *et al.*, 1988) (i.e. comparable with what is found in crustaceans only the ecology is the opposite way round).

Even though not as well developed as in the adult stages, the newly hatched nauplii of *A. franciscana* exhibited a relatively well developed pattern of respiratory regulation during exposure to progressive hypoxia, relative to adult brine shrimp and compared with the adults of many other crustacean species (Johansen *et al.*, 1970; Mangum & Van Winkle, 1973; Taylor *et al.*, 1973; Childress, 1975; McMahon & Wilkens, 1975, 1977, 1983; Taylor, 1976; Butler *et al.*, 1978; Jouve & Truchot, 1978; Bridges & Brand, 1980a,b; Bradford & Taylor, 1982; Morris & Taylor, 1983; Aldrich, 1986; Taylor & Spicer, 1989; Zainal *et al.*, 1992). Although detailed interspecific comparisons of P_c are fraught with difficulties (and as such have been avoided here) what seems clear is that adult brine shrimp do have one of the best developed abilities to regulate oxygen uptake during exposure to progressive hypoxia of all the Crustacea, perhaps with the exception of the thalassinids (Thompson & Pritchard, 1969; Anderson *et al.*, 1991, 1994; Astall *et al.*, 1997) which often inhabit chronically hypoxic environments (Atkinson & Taylor, 1988). Although one might have predicted that newly hatched and adult *A. franciscana* should have similar patterns of respiratory regulation this was clearly not the case, even though both may be present simultaneously in potentially hypoxic brine ponds (but see Section 4.4.2 below). The most pronounced increase occurred between hatching and achieving Stage 6. This coincided with the appearance and development of the gills on the newly forming thoracic region, and the appearance of a functional heart (Spicer, 1994; Spicer & Morritt, 1996), as well as an ontogenic shift in the oxygen binding properties of the hemoglobin in the hemolymph (Moens *et al.*, 1991). It is not inconceivable that the appearance of specialised gas exchange areas, a functional circulation, and a respiratory pigment sensitive to hypoxic stress, provided the physiological mechanisms for allowing the development and improvement of respiratory regulation we see in Stage 6 individuals. Certainly this 'improvement' of respiratory performance with development could be 'prevented' by culturing nauplii of *A. franciscana* in heavy metal contaminated sea water (Spicer, 1995c). In this case newly hatched and Stage 6 individuals possessed similar P_c

values and nauplii did not develop beyond Stage 6, even although the development of cardiac function was identical to that observed in control animals. Presumably the heavy metal interfered with gill structure or function or with the ability of the respiratory pigment to load and unload oxygen (see Chapter 6 for further discussion).

Taking all of this into account, together with the results of the experiments examining hemoglobin function reported above (Section 4.3.2), it is suggested that improvement of respiratory performance during development, at least in normoxic animals, is not dependent upon the presence of a functional respiratory pigment or circulatory system (see Chapter 6 for a more full discussion). This is not necessarily to say that these organ systems are not important - it would appear that other physiological mechanisms (e.g. increased ventilation, which I have not examined) can make up for their 'absence'.

4.4.2 Improvement of respiratory regulation in hypoxia-cultured individuals

Culture under conditions of either periodic or chronic hypoxia had no significant effect on oxygen uptake when *A. franciscana* were tested under normoxic conditions. Even if we compare rates of oxygen uptake, estimated at the oxygen tension of the respective culture media there was still no difference. This stands in contrast to the work of Gilchrist (1954) who found that for *A. salina* not only did the hemoglobin function at low oxygen tensions, but that at high (normoxic) tensions, the oxygen uptake of control individuals was greater than that of C0 treated individuals. It is difficult to compare the results of this study with those of the current work. It could be that Gilchrist (1954) had not only blocked hemoglobin function but also poisoned the cytochrome system, in a way that produced low rates of oxygen uptake (see below for defence of CO method). However, on the basis of the data presented in this chapter the hypothesis that *A. franciscana* may

respond to hypoxia by reducing their metabolism, which would be indicated by a decrease in oxygen uptake, can be rejected.

However, culture under conditions of chronic hypoxia did result in the 'improvement' in respiratory performance (decrease in P_c) appearing earlier in development than was recorded for normoxic controls. This has also been recorded for newly hatched planktonic Norwegian lobsters *Nephrops norvegicus* although not for the newly hatched amphipod crustacean *Echinogammarus pirloti* (Spicer, 1995b). Improvement of respiratory performance during exposure to declining oxygen tensions has been recorded before for brine shrimp, but only in sexually mature individuals (Gilchrist, 1959; Vos *et al.*, 1979; Decleir *et al.*, 1989; DeWachter & Abbeele, 1991) Furthermore the P_c for hypoxia cultured individuals was significantly less than that for normoxia cultured individuals at any given developmental stage. In other words the former were better able to regulate their rates of oxygen uptake over a wider range of environmental oxygen tensions than the latter. Interestingly, this means that, with the exception of some of the very early developmental stages, most of the brine shrimp present in a hypoxic pool will possess similar extremely well-developed patterns of respiratory independence from oxygen.

Whilst, above (Section 4.4.1), we rejected the hypothesis that all developmental stages of *Artemia* should display the same degree of respiratory independence we see that data from individuals cultured under hypoxia come closer to supporting this hypothesis. Consequently, it is suggested that when cultured under hypoxic conditions brine shrimp assume the well developed adult pattern of respiratory regulation as soon as possible after hatching, i.e. approximately Stage 3. Before this point, developmental constraints (i.e. the 'order' in which the nauplius is 'put together' and the way in which this interacts with the environment) presumably prevent brine shrimp from hatching with the 'adult' pattern of physiological regulation. In this way newly-hatched brine shrimp remain more sensitive to hypoxia than adults for only a few hours (see Chapter 6 for more full discussion).

Of considerable interest is the fact that individuals cultured under periodic hypoxia showed a respiratory response to acutely declining oxygen tensions that was intermediate to the normoxic and chronically hypoxic patterns. This strongly suggests that the effect of hypoxic acclimation on respiratory performance is dependant on the time exposed to hypoxia and not necessarily the pattern of hypoxic/normoxic exposure. The effects of cyclical or periodic hypoxic stress are only beginning to be explored although, to date, most (although still very few) studies have considered mortality rather than examining the effect on physiological mechanisms. In general cyclical exposure of larval and adult crustaceans resulted in an increase in hypoxic tolerance (e.g. Vargo & Sastry, 1977) supporting the data presented here.

4.4.3 Mechanisms underlying hypoxia-related improvement in respiratory performance

There are a number of physiological mechanisms that could explain the 'improvement' in respiratory regulation during exposure to declining oxygen tensions found for *A. franciscana* cultured under both periodic and chronic hypoxia.

It has been noted that species which show very well developed powers of respiratory regulation (i.e. have low P_c points) are characterised by low metabolic rates, e.g. thalassinid shrimps (Thompson & Pritchard, 1969; Anderson *et al.*, 1991; Astall *et al.*, 1997). It is conceivable then that a lower P_c point, as a result of hypoxic acclimation, may be achieved by an individual having a reduced metabolism. However, as noted at the beginning of this section, the oxygen uptake of hypoxic and normoxic cultured brine shrimp was not significantly different. Therefore, lowering of P_c due to hypometabolism, as way of coping with hypoxic stress, seems unlikely in *A. franciscana*.

We have seen previously that treatment of normoxic cultured brine shrimp with CO, to inactivate the oxygen binding properties of the hemoglobin in their hemolymph, did not significantly affect the overall respiratory performance of the *A. franciscana*. However, this was not the case for hypoxic cultured individuals. Respiratory independence was compromised by the CO treatment in a way not seen in normoxic controls. Indeed most of the respiratory improvement resulting from culture under hypoxia was negated by treatment with CO, although it is significant that respiratory independence did not disappear altogether. This strongly suggests that the improved respiratory independence of hypoxia cultured brine shrimp is due to the presence of respiratory pigment.

In contrast with the situation in vertebrates where hemoglobin is considered essential for gas exchange to occur, the role of hemoglobin in invertebrates has been disputed and is still controversial (Weber, 1980). For example, even the early work of Fox (1948) questioned whether or not *Daphnia* survival was independent of the individual hemoglobin content. However, three years later he thought that the extent of hypoxia-related induction of hemoglobin in Daphnia could be related to survival times when exposed to asphyxia. Also in the branchiopod *Triops* inactivation of hemoglobin by CO reduced oxygen uptake only in the largest specimens. Part of the 'problem' lies in the fact that the CO method has been severely criticised, as it may interfere with cellular oxidases, resulting in an overestimation of hemoglobins role as an oxygen carrier or store (Weber, 1980). However, Weber (1980) defending the method, when properly used, pointed out that the affinity of most invertebrate hemoglobins was quite different for CO and oxygen than for cytochrome oxidases. Also the results of CO dosing experiments are often confirmed by alternative experimental approaches, e.g. examining the oxygen uptake of *Daphnia* from oxygen rich (low hemoglobin concentration) and oxygen poor (high hemoglobin) environments (Hoshi & Inada, 1973) or measuring respiratory performance of control versus gill ligatured amphitrite worms (Mangum *et al.*, 1975). Certainly in my own

experiments the fact that CO compromised respiratory regulation but not oxygen uptake is good evidence for the validity of using the CO technique following the method described above (Section 4.2.2). Furthermore, rather than overestimating the importance of the presence of a respiratory pigment, it is more likely, as pointed out by Mangum *et al.* (1976) that use of the CO technique will result in an underestimation of its importance in view of possible ventilatory and compensatory responses to the pigment becoming dysfunctional.

It may be considered surprising that the presence of a respiratory pigment is essential for the respiratory function of brine shrimp, as one might have assumed that diffusion would be sufficient for oxygen uptake in such a small animal. However, fairly good evidence has been presented that in *Daphnia*, which are of a similar size range, hemoglobin played an important role in gas exchange, particularly under conditions of environmental stress, such as hypoxia or activity (Kobayashi & Nezu, 1986; Kobayashi & Yoshida, 1986; Kobayashi & Tanaka, 1991). Therefore the conclusion that hemoglobin is vital for the well being of *Artemia* cultured under hypoxia is not an unreasonable one.

In the previous chapter we saw that, in common with many other crustaceans culture under hypoxia resulted in a dramatic increase in the respiratory pigment content of individual brine shrimp. However, we cannot say definitively it is only the increase in the amount of oxygen carried in the hemolymph that accounts for the difference between normoxic and hypoxic cultured brine shrimp. We know in the case of *Nephrops norvegicus*, already referred to above, that increased respiratory regulation was accompanied by a shift in the affinity for oxygen of the respiratory pigment (Spicer, 1995b). Given what we know of changes of brineshrimp hemoglobin content and affinity during development, and as a response to hypoxic stress (in adults), it seems likely that both hemoglobin concentration and oxygen affinity are likely to be important in hypoxic acclimation.

In the light of what has gone before it may appear puzzling that there was no effect of CO on the oxygen uptake of newly hatched larva either from adults cultured under hypoxia or adults cultured under normoxia, despite the fact that hypoxic cultured adults were considerably more hemoglobin rich than the controls (see Section 3.3.3). However, in detailed studies on the 'embryonic' hemoglobin of *Artemia* Gilchrist & Green (1959) found that dark brown eggs contained x 3.5 - 4 as much haem as pale cream coloured eggs: when they measured the total haem content of newly hatched nauplii from both types of egg, there was no difference between them. The haem, it was deduced, was concentrated purely in the discarded shell. This was also the case in *Triops* sp. eggs which are surrounded, when in maternal oviduct, by a bright red liquid containing a protohaemochromogen, where this pigment passes into the egg shells but not into the ova (Fox, 1955). Horne & Beyenbach (1971) confirmed this when found that there was no function of the hemoglobin in small *Triops*, but the function is only important in specimens larger than 1g. Furthermore examining *Simocephalus* and *Daphnia* Kobayashi *et al.* (1990) concluded that hemoglobins in new hatchlings were not very important in gas exchange and the primary function of the hemoglobin was as a fuel to meet the metabolic costs of accelerated development (as when treated with CO daphnids require a longer time to complete their development) (cf. Fox, 1955).

It is concluded that hemoglobin plays an essential role in the development of respiratory independence in brine shrimp cultured under hypoxia, and its presence may (at least partially) explain the difference in respiratory performance between hypoxic and normoxic cultured brine shrimp. As we have seen above, if hemoglobin oxygen transporting function is important for normoxic cultured individuals, it is not indispensable as, if it is removed, other compensatory physiological mechanisms are able to maintain the overall pattern of respiratory independence (Section 4.4.1). This was not the case for hypoxia cultured individuals where other possible physiological mechanisms (e.g. circulatory and

ventilatory) could not maintain the same degree of respiratory independence once respiratory pigment function was disabled.

4.4.4 Anoxia tolerance

Artemia franciscana showed a tolerance to anoxia (LT_{50} > 35 h) similar to that of the most anoxic tolerant crustaceans studied, i.e. thalassinids and some isopod species (Anderson *et al.*, 1994; Hagerman & Szaniawska, 1990). This was considerably greater than that recorded for many of the crab, shrimp and lobster species studied such as the shrimp *Palaemon* spp. and the crab *Carcinus maenas* (2-4 h and 17h for 100 % mortality respectively (Taylor & Spicer, 1987; Hill *et al.*, 1991) or the shrimp *Crangon crangon* (LT_{50} = 2.5 h, Hagerman & Vismann, 1995). Unfortunately we know little of the anaerobic pathways that operate in brine shrimps (Vos *et al.*, 1979; Conte *et al.*, 1980; Decleir *et al.*, 1980)), although it would not be too surprising if, in common with most other crustaceans, L-lactate were still the principle (if not the only) metabolic substrate (Bridges & Brand, 1980b; Hill *et al.*, 1991).

Even more interestingly adult *A. franciscana* cultured under hypoxic conditions showed an enhanced tolerance to anoxia with LT_{50} values estimated to be considerably greater than 56 h. There are two possible explanations for this difference. Firstly there could be some modification of the capacity for anaerobic metabolism. While this is a possibility it is not one that was examined in the present study. Secondly, the increase in hemoglobin concentration found for individuals cultured under hypoxic conditions may extend the amount of time aerobic metabolism can be sustained (Decleir *et al.*, 1989). In this case hemoglobin would act as an oxygen store, slowly releasing oxygen to the tissues as the oxygen tension within the animal decreased as a result of aerobic metabolism. This suggestion is strengthened by the fact that here, and also in the respirometry experiments

recorded above, mortality only occurred after the hemoglobin in the hemolymph became visibly deoxygenated.

4.4.5 Conclusions

The ability of *Artemia franciscana* to maintain respiratory independence under conditions of declining oxygen tension was present upon hatching but still improved dramatically in early development until the 'adult' pattern was established. This coincided with the development of the gills, the appearance of a functional heart and a change in the oxygen binding properties of the respiratory pigment hemoglobin. While hemoglobin may have been important in the improvement of respiratory performance, it was not indispensable.

The oxygen uptake of *A. franciscana* was dramatically altered by culture under hypoxic conditions. While the presence of hemoglobin early in development appeared to be of little respiratory significance, with continuing development it increasingly played a more important role in oxygen exchange. The development of respiratory independence which was characteristic of normoxia cultured brine shrimp was 'brought forward' in hypoxia cultured individuals and so they assumed the 'adult' pattern much earlier in development. Furthermore the 'adult' ability to regulate oxygen uptake in the face of declining oxygen tensions was better developed in hypoxic than in normoxic cultured brine shrimp, with both of these features being negated if animals were treated with CO, eliminating the role of hemoglobin as a respiratory pigment. Thus hemoglobin played a critical role in acclimation to chronic hypoxia and may also have been responsible for enhancing the survival of these animals when exposed to anoxia, the molecule acting as an oxygen store.

Chapter 5 Subcellular Responses to Hypoxia: Effect of Culture under Chronic Hypoxia on Structure and Function of Mitochondria

5.1 Introduction

5.1.1 Effect of chronic hypoxia and development on mitochondrial structure and function

While the effects of hypoxia on whole animal respiration (organismal level) are comparatively well documented for aquatic invertebrates, and crustaceans in particular (See Sections 1.2 & 4.1.1), the effects of hypoxia on cellular and subcellular structure and function are not so well known. Given that we have seen that ability to regulate oxygen uptake during progressive hypoxia improves in brine shrimp *Artemia franciscana* during development and also as a result of culture under chronic hypoxia (Chapter 4), it would be interesting to examine cellular and subcellular responses that accompany this improvement. Mostly long term improvements in respiratory function are explained at the level of whole animal response (increased perfusion, increased respiratory pigment concentration etc., cf. Chapter 4) and little attention is given to cellular responses. As mitochondria are inextricably linked to the process of aerobic metabolism, the question arises as to what effect culture under chronic hypoxia has on the structure and function of these subcellular organelles.

The outer membrane of all mitochondria possess the same, very stable smooth form while the inner membrane, the cristae, can appear in a variety of different forms and can vary with time and with function (Threadgold, 1967). This latter feature is of interest as there would appear to be a correlation between the surface area of cristae in a mitochondrion and its general metabolic or enzymatic activity: the more cristae, the more active in aerobic metabolism (Threadgold, 1967).

Most of our literature on the effects of development and hypoxia (separately) on mitochondria distribution, form and function comes from studies of mammalian cells.

Generally speaking mitochondrial energy metabolism seems to decrease with age (e.g. Capozza *et al.*, 1994). This is borne out by the results of a recent study in which liver mitochondria from 'old' rats when injected into liver cells of 'young' rats were less able to fulfil cell energy requirements. In a recent study of changes in synaptic morphology with development while the total mitochondrial density was independent of age, there was an increase in total numbers, i.e. the average volume of mitochondria decreased with age (Bertoni-Freddari *et al.*, 1993 also Brooks *et al.*, 1995 on tropical fish).

We know little of changes in mitochondria structure and function during development in invertebrates. The workers that have used *Artemia* as their experimental animal have focused almost entirely on the anoxia and role of mitochondria in encystment of the blastula and on subsequent emergence from quiescence (Marco *et al.*, 1980; Kwast & Hand, 1993, 1996a,b; Kwast *et al.*, 1995); mitochondrion protein synthesis is depressed during anoxia-induced quiescence, *via* a decrease in intracellular pH in embryos of *Artemia franciscana*. It has been shown that there is a dramatic increase in activity of cytochrome *c* oxidase, an enzyme integral to mitochondrial respiration (Wikstrom *et al.*, 1981) during preemergence and this increase could be prevented either by anoxia or aerobic acidosis (Hofmann & Hand, 1990) The biogenesis of mitochondria after cysts were rehydrated has been described (Schmitt *et al.*, 1973). Here the hydration of the cyst induced marked biochemical and morphological changes in the mitochondria, namely increased capacity for aerobic respiration and the formation of cristae: the increase in cristae surface area in early embryonic (but free-living) development was by the same order of magnitude of changes in whole animal oxygen uptake. As it stands, however, we know little about changes in mitochondrial form and function with development in brine shrimp except of in these very early developmental stages.

The effects on mitochondria of acute (short term) exposure to hypoxia are relatively well understood for mammalian cells. Mitochondrial and cellular respiration appears to be controlled by oxygen (Gnaiger *et al.*, 1995); incubation of isolated mitochondria for 4 h at low oxygen resulted in reversible suppression of respiration (Chandel *et al.*, 1996); there is mitochondrial swelling (Morton *et al.*, 1994) and an increase in mitochondrial Ca^{2+} which

impaired function (Silverman, 1993); specific hypoxia-stress proteins were generated to deal with oxidative damage due to severe tissue hypoxia (Corbucci *et al.*, 1995); there was no immediate change in mitochondria number (Russell & Jackson, 1994). Exposure to chronic hypoxia, however, produced a different suite of (sometimes contradictory) responses, which included an increase in volume density of mitochondria (Desplanches *et al.*, 1993), in some tissues although not others (where there was a decrease (Ferretti, 1990; Van-Ekeren *et al.*, 1992), the production of smaller mitochondria (Lennard & Huddart, 1992); an increase in the activity of cytochrome *c* oxidase (Korzeniewski, 1996) and an increased deletion rate of mitochondrial DNA (Merril *et al.*, 1995). Apart from the effect of anoxia on mitochondrial structure and function in arrested embryos, we know nothing of the effect of hypoxia on mitochondria in the cells of brine shrimp, exposed to, or cultured under, chronic hypoxic conditions.

5.1.2 Aims of Study

On the basis of what has gone before the aims of this chapter are as follows.

1. The density and structure of mitochondria will be examined qualitatively in a wide range of different tissues from brine shrimp cultured under either normoxic or chronic hypoxic conditions and at a number of critical developmental stages: Stages 3 and 6 as stages in which improvement of respiratory performance during exposure to acute hypoxia is taking place; Stages 9, 13 and late being representative of the different levels of respiratory independence achieved by individuals from normoxic as opposed to individuals from hypoxic culture. The hypotheses to be tested are that a) as in mammalian cells there will be an increase in number, but a decrease in individual volume of mitochondria with development b) with the improvement in respiratory independence during exposure to acute hypoxia which takes place early in brine shrimp development there will be an improvement in the aerobic capacity of mitochondria, in terms of increased number or increase in cristae surface area (or both) c) as culture under hypoxic conditions resulted in an improvement in respiratory independence,

there will be an improvement in the aerobic capacity of mitochondria from hypoxic culture, when compared with normoxic culture, in terms of increased volume, number or cristae surface area (or a combination of all three).

2. Cytochrome *c* oxidase is the final enzyme regulating the flow of electrons through the mitochondrial electron transport chain to the terminal electron acceptor oxygen in aerobic respiration (Wikstrom *et al.*, 1981). Therefore the activity of cytochrome *c* oxidase will be measured for whole brine shrimp at different stages of development, cultured under either hypoxic or normoxic conditions (i.e. comparable with material examined using TEM above), to give a good quantitative estimate of the potential (as a measure of capacity) of tissues to respire aerobically. The hypotheses to be tested are that a) as with mammalian tissues weight-specific enzyme activity should decrease with age b) individuals cultured under chronic hypoxia should possess greater enzyme activities than individuals cultured under normoxia, at an equivalent developmental stage. The effect of chronic hypoxia on the distribution on cytochrome *c* oxidase within individuals was qualitatively examined to gauge the extent to which this could be correlated with changes in mitochondrial density and structure and measures of activity of the enzyme for whole animals.

5.2 Material and methods

5.2.1 Origin of animal material

Brine shrimp were cultured under normoxia and chronic hypoxia following exactly the culture techniques and methods outlined in Section 3.2.1. Individuals at developmental Stages 3, 4, 5, 6, 8, 9, 13 and late Stage were removed when, and as, required and used for examination by light and electron microscopy and determination of cytochrome *c* oxidase activity as described below.

5.2.2 Electron microscopy

The structure and occurrence of mitochondria in different tissues (limb muscle, somatic muscle, visceral muscle, heart, gut wall, hemolymph cells) of individuals (Stages 3, 4, 5, 6, 8, 9, 13 and late Stage) cultured under either normoxic or hypoxic conditions, was examined using transmission electron microscopy of serial sections, and was carried out as follows.

Individuals were fixed in either gluteraldehyde for 60 min or Karnovsky's fixative for 180 min, both at approximately 5°C. Stage 9, 13 and late stage individuals were cut transversely into two pieces in order to facilitate effective tissue penetration by the fixative. After this the fixative was exchanged for sucrose in phosphate buffer. Separate pieces and whole mounts were postfixed with 2 % osmium tetraoxide, in the same buffer for 90 min. Pieces or whole mounts were washed in deionised water, dehydrated using ethanol dilutions and then finally fixed in a mixture (50:50) of propylene oxide and resin. Thereafter specimens were embedded in resin and dried at 60°C before sectioning. Thin sections (70 - 90 nm thick) were cut using a microtome (Reichert Ultracut) and then stained first in uranyl acetate (3 % in 50 % ethanol) for 15 min and secondly in Reynold's lead citrate for 2 min. Stained sections were examined using a transmission electron microscope (Philips CM 10).

5.2.3 Light microscopy and cytochrome *c* oxidase localisation

Material was prepared for examination by light microscopy exactly as outlined previously in Section 3.2.4. The material presented in Sections 3.3.3 and 3.3.4 which deals with hemoglobin staining is, therefore, directly comparable with that presented here. Staining for cytochrome *c* oxidase was carried out using Gräff's G - Nadi reaction 1916 as modified by Kiernan (1990). This procedure is highly specific for this enzyme as the 'Nadi' reaction depends upon the fact that cytochrome *c* oxidase is required for the oxidation reaction between α-naphthol and a dimethyl-*p*-phenylaminediamine hydrochloride to form the blue-violet coloured 'stain' (indophenol blue). Sections of fresh, unfixed tissue were incubated in Nadi reagent for 1 h at 37°C, before being mounted in potassium acetate solution (20 %).

5.2.4 Enzymatic assay of cytochrome *c* oxidase

The activity of cytochrome *c* oxidase was determined for Stage 3 and late Stage individuals (males and females) using the enzymatic technique described by Hendry & Grime (1993). This assay depends on the fact that when reduced cytochrome *c* is added to a mitochondria-containing tissue preparation, it will become oxidised at a rate that is dependent on the activity of cytochrome *c* oxidase present.

Reduced cytochrome *c* was prepared as follows. An ion exchange column (1.3 x 5 cm, Sephadex G50) was first washed with KPi buffer (50 mmol.l^{-1}). Both cytochrome *c* and ascorbate were mixed in excess, dissolved in 0.5 ml of KPi buffer, loaded onto the column and eluted using excess buffer. The visibly pigmented fraction (1.5 ml), eluted after the void volume was collected, and stored on ice until required.

Either 300, 100 or 100 individuals for each developmental Stage (Stage 3, late Stage male and late Stage female respectively) and each experimental treatment (normoxia or hypoxia) were washed and homogenised in an ice cold KPi - Sorbitol buffer. The buffer was constructed by adding sorbitol (250 mM) and NaEDTA (0.2 mM) to 0.1 mol.l^{-1} KPi buffer, and adjusting the final mixture to pH = 7.5. The homogenate was then centrifuged at 3 000 g for 1 min to remove cellular debris and then centrifuged at 15 000 g for 3 min. After this time the resultant pellet was separated from the supernatant, before being resuspended in fresh ice cold KPi buffer (1.1 ml).

To the resuspended pellet solution (1 ml) was added 25 µl of reduced cytochrome *c*. The resultant mixture was placed in a quartz cuvette (1 cm pathlength) and the rate of decrease in absorbance measured at a wavelength of 550 nm. The activity of cytochrome *c* oxidase was then calculated using the extinction coefficient given by Hendry & Grime (1993) The total protein content of a subsample (0.1 ml) of the resuspended pellet solution was measured using a commercially available Coomassie Blue technique (Sigma). Consequently specific activity of cytochrome *c* oxidase was expressed per unit (mg) fresh weight protein.

5.3 Results

5.3.1 Effect of chronic hypoxia on mitochondria structure

5.3.1.1 Limb muscle and hemolymph cells mitochondria

Presented in Figure 5.1 is a representative TEM of a developing limb muscle from a Stage 3 individual. While mitochondria were present they were small and widely scattered. At greater magnification of this section we see that the mitochondria present are 'typical' in terms of their shape and the extent of their cristae surface area (Figure 5.2). However, if we examine an equivalent section from a Stage 3 individual cultured under chronic hypoxia, we find that not only was mitochondrial density noticeably increased (Figure 5.3) but the mitochondria present were megamitochondria (Figure 5.4). These megamitochondria could be up to three times the length and four times the thickness of mitochondria from limb muscle tissues of control individuals. Furthermore the cristae surface area was greatly enlarged and exhibited more intense folding. The differences between hypoxic and normoxic Stage 6 individuals were also noted in limb muscles from Stage 3, 9 and late Stage individuals. There were no obvious changes in mitochondrial density or appearance with increased development although it should be noted that no quantitative assessment was attempted. The presence of megamitochondria was also noted in many of the hemolymph cells examined from individuals cultured under chronic hypoxia.

5.3.1.2 Heart muscle mitochondria

Mitochondria present in the heart muscle of individuals (cultured under normoxia) at different stages of development (Stages 3, 6, 9 and late) were similar in appearance to those of the developing limb muscles (Figure 5.5). They were, however, considerably smaller (approximately x 4) and there were fewer of them. Both the density and appearance of these mitochondria were altered by culture of individual animals under chronic hypoxia, and the

Figure 5.1 Representative TEM of limb bud muscle (transverse section) of Stage 3 *A. franciscana* cultured under normoxia (Magnification x 7 800): LMC = limb muscle cells; MF = muscle filament; MT = mitochondrion; N = nucleus.

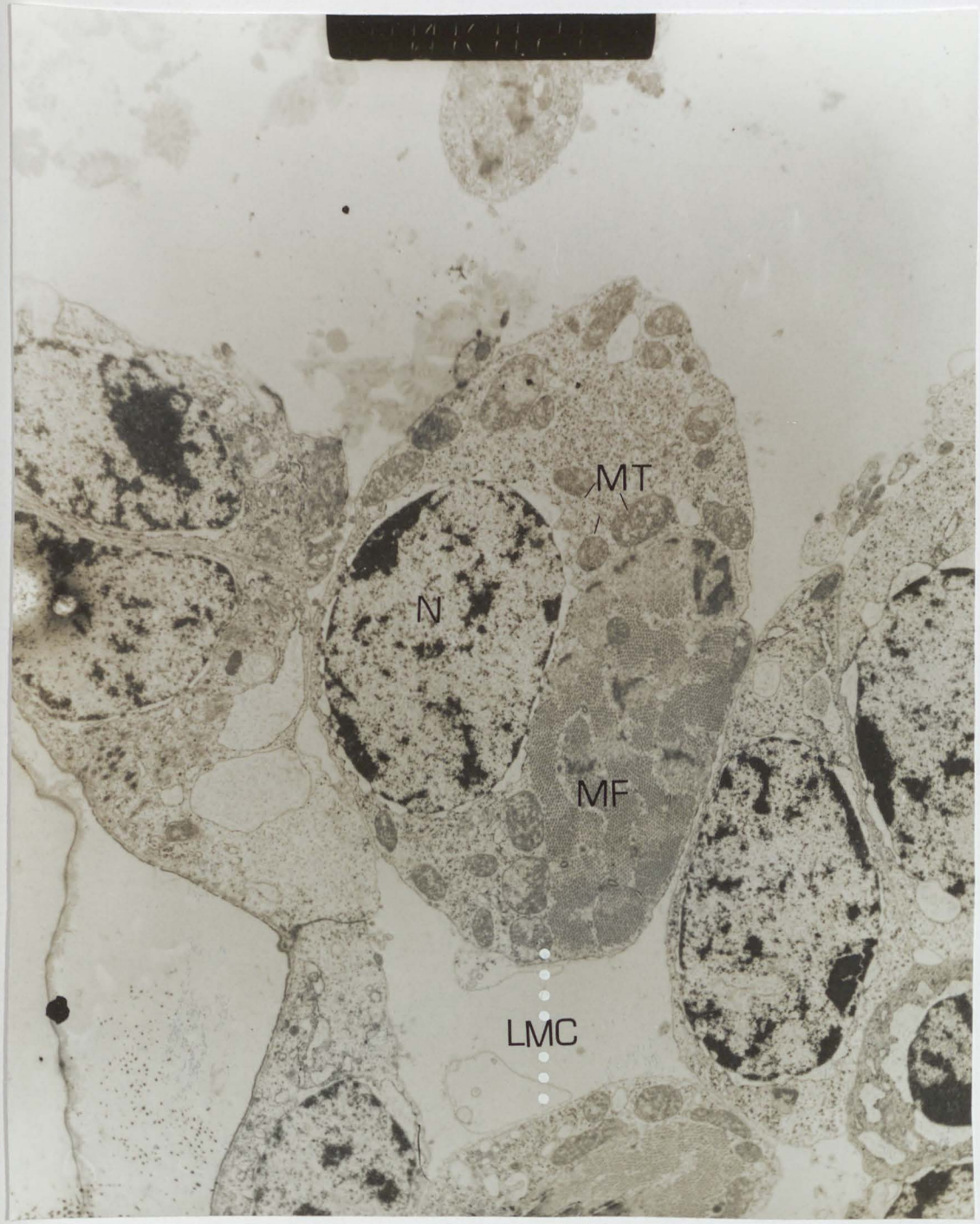

MT
N
MF
LMC

Figure 5.2 Enlargement, to show mitochondrial detail, of representative TEM of limb bud muscle (transverse section) of Stage 3 *A. franciscana* cultured under normoxia (Magnification x 31 000): C = cristae; IM = inner mitochondrial membrane; OM = outer mitochondrial membrane; MF = muscle filament; MT = mitochondrion; N = nucleus.

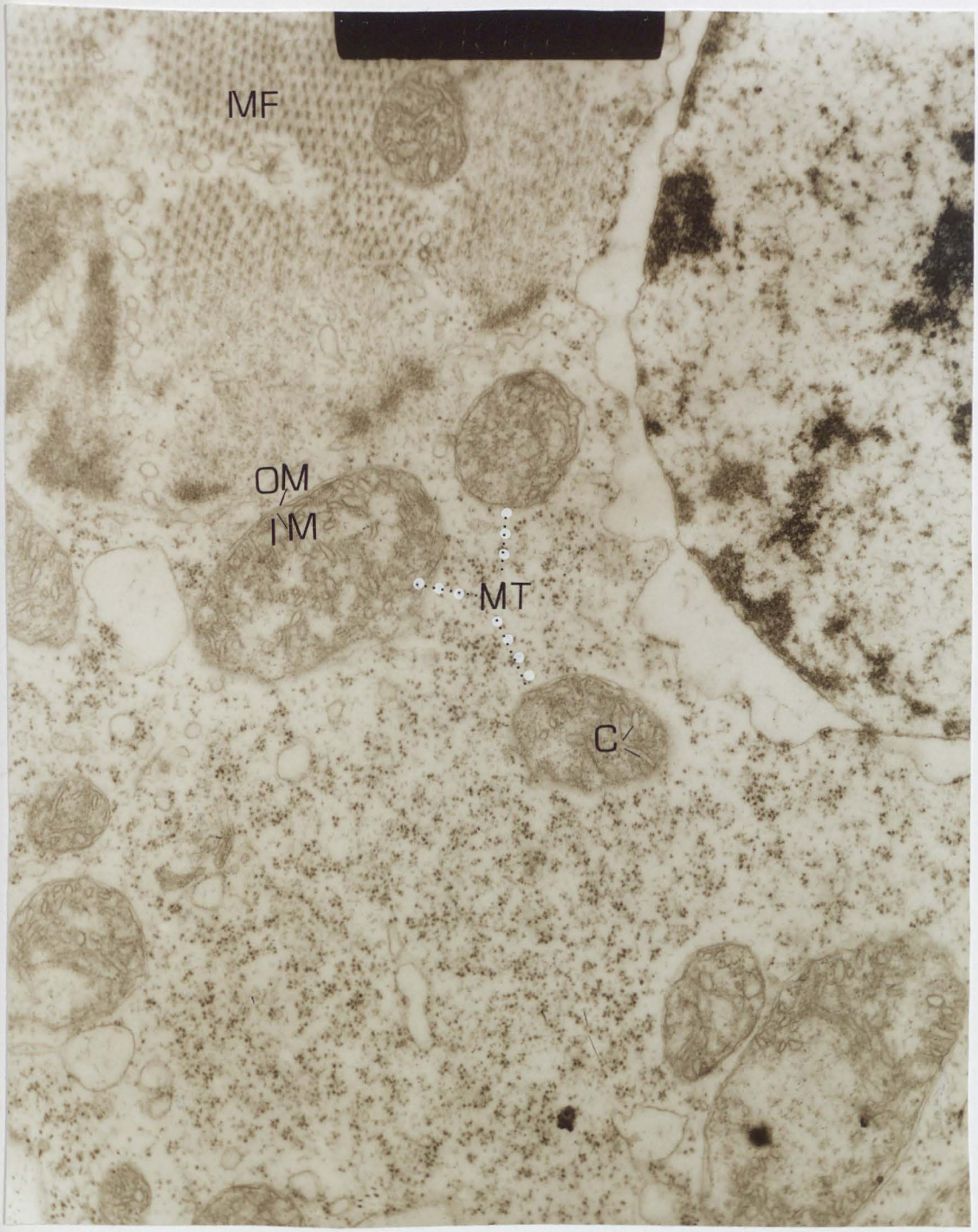

MF
OM
IM
MT
C

Figure 5.3 Mitochondrial detail, of TEM of limb bud muscle (transverse section) of Stage 3 *A. franciscana* cultured under chronic hypoxia (Magnification x 13 200): C = cristae; IM = inner mitochondrial membrane; OM = outer mitochondrial membrane; MF = muscle filament; MT = mitochondrion.

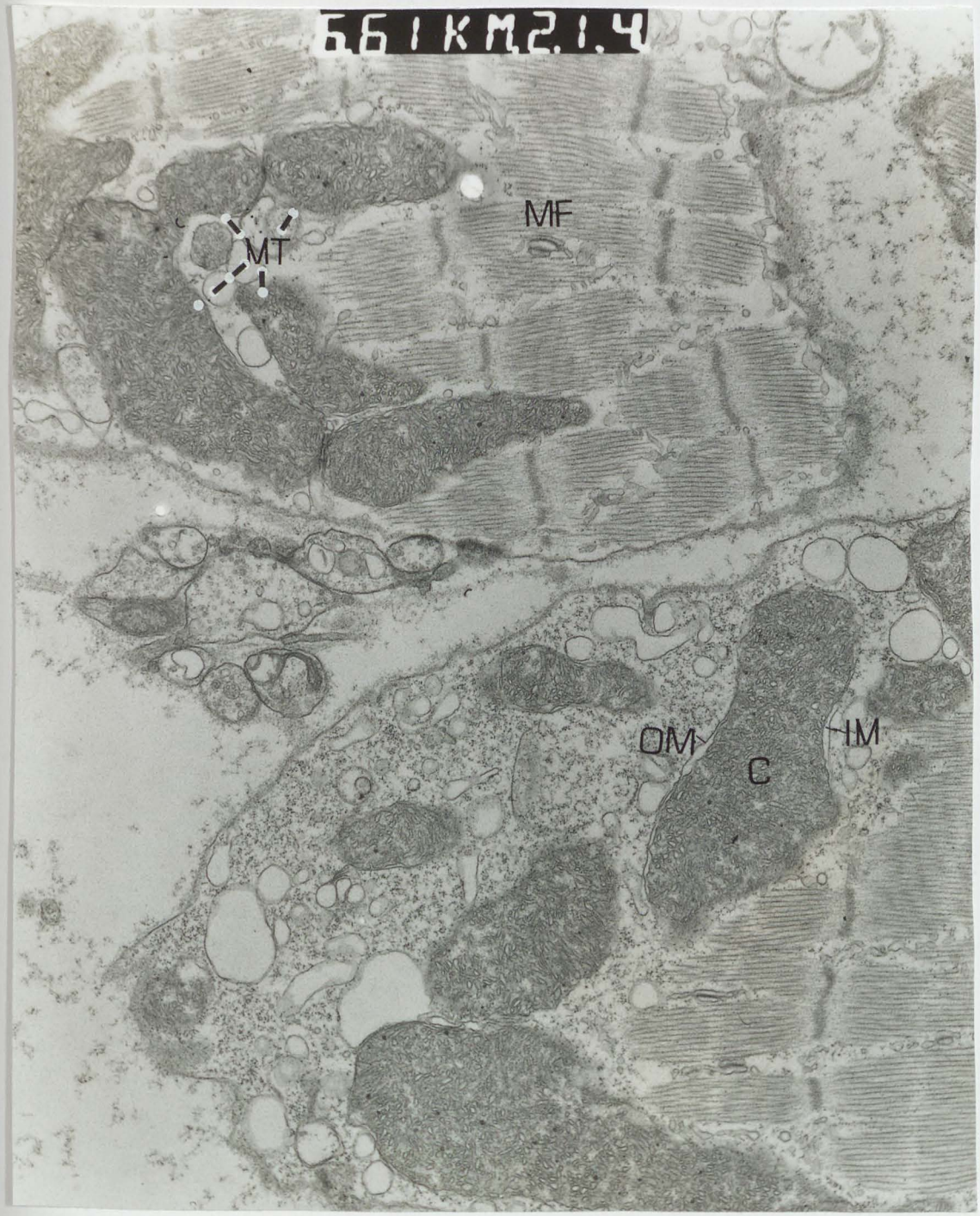

5.6 1 K M 2.1.4
MF
MT
OM
C
IM

Figure 5.4 Enlargement, to show mitochondrial detail, of representative TEM of limb bud muscle (transverse section) of Stage 3 *A. franciscana* cultured under chronic hypoxia (Magnification x 31 000): C = cristae; IM = inner mitochondrial membrane; OM = outer mitochondrial membrane; MF = muscle filament; MT = mitochondrion.

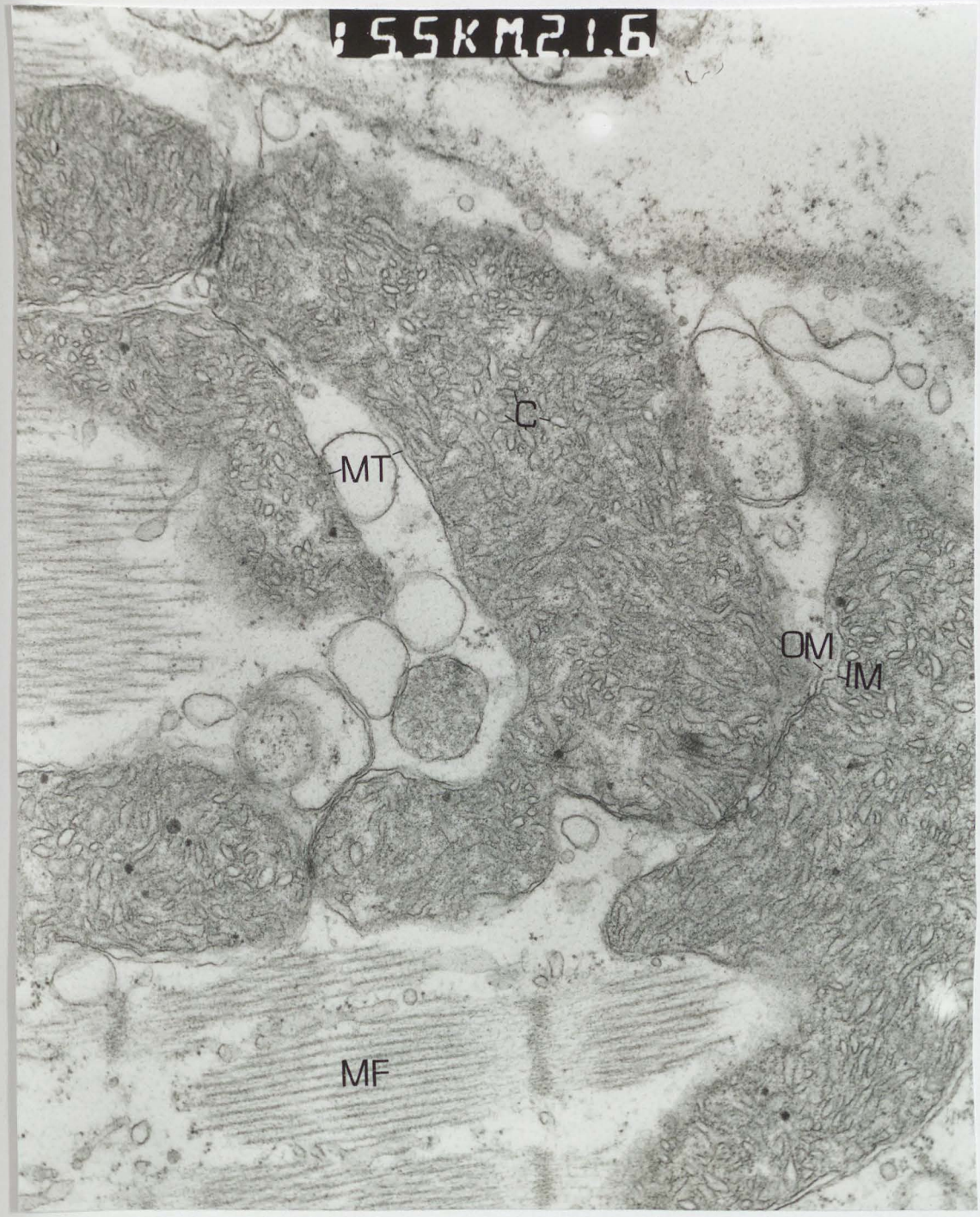
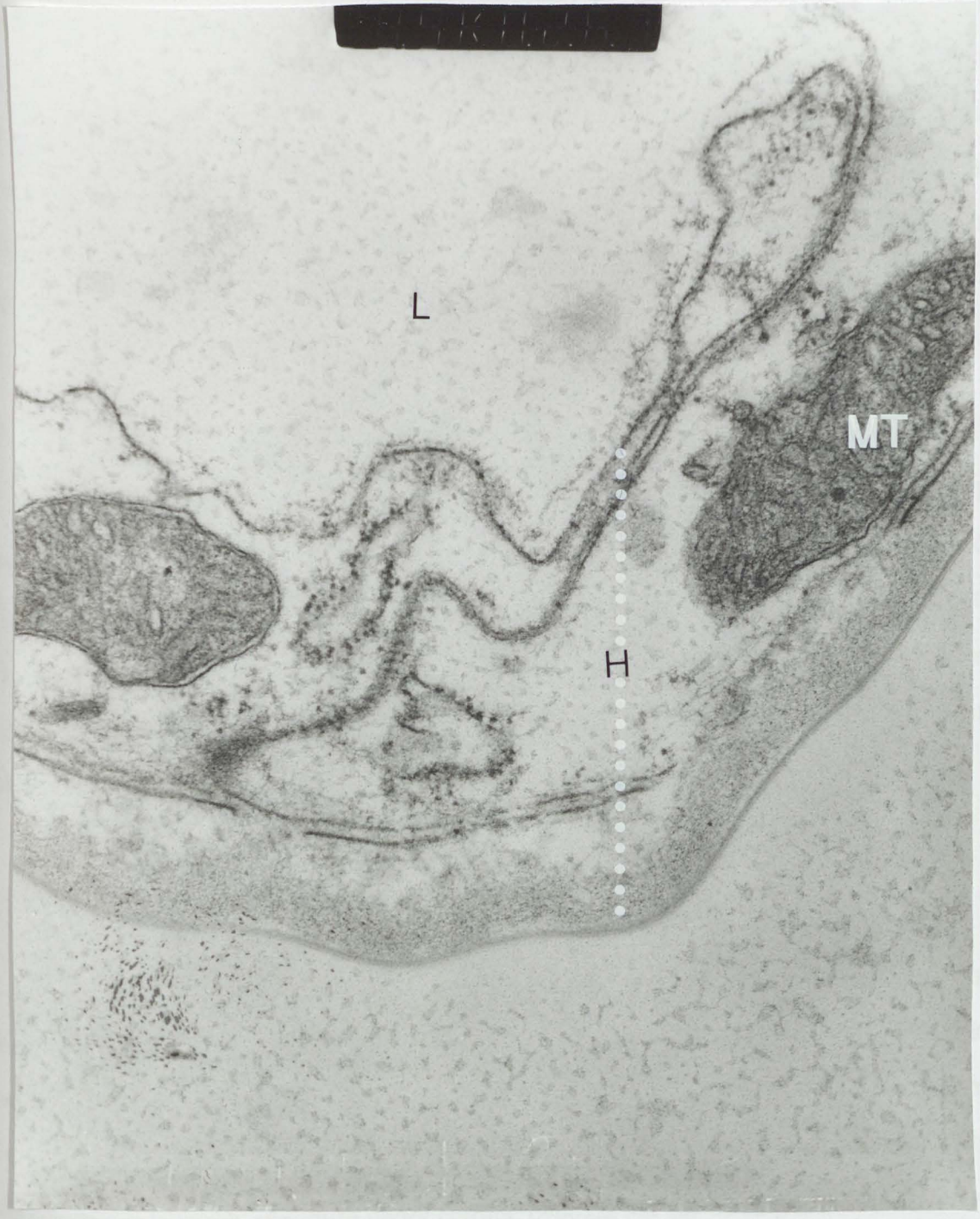

15.5KM2.1.6
C
MT
OM
IM
MF

Figure 5.5 Representative TEM of heart section (transverse section) of Stage 3 *A. franciscana* cultured under normoxia (Magnification x 57 000): H = heart wall; L = heart lumen; MT = mitochondrion.


L
MT
H

nature of the alterations were development-specific. In heart muscle from Stage 3 and 6 individuals cultured under chronic hypoxia the mitochondria were more oval in shape than found in normoxic controls and their cristae were either greatly underdeveloped or in some cases absent altogether (Figure 5.6). In differentiated heart muscle (Stages 9 and late), however, the density of mitochondria from hypoxic cultured individuals had increased (Figure 5.7) when compared with normoxic controls (Figure 5.8), although individual mitochondria were still small. Cristae were certainly more pronounced than was noted for chronic hypoxia-cultured Stage 3 or 6 individuals although still not as well developed as those of mitochondria found in limb muscle of normoxic controls.

5.3.1.3 Somatic and visceral tissue mitochondria

Mitochondria present in somatic and visceral tissues were similar in appearance, although generally less densely packed than those found in the limb bud muscles of normoxic controls (Figure 5.9). Culture of individual animals under chronic hypoxia did not markedly affect the density of mitochondria but it did affect their form (Figure 5.10); 'hypoxic' mitochondria were slightly smaller, more oval and contained fewer and less convoluted cristae than normoxic controls.

5.3.1.4 Gut wall

Mitochondria present in the gut wall generally were the same size, although more numerous than in those present in the limb bud muscles in normoxic controls: The cristae were characterised by a greater surface area. This was the case for all of the developmental Stages studied (Figure 5.11). The appearance of the mitochondria in the gut wall of individuals did not alter as a result of culture under chronic hypoxia but the density did seem to increase (Figure 5.12).

Figure 5.6 Representative TEM of heart section (transverse section) of Stage 3 *A. franciscana* cultured under chronic hypoxia (Magnification x 57 000): H = heart wall; L = heart lumen; MF = muscle filament; MT = mitochondrion.

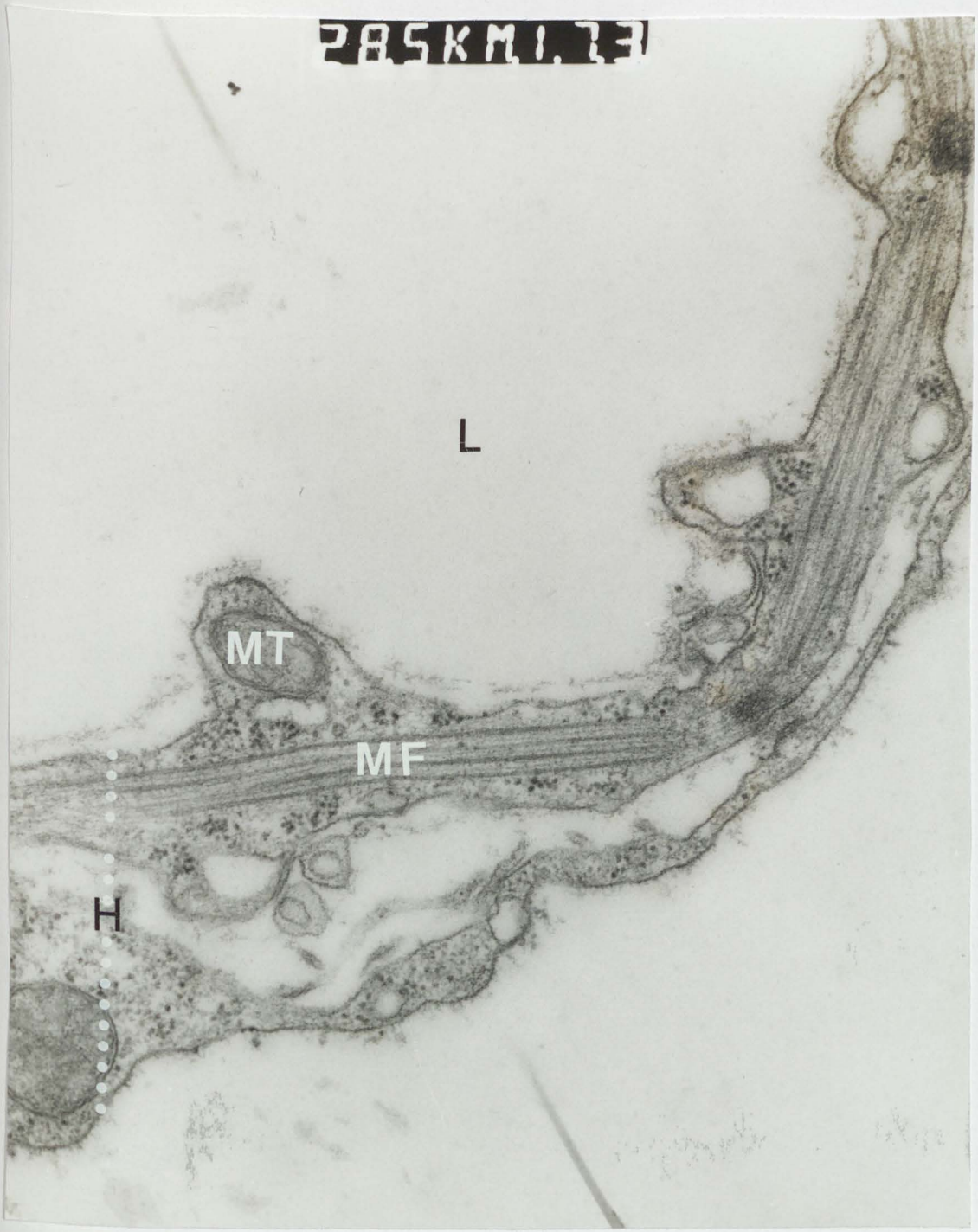

L
MT
MF
H

Figure 5.7 Representative TEM of heart section (transverse section) of Stage 9 *A. franciscana* cultured under chronic hypoxia (Magnification x 42 000): C = cristae G = gut wall; H = heart wall; L = heart lumen; MF = muscle filament; MT = mitochondrion; SG = secretory granules.

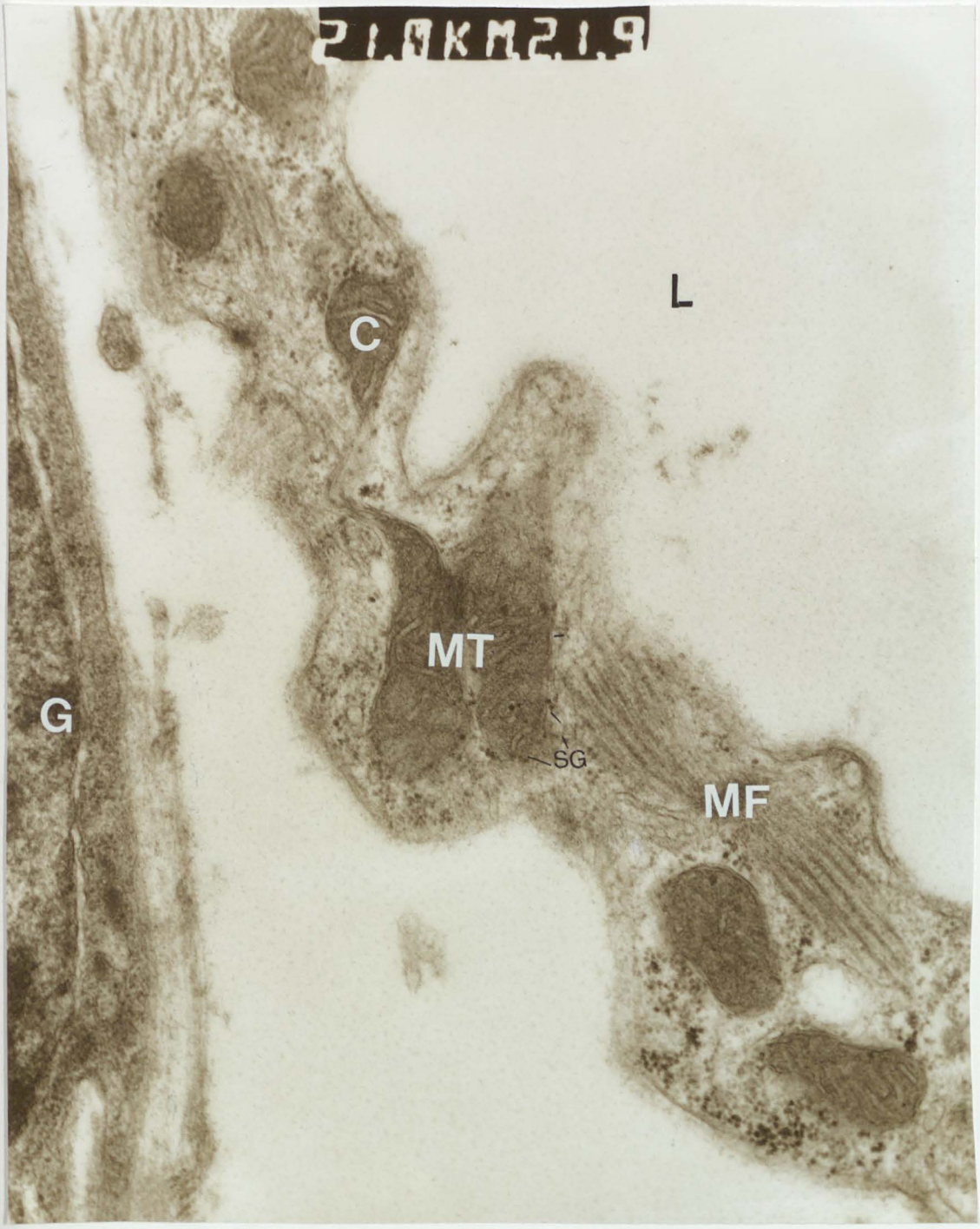

L
C
MT
G
SG
MF

Figure 5.8 Representative TEM of heart section (transverse section) of Stage 9 *A. franciscana* cultured under normoxia (Magnification x 42 000): C = cristae; H = heart wall; L = heart lumen; MF = muscle filament; MT = mitochondrion.

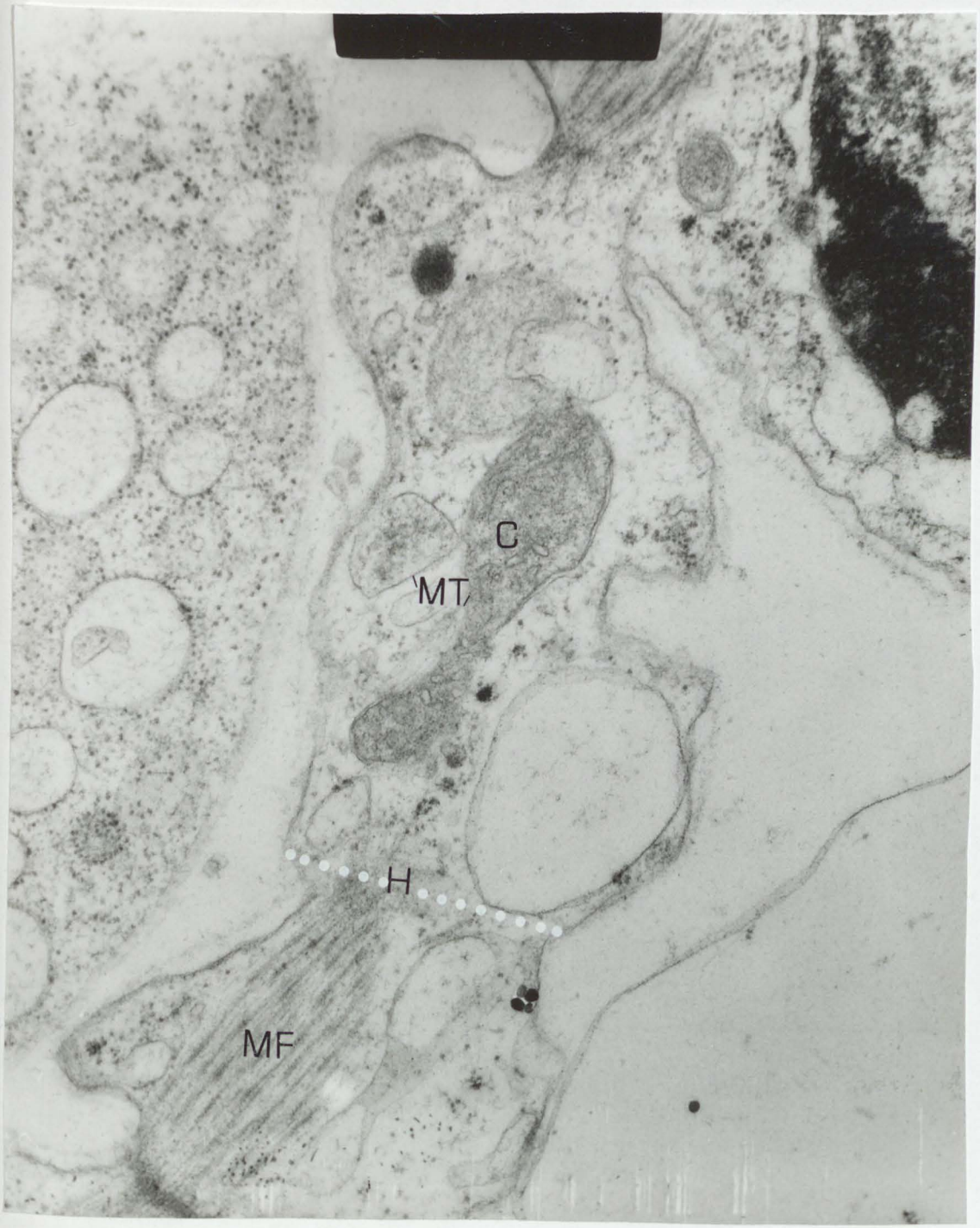

C
MT
H
MF

Figure 5.9 Representative TEM of thoracic somatic muscle, (transverse section) of Stage 8 *A. franciscana* cultured under normoxia (Magnification x 57 000): MF = muscle filaments; MT = mitochondrion.

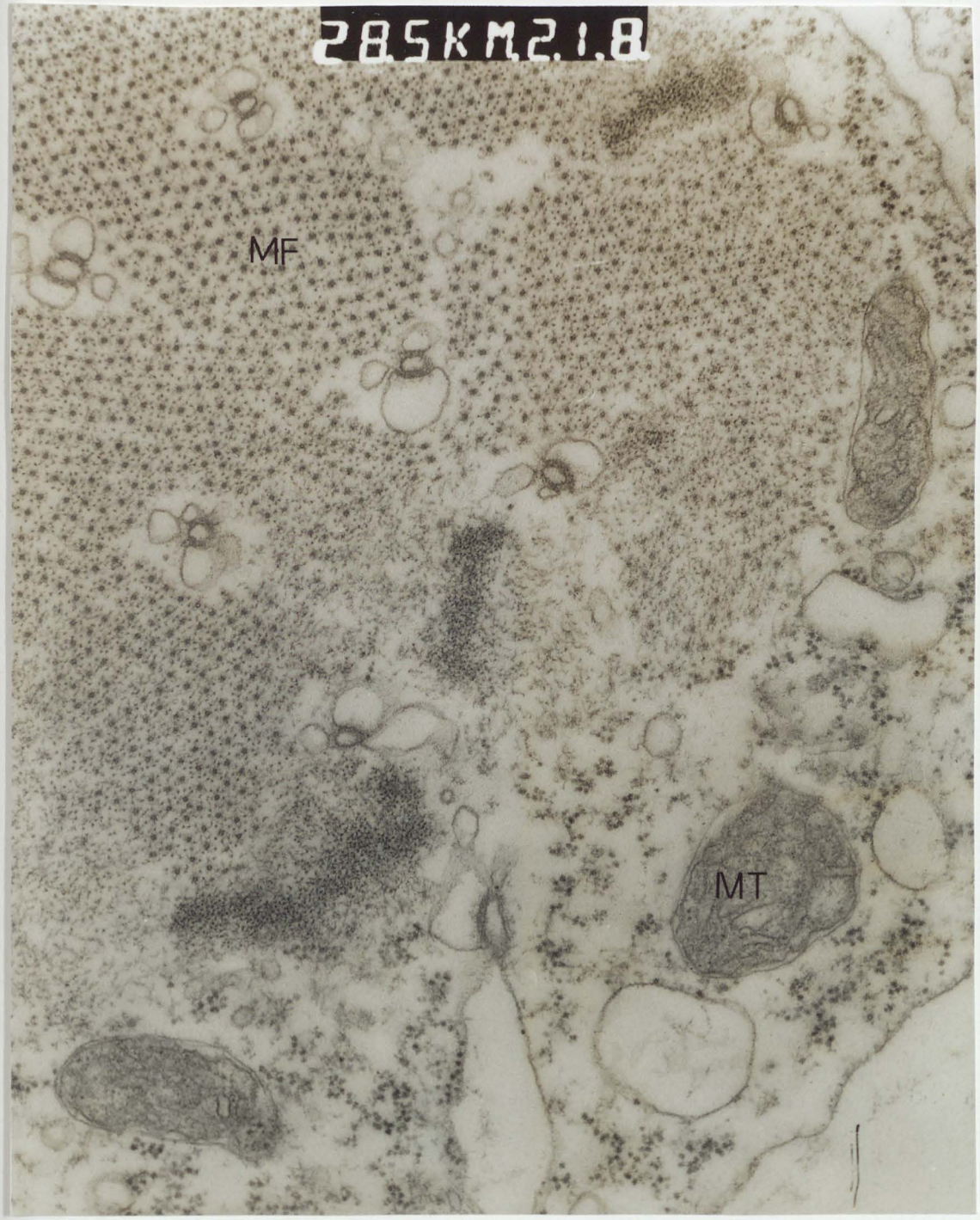

28.5KM2.1.8
MF
MT

Figure 5.10 Representative TEM of thoracic somatic muscle, (transverse section) of Stage 9 *A. franciscana* cultured under chronic hypoxia (Magnification x 57 000): MF = muscle filaments; MT = mitochondrion.

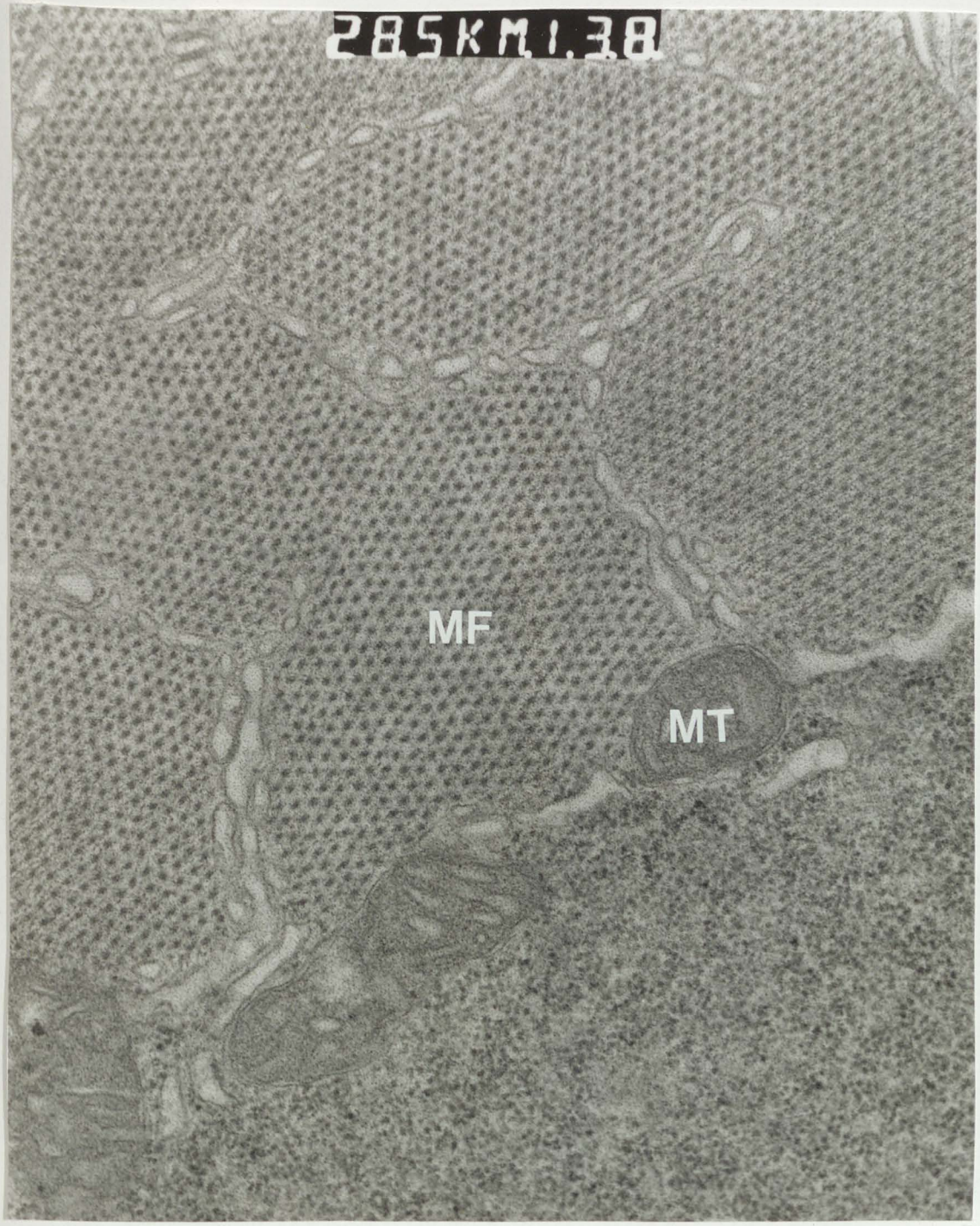

28.5KM1.38
MF
MT

Figure 5.11 Representative TEM of gut wall, (transverse section) of Stage 8 *A. franciscana* cultured under normoxia (Magnification x 9 900): L = lumen; MF = muscle filaments; MT = mitochondrion; N = nucleus.

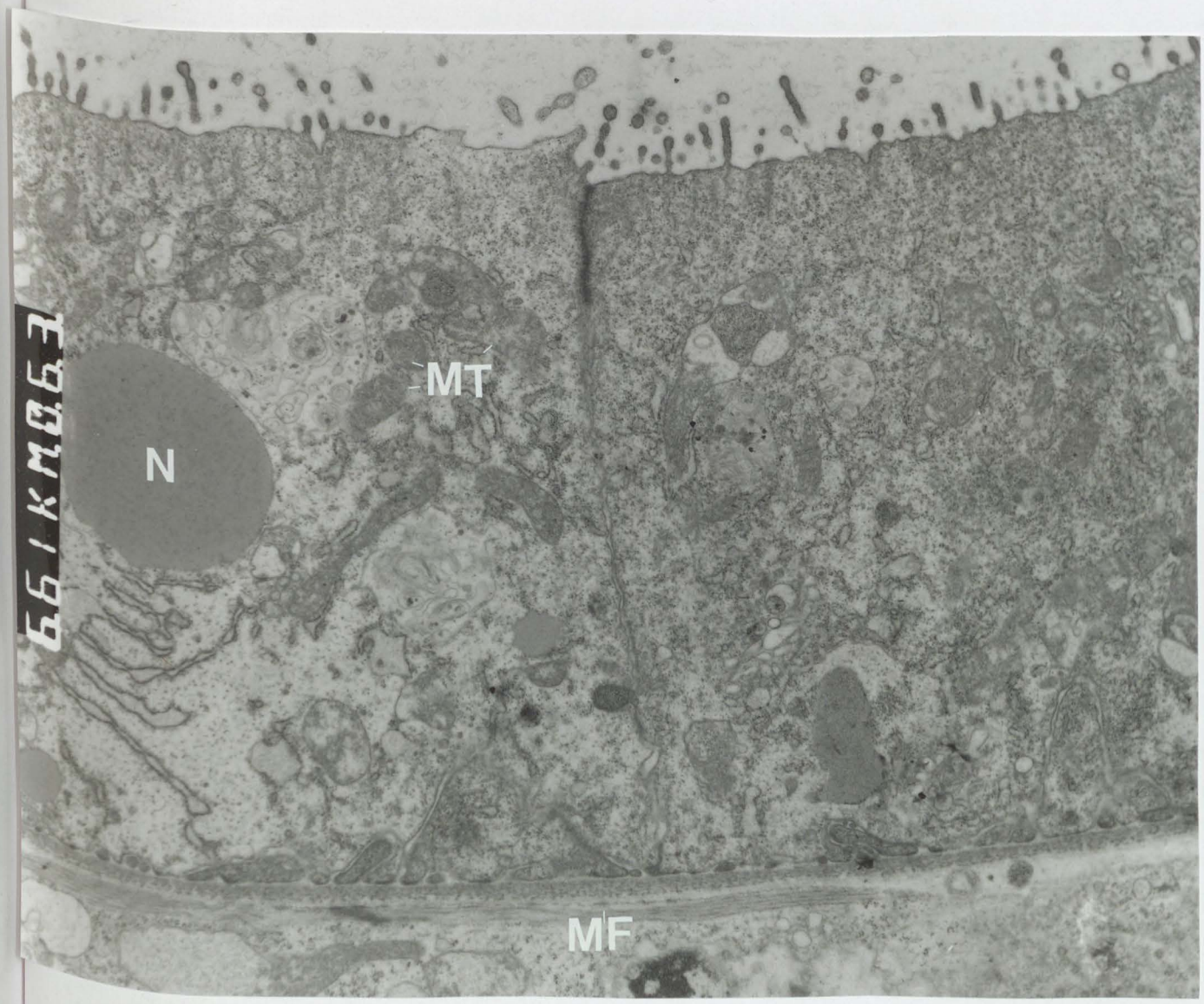
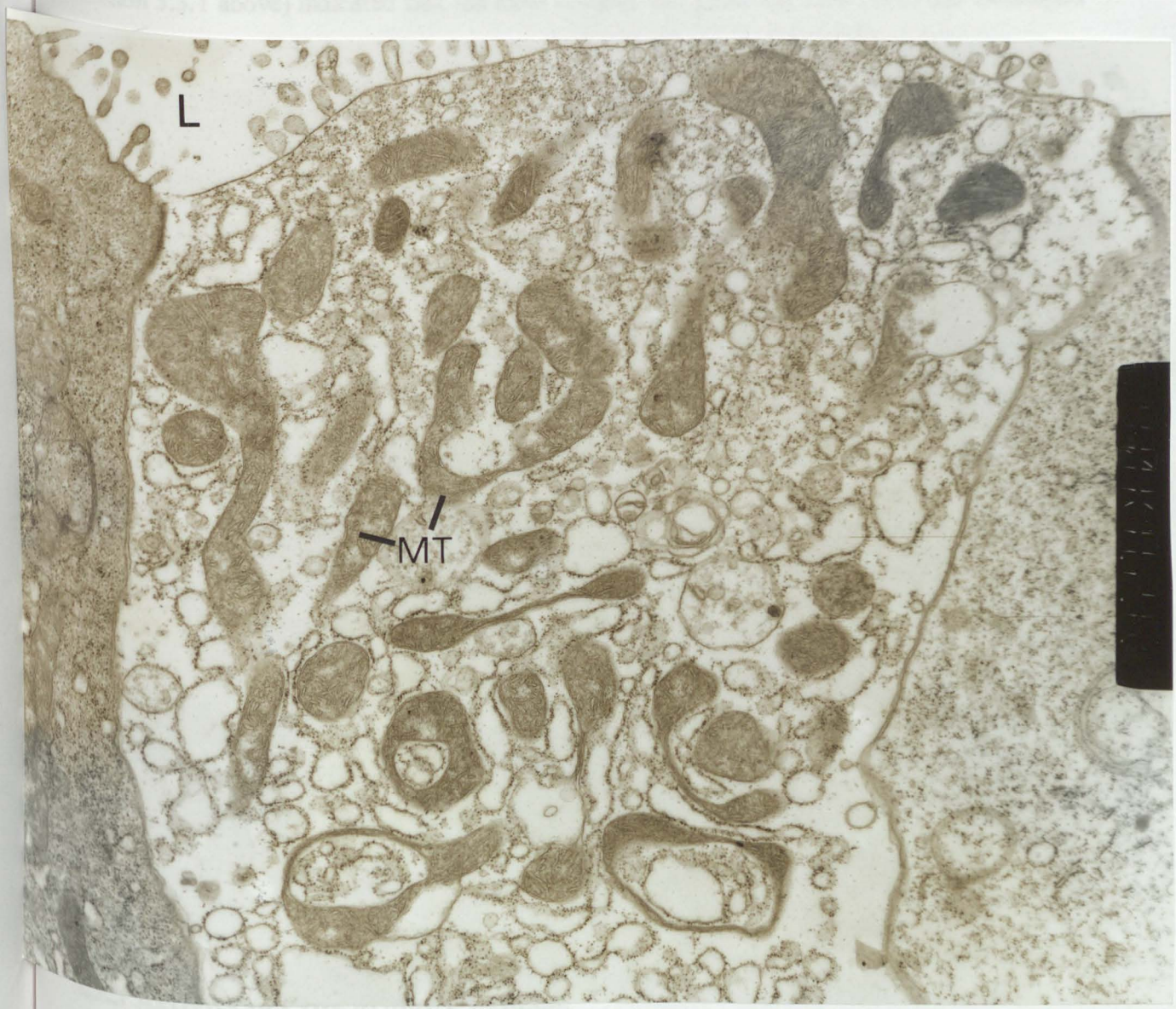

N
MT
MF

Figure 5.12 Representative TEM of gut wall, (transverse section) of Stage 8 *A. franciscana* cultured under chronic hypoxia (Magnification x 17 800): L = lumen; MT = mitochondrion.


L
MT

5.3.2 Localisation of cytochrome *c* oxidase

Presented below is a set of light micrographs, showing the localisation of cytochrome *c* oxidase in a range of different tissue types in normoxic and hypoxic cultured *A. franciscana*. Only late stage individuals have been examined as the TEM work on the mitochondria (Section 5.3.1 above) indicated that the most obvious and profound differences had developed by this time.

Presented in Figure 5.13 are a pair of light micrographs depicting the swimming limbs (1 to 4) of late stage *A. franciscana* cultured under normoxic and hypoxic conditions. The density of the blue staining represents the presence of cytochrome *c* oxidase. While cytochrome *c* oxidase was obviously present in the limbs of individuals cultured under normoxia, culture under chronic hypoxia resulted in a dramatic increase in the amount of this enzyme, as evidenced by the increase in the intensity of the blue stain. A similar pattern of staining was observed for the other limbs examined namely the mandibles (not figured).

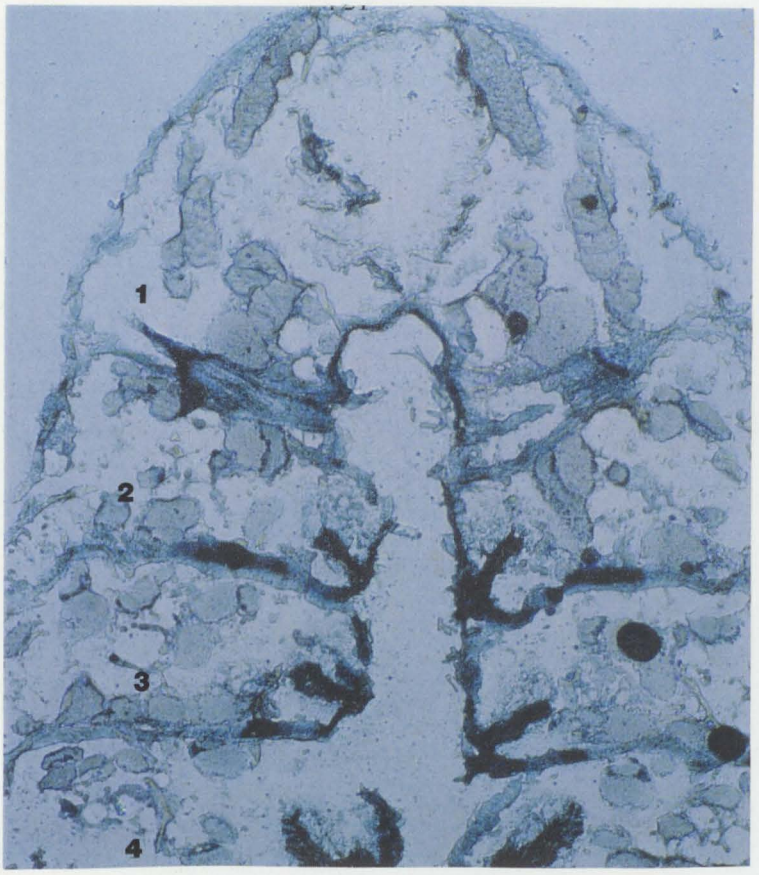
As was the case with the limbs, there was a dramatic increase in the concentration of cytochrome *c* oxidase in the dorsal epidermis and the gut (Figure 5.14), and the lining of the ovisac and uterus in the female (Figure 5.15), of *A. franciscana* as a result of culture under hypoxia.

Both the outer shell, as well as the arrested embryo (late gastrula), of cysts from mother cultured under hypoxia stained very heavily for cytochrome *c* oxidase when compared with normoxic controls (Figure 5.16). The shell in particular was very densely stained.

5.3.3 Activity of cytochrome *c* oxidase

The activities of cytochrome *c* oxidase from Stage 3 and late Stage, males and females, cultured under either normoxia or chronic hypoxia, are presented graphically in Figure 5.17. There was no significant difference in activity between normoxic Stage 3 and late stage males (Student's 't' test, $t = 0.12$, d.f. $= 4$, $P > 0.05$) or females (Student's 't' test, $t = -1.20$, d.f. $= 3$, $P > 0.05$) For each developmental stage examined, i.e. Stage 3, late Stage male, late Stage

Figure 5.13 Representative light micrograph (longitudinal section) of limbs of (A) normoxic and (B) hypoxic cultured late Stage *A. franciscana* stained for the presence of cytochrome *c* oxidase using Gräff's G- Nadi reaction. (Magnification x 125 for normoxia, x 160 for hypoxia): Appendages are labelled 1 to 4.


1
2
3
4

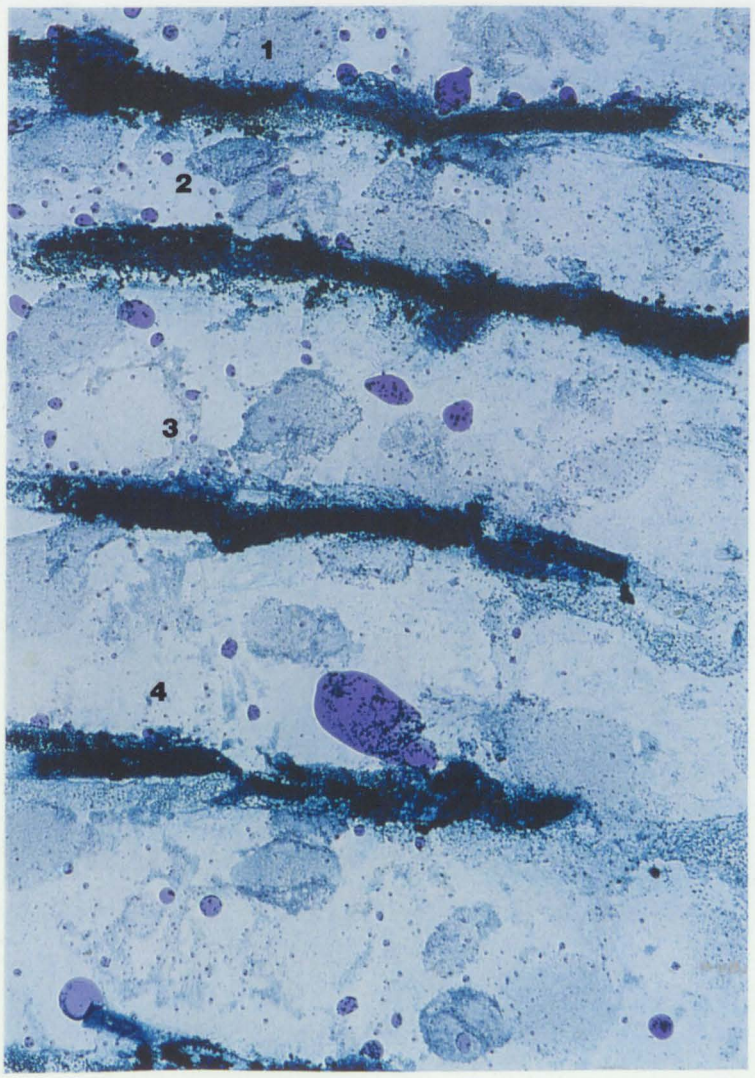
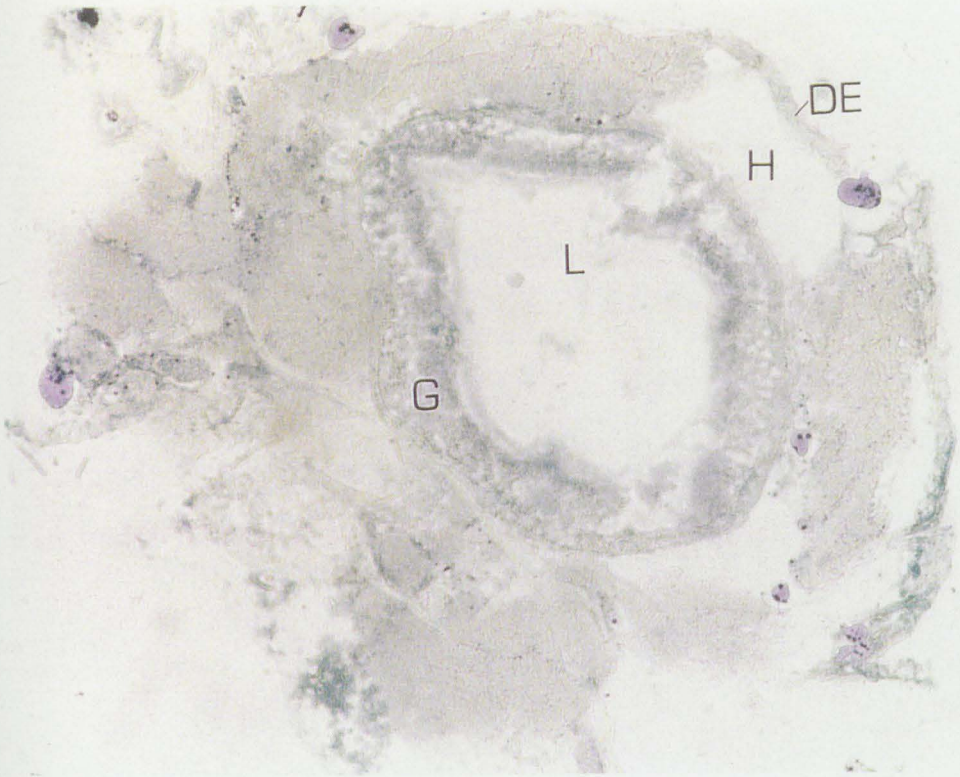

1
2
3
4

Figure 5.14 Representative light micrograph (transverse section) through the thorax of (A) normoxic and (B) hypoxic cultured late Stage *A. franciscana* stained for the presence of cytochrome *c* oxidase using Gräff's G- Nadi reaction. (Magnification x 160): DE = dorsal epidermis; G = gut wall; H = heart; L = lumen.


DE
H
L
G

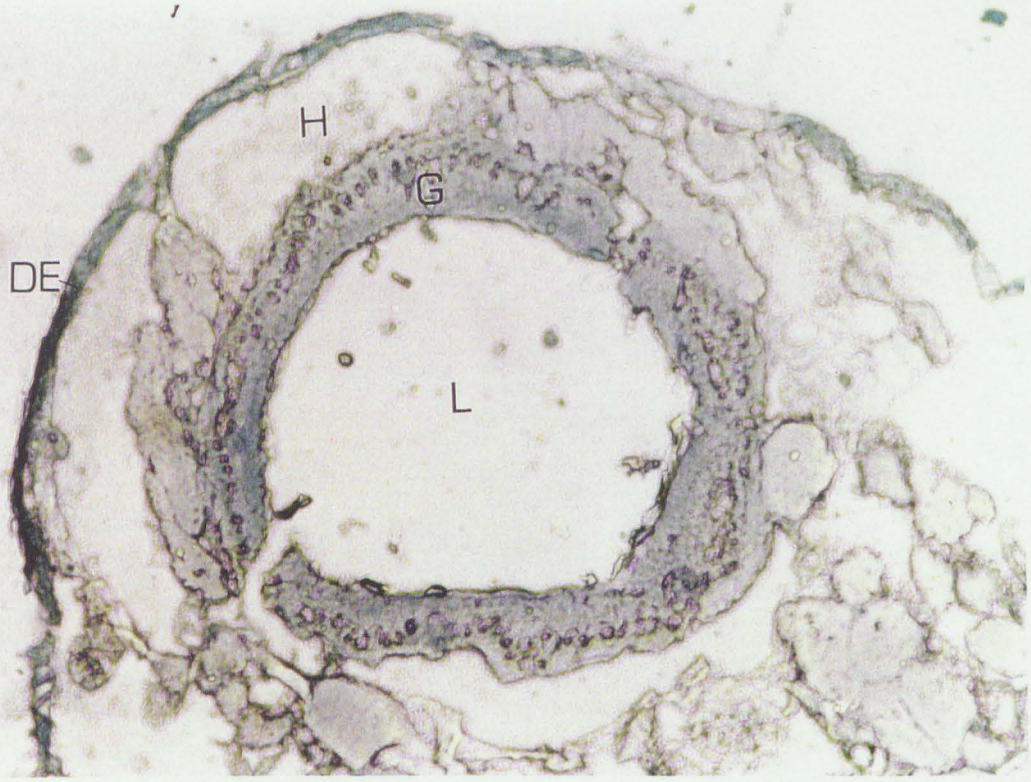
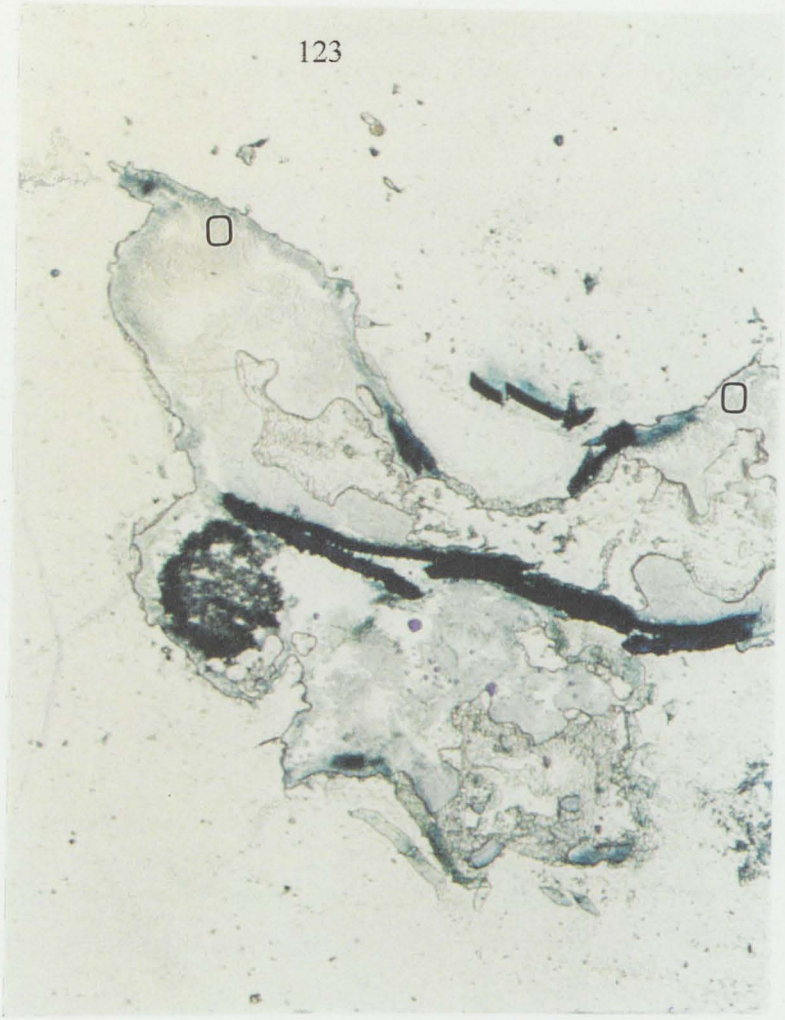

H
G
DE
L

Figure 5.15 Representative light micrograph (sagittal section) through the uterus and ovisac of (A) normoxic and (B) hypoxic cultured late stage *A. franciscana* stained for the presence of cytochrome *c* oxidase using Gräff's G- Nadi reaction. (magnification x 48 for normoxia and x 32 for hypoxia): O = ovisac.



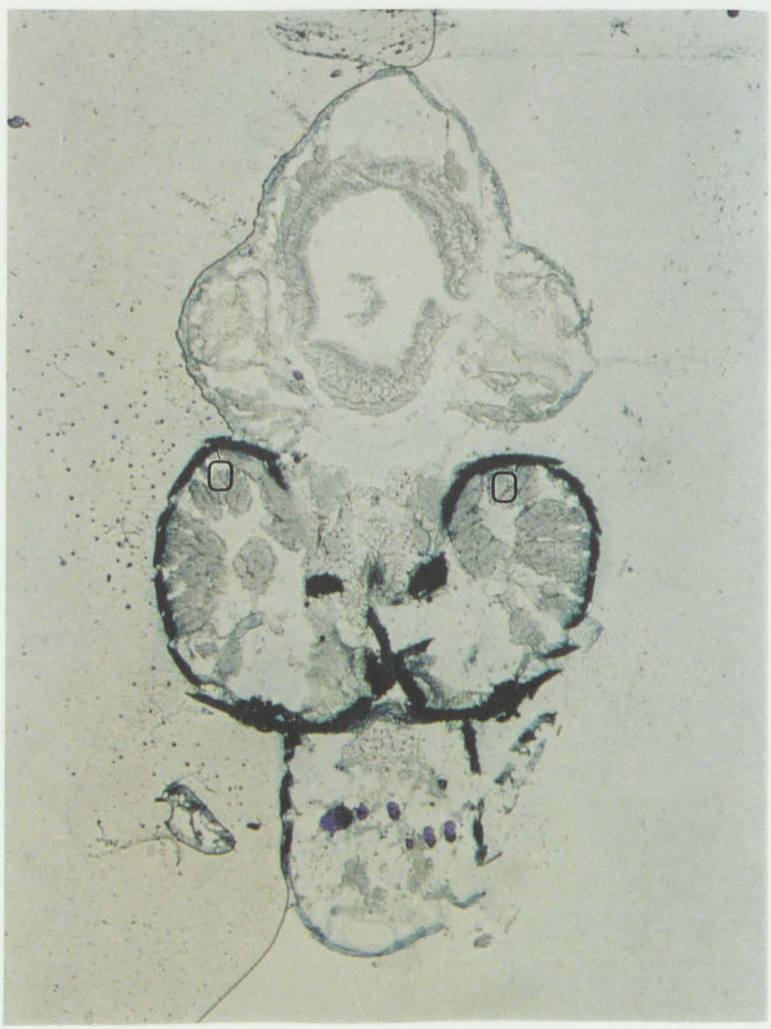
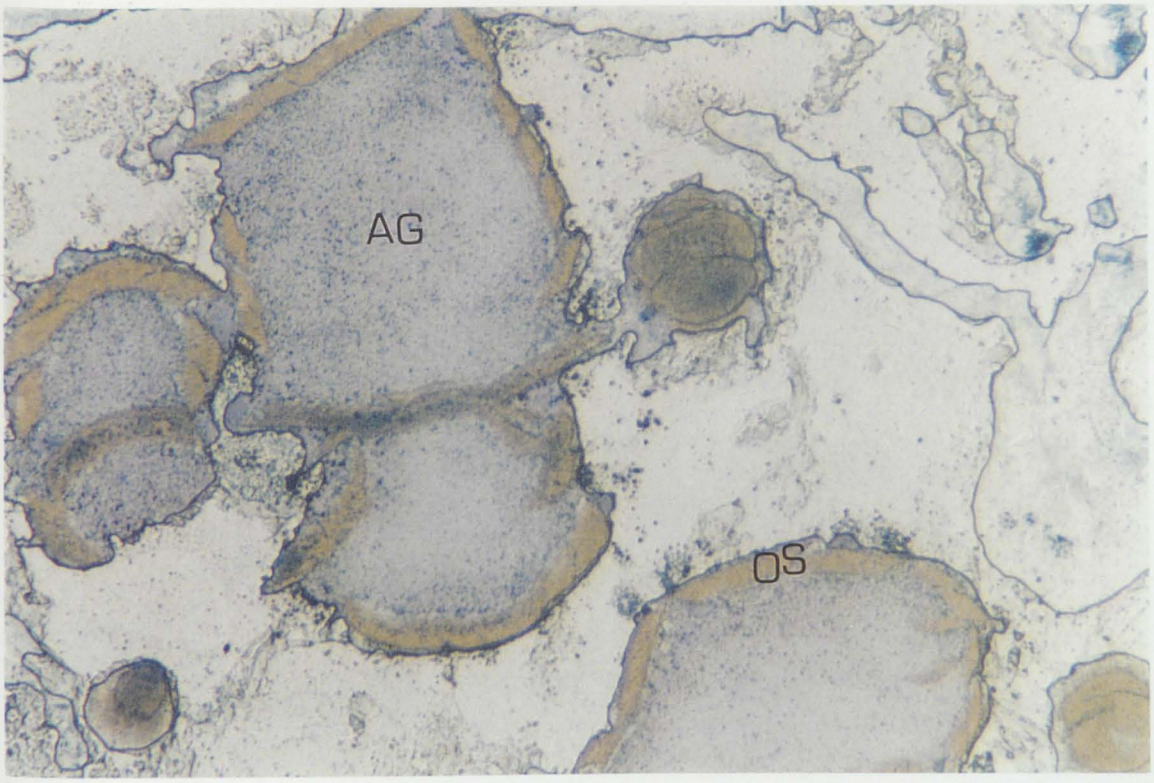


Figure 5.16 Representative light micrograph (transverse section) through cysts of *A. franciscana* taken from (A) normoxic and (B) hypoxic cultured females stained for the presence of cytochrome *c* oxidase using Gräff's G- Nadi reaction. (Magnification x 144): OS = outer shell; AG = arrested gastrula.


AG
OS

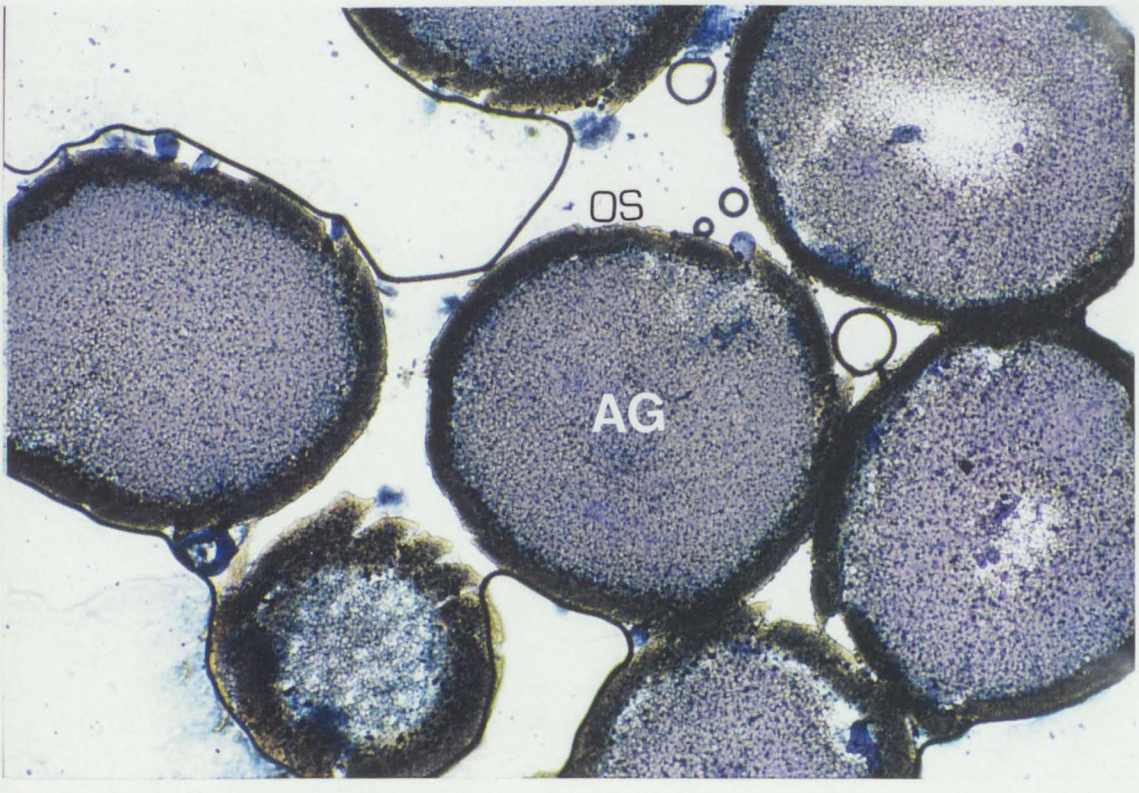
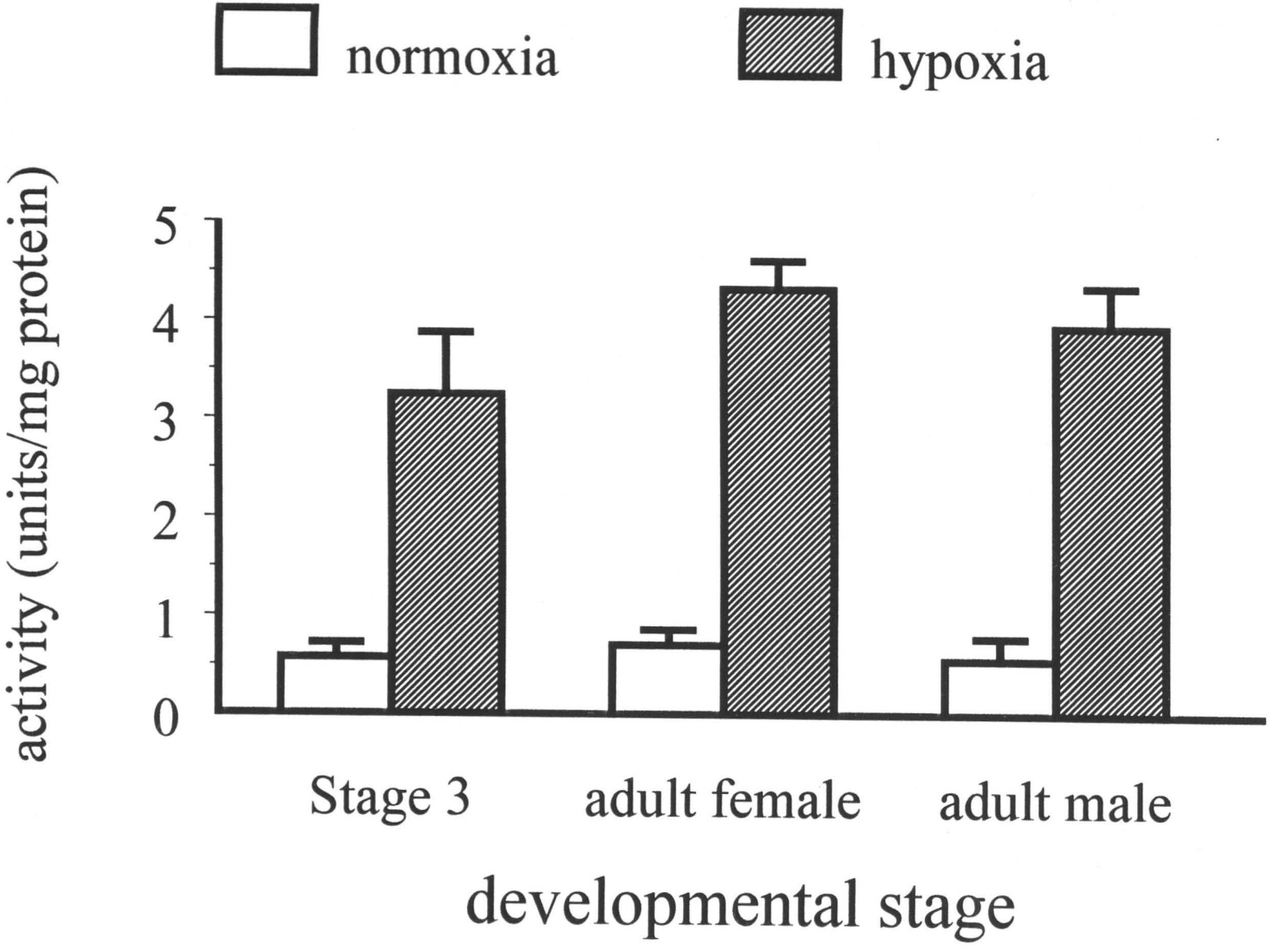

OS
AG

Figure 5.17 Specific activity of cytochrome *c* oxidase in different developmental stages of *A. franciscana* cultured under normoxia or chronic hypoxia. Values are given as means (n = 3 - 5 pooled samples) ± 1 S.D,.


normoxia
hypoxia
activity (units/mg protein)
0
1
2
3
4
5
Stage 3
adult female
adult male
developmental stage

female, there was a significant effect of chronic hypoxia on enzyme activity (Student's t test, t = -3.51, d.f. = 3, $P < 0.01$; t = -10.05, d.f. = 5, $P < 0.001$; t = -18.98, d.f. = 3, $P < 0.001$ respectively). The activities recorded for hypoxic animals were approximately x 6 - 7 greater than that of normoxic controls. For individuals cultured under chronic hypoxia there was a significant difference between activities for Stage 3 and those for late Stage females (Student's 't' test, t = -3.51, d.f. = 4, $P < 0.05$) but not males (Student's 't' test, t = -1.16, d.f. = 6, $P > 0.05$).

5.4 Discussion

5.4.1 Changes in mitochondria form and density with development

In general the appearance and distribution of mitochondria in brine shrimp *Artemia franciscana* changed little during development, *once the tissues containing the mitochondria had differentiated.* This stands in contrast to, for example, some recent work on fresh water fish where a dramatic decrease in both the volume and density of mitochondria accompanied development (Brooks *et al.*, 1995 also see Section 5.1.1 above for references). Before maturation, in *A. franciscana*, most mitochondria (and oxidative activity as indicated by cytochrome *c* oxidase concentration) were concentrated in the contractile muscle cells of the limbs and throughout the gut wall. This is probably related to the heightened aerobic activity required in these tissues for movement and/or to fuel active transport mechanisms (Hogeboom *et al.*, 1957 and Schneider, 1959) as well as possibly those tissues acting as an osmometer, functioning in the uptake and extrusion of intracellular fluid (Hootman & Conte, 1974). Typically the rest of the main tissue types examined displayed low oxidative activity and comparatively fewer mitochondria. For example, the somatic and vesicular muscle fibres contained relatively few mitochondria with underdeveloped cristae and were very similar in appearance to subcellular descriptions of poorly active muscle fibres such as the smooth, tonus-maintaining muscle fibres (Edwards & Ruska, 1955).

The form of the mitochondria present in the heart muscle of *A. franciscana* , one of the few tissues for which we have good comparative information, differed greatly from those described from the heart of some, but not all, of the closely-related species examined to date. The heart of *Lepidurus arcticus* (Tjønneland *et al.*, 1980) was characterised by a high density of mitochondria, and *Daphnia pulex* apparently possessed giant mitochondria even when the animals have been kept under normoxic conditions (Stein *et al.*, 1966). However, in common with *A. franciscana* studied here the heart tissues of *Artemia salina*, *Branchinecta paludosa*, *Branchipus schaefferi*, and *Streptocephalus* were characterised by low numbers of highly dispersed mitochondria (Økland *et al.*, 1982).

In this present study both the density of mitochondria and the surface area of the cristae, in cardiac tissue increased with increasing development; there was, however, little consistent trend observed in changes in the size of individual mitochondria. The reason the heart is different from many of the other tissues examined may, however, be related to the fact that this tissue takes longer to differentiate than most of the other tissues examined, appearing and beginning to differentiate in Stages 3 - 5 but only switching to growth by elongation after Stages 11 - 12 (Spicer, 1994). If this were the case we could say that there were no significant changes in the form or distribution of differentiated tissues during development. In conclusion the evidence we have, qualitative though it is, is not strong enough to support the hypothesis that there was an increase in number, but a decrease in mitochondial volume with development, in terms of the entire life cycle of the animal. However, it may be a different story if we replace the word development with differentiation.

There are profound implications for the fact that *Artemia* does not hatch as a completely differentiated miniature 'adult'. One of the key periods of intensive differentiation is during the formation of the heart, the gills, and the thoracic segments, each bearing a swimming appendage; Much of this developmental activity is crammed into Stages 4 - 13 (Weisz, 1946, 1947; Anderson, 1967; Criel, 1991a,b). This coincided with the dramatic increase during development in the ability to maintain respiratory independence on exposure to acutely declining oxygen tensions of normoxia cultured *A. franciscana.* (Section 4.4.1). In other

words our hypothesis, that there would be an increase in the aerobic capacity of the tissues as a result of an increase in the number of, and the cristae area within, mitochondria, was supported. However, this was not due to the modification of existing structures but to the process of mitochondrial biogenesis itself, in which the mitochondria form initially as spherical objects lacking cristae, and then increased in size and developed a large cristal surface area in a very short space of time (Schmitt *et al.*, 1973; Mattisson & Birch-Andersen , 1962).

5.4.2 Effect of chronic hypoxic culture on mitochondria form and density

There were profound differences in mitochondria form, distribution and density as a result of culture under chronic hypoxic conditions. However, the differences varied between tissues.

In those tissues which were already densely populated by mitochondria (limb muscle and gut wall) there were dramatic increases in the density of mitochondria, and in the case of the limb muscle the size, and folding of cristae (i.e. surface area) of the mitochondria was greatly enlarged too. Increase in mitochondrial density as a result of culture under chronic hypoxia, lends support to our hypothesis, based on mainly mammalian work (see references above), that hypoxic exposure should result in the density, size and or cristae area of mitochondria. This would appear to be different from the situation very early in *Artemia* development (late blastula) where hypoxia or anoxia resulted in a decrease in both size and protein synthesis of mitochondria (Kwast & Hand, 1996a,b).

The presence of megamitochondria was an obvious and characteristic feature of limb muscles in *A. franciscana* cultured under hypoxic conditions. They resembled similar structures described in flight muscles of insects , where mitochondria were very large and dense and had many cristae or tubules lying one next to the other (Moor & Ruska, 1957; Vogell, 1963). Similarly in the submandibular gland of the rabbit, in the resting state the mitochondria were few, small and slender, and scattered randomly in the cytoplasm, but in their synthesising stage the mitochondria increased in diameter two or three times (Threadgold, 1967). Megamitochondria are a common feature of tissues that require high

rates of oxidative metabolism such as spermatozoa , mammalian striated muscles and retinal rods (Threadgold, 1967). What is interesting in the case of *A. franciscana* is that megamitochondria were not a feature of normoxic animals but their formation was induced by applying an environmental stimulus in this case chronic hypoxia. Induced formation of megamitochondria has been recorded in rat pancreatic tissue, as a result of exposure to ethanol or iron (Tandler *et al.*, 1996) and mouse liver tissue by the chemical chloramphenicol (Matsuhashi *et al.*, 1996). Unfortunately the present experiments on *A. franciscana* tell us little about the mechanism of formation, e.g. formation of megamitochondria in the retinal cone of the fish *Tupaia belangeri* was by growth of a single mitochondrion not by the fusion of smaller mitochondria (Knabe & Kuhn, 1996).

Although the cristae in the mitochondria of limb muscles became more folded and possessed a greater surface area as a result of culture under hypoxic conditions in essence their form was still tubular and comparable in morphology to similar structures not only in other animal tissues but also in many protozoan and algal species (Threadgold, 1967).

In contrast to the situation in limb muscle, the effect of chronic hypoxia on the mitochondria found in somatic and visceral tissues was mainly negative. While there was no change in density, which was already low compared with limb muscle, cultured under chronic hypoxia, the mitochondria were much smaller than controls and the cristae were much less convoluted. Clearly culture under chronic hypoxia had a beneficial effect on mitochondrial density and form in some tissues but not others. While in limb muscle there were increases in mitochondrial size, cristal surface area and density, only increases in density and cristae areas (not mitochondrial volume) were seen in heart tissue and an increase in density alone was noted in the gut. This may give some indication of the aerobic nature of some tissues (muscle & heart) and the capacity for anaerobic metabolism in others. Certainly if this is true then the fact that the volume and cristal area of mitochondria in somatic and visceral tissues decreased under chronic hypoxia implies that these tissues have a greater capacity for anaerobic metabolism than the others and/or a lower metabolic rate as a result of hypoxic exposure,

would seem to indicate that the tissues of chronic hypoxia-cultured brine shrimp should have a greater capacity for aerobic metabolism than normoxia-cultured controls.

5.4.3 Effect of chronic hypoxia on cytochrome *c* oxidase concentration and activity

The results of the cytochrome *c* oxidase staining and activity experiments strongly support what has gone before on mitochondria form and density, if we take it that anything that enhances mitochondrial size and/or area, increases the capacity for aerobic metabolism. All the tissues examined using the TEM stained positive for the presence of cytochrome *c* oxidase and furthermore the concentration increased dramatically as a result of culture under chronic hypoxia. The early work of Keilin (1925) showed that staining for cytochrome *c* oxidase, the greatest concentrations were present in the most (aerobically) active tissues, e.g. in the pharyngeal muscles of molluscs, the heart muscle of crabs and mammals and the thoracic (wing bearing) muscles of flying insects.

Equally as interesting was the fact that the 'internal environment' of the brine shrimp as well as the embryo and cyst shell, showed an increase in the amount of cytochrome *c* oxidase present as a result of exposure to chronic hypoxia, which correlated strongly with the induction of hemoglobin in these tissues. The implications of these findings for the developing embryo will be discussed more fully in the next chapter (Section 6.1.4). For the moment we will be content with noting that in many parasitic worms, such as *Ascaris lumbricoides*, the highest concentration of cytochrome is found in the spermatozoa and eggs and in the mollusc, *Helix aspersa* in the genital glands and in frogs in the testes and heart (Keilin, 1925).

Turning from the concentration of cytochrome *c* oxidase to look at the activity of this enzyme in whole animal preparations, there was a dramatic increase in total enzyme activity as a result of culture under chronic hypoxia. This supports the hypothesis that activity should increase with chronic hypoxia and fits well with the work of Korzeniewski (1996) who predicted, on theoretical grounds, that the activity of cytochrome *c* oxidase should increase

with a decrease in available oxygen. However, the hypothesis that weight-specific enzyme activity should decrease with age seemed to be held in males but not females. It could be suggested, however, that the cytochrome *c* oxidase activity associated with the ovisac and egg sac is so pronounced as to elevate the total body activity of females over that of males.

5.4.4 Conclusions

Rosenthal & Drabkin (1942 quoted in Drabkin, 1950) showed, more than 50 years ago, that there was a strong relationship between oxygen uptake, mitochondrial presence, the concentration of cytochrome *c* and the activity of cytochrome *c* oxidase in the tissues. This was, however, questioned by Drabkin (1950) who suggested that while the oxidase is present in excess in the tissues the concentration of cytochrome *c* is the limiting factor. Similarly some have questioned the relationship between the number of mitochondria and the activity of cytochrome *c* oxidase activity by pointing out that other factors come into the equation, e.g. the decline in cytochrome *c* oxidase activity in the proximal convoluted tubules of the frog nephron was not due to a decrease in mitochondria number, but to the reorientation of the cristae within the organelle (Karnovsky, 1965, quoted in Threadgold, 1967) .

What is clear from this present study on the brine shrimp *A. franciscana* is that, backing up the early study of Rosenthal & Drabkin (1942 as quoted in Drabkin, 1950), there was fairly good agreement between TEM studies of mitochondria density and form, localisation of cytochrome *c* oxidase and the activity of the enzyme in whole body preparations, in what is admittedly a qualitative approach to this question. Culture under chronic, but comparatively moderate, hypoxia generally increased mitochondria volume, number and cristae surface area, in the already most mitochondria rich tissues, and also increased the concentration and activity of cytochrome *c* oxidase. This was most noticeable for the most active (aerobic) tissues. This is a slightly different picture, from that based on exposure to severe chronic hypoxia or anoxia which sees the subcellular responses of brine shrimp, and animal tissues in general, as being very negative in effect, e.g. resulting in depression of protein synthesis (Kwast & Hand, 1996a,b), generation of stress proteins to deal with hypoxia-related oxidative damage

(Corbucci *et al.*, 1995), deletion of mitochondrial DNA in nervous tissue (Merril *et al.*, 1995), and depressed aerobic metabolism of isolated mitochondria (Chandel *et al.*, 1996). Obviously the level and duration of hypoxia must be taken into account when assessing the impact of chronic hypoxia on subcellular respiratory structure and function. In the case of the moderate, but prolonged, hypoxia experienced by *A. franciscana* in this present study, it could be argued that the subcellular responses recorded are quite positive and perhaps adaptive.

Therefore it is concluded that brine shrimp show subcellular responses to hypoxic culture in that they enhance the aerobic capability of their most active tissues such as limb and cardiac muscle; so far from shutting down aerobic metabolism in favour of recourse to anaerobic metabolism, it would seem that under moderate chronic hypoxia, the efficiency of aerobic metabolism was increased and enhanced.

Chapter 6 General discussion - putting the pieces together

6.1 Physiological responses to hypoxic culture and subsequent hypoxic exposure in *Artemia franciscana*: patterns and mechanisms

6.1.1 Changing the physiological itinerary - 'telescoping'

One of the main findings of this study is that *Artemia franciscana*, cultured under normoxic conditions, displayed a dramatic improvement in respiratory performance when exposed to acutely declining oxygen tensions, as development proceeded (Section 4.4.1). The ability to regulate oxygen uptake during progressive hypoxia developed early in ontogeny (Stages 1 - 6) and had attained the 'adult pattern' by the time the gills and heart had formed in the thoracic stage of development. For *A. franciscana* cultured under chronic hypoxia, this improvement in respiratory performance was achieved at a much earlier developmental stage (Section 4.4.2). In other words, physiological development and morphological development were proceeding at different rates. Furthermore the effect of hypoxic culture of bringing development forward in time, i.e. hypoxia cultured brine shrimp developed faster than normoxic controls, at least during early ontogeny (Section 3.4.1) meant that the hypoxia-induced improvement in respiratory performance was brought even further forward in real time. This telescoping of respiratory physiological development means that the adult pattern of respiratory regulation was achieved considerably sooner than was found for normoxic controls. We will now discuss some of the possible mechanisms underlying the improvement in respiratory performance of *A.*

franciscana with development as well as those underlying the hypoxia-related, telescoping of respiratory improvement.

6.1.2 Gas exchange surfaces, ventilation and perfusion

We have noted that respiratory improvement during normal development co-occurred with the development of the gills in *Artemia franciscana*. This has been recorded before by Spicer (1995c). He suggested that if the development of the gills was responsible for respiratory improvement then if he used heavy metal exposure to damage gill structure (and thereby compromise gill functions such as gas exchange and osmoregulation) then he could negate any potential improvement. This is exactly what did happen, implicating the formation of the gills in the improvement in respiratory performance, although Spicer (1995c) did admit that he could not discount any effect that heavy metal exposure might have on hemoglobin function and impairment of hemoglobin function possibly giving the same result. So we conclude that it would not be surprising if the development of the gills were implicated in the improved respiratory performance which takes place during their formation. What is certain is that the further hypoxia related improvement in respiratory performance that takes place earlier in development cannot be attributed to the early formation of the gills. The gills of hypoxia-cultured individuals develop earlier in time than normoxic controls, but not at an earlier developmental Stage (Section 3.4.1). Indeed this is physically impossible as the gills require limbs to bear them and the limbs require thoracic segments to bear them (Sections 3.1.1 and 3.4.1). Thus the developmental iternary is an important developmental constraint in the ability to bring some structural features forward in ontogeny.

As the gills are located on the swimming appendages of *A. franciscana*, their ventilation is achieved during normal swimming activity (Section 2.2). Although ventilation was not specifically quantified during this study, there was no obvious increase in limb beating

frequency either as a result of culture under hypoxia or as a result of exposure to acutely declining oxygen tensions. In fact in the case of the latter, after the P_c point was reached there was often a decrease in swimming activity noted (Section 4.3.1.3). In conclusion, although we have little evidence of changes in ventilatory performance being related to the maintenance of oxygen uptake during culture under chronic hypoxia or when exposed to acutely declining oxygen tensions, we cannot rule such changes out. On the basis of what we know of respiratory responses of other crustaceans to hypoxia (Section 4.1.1), ventilatory changes are more likely to be involved in short term responses rather than the more long term responses we consider when we look at respiratory improvement during development or hypoxia-related improved respiratory performance earlier in development (Section 4.4.2).

It is conceivable that changes in the patterns of tissue perfusion during development, and as influenced by hypoxic culture, could be implicated in changes in respiratory performance noted during this study. However, as was the case with the gills (see above) heart formation only takes place during stages 3 - 4 (Section 3.1.1) and a fully functioning differentiated heart is not present until well after the adult pattern of respiratory regulation has been established (Spicer, 1994, 1995a,b, Spicer & Morritt, 1996). So, development of cardiac function could be implicated in the late stages of the improvement of respiratory regulation in normoxic, but not hypoxic, cultured brine shrimp for the same reasons we came to this conclusion about the gills (you cannot develop a heart before you develop somewhere, i.e. thoracic segments, to keep it). However, there is a possible confounding factor, as we know that in *A. franciscana* before a functional heart is present circulatory function is provided by the muscle movements resulting from swimming activity and also from peristaltic movements by the gut (Spicer, 1994). Furthermore we know that the rate of gut peristalsis can be increased by restraining the swimming limbs, thereby eliminating the role of their muscle movements in circulation (Spicer, 1994). Consequently, it is not

inconceivable that modification of such gut movements could play a role in improving the respiratory performance of hypoxic cultured brine shrimp even before the appearance of a functional heart. This possibility deserves more careful study.

6.1.3 The respiratory pigment hemoglobin

That there are changes in the affinity for oxygen of brine shrimp hemoglobins during development is not new and is relatively well documented (Section 4.1.3). As noted by Spicer (1995c) the change in affinity takes place at the same time as the gills and heart develop and co-occur with the improvement in respiratory performance that takes place during development in *A. franciscana.*

However, the developmental shift in oxygen affinity observed by previous studies appears to be going in the wrong direction (from high to low) to account for the improvement in respiratory performance which develops during the early thoracic period of development. A pigment with a high affinity for oxygen is generally seen as being more advantageous for oxygen uptake under conditions of declining oxygen tensions than a low affinity pigment (Weber, 1980; Mangum, 1983, 1990). Certainly there was no detectable increase in total hemoglobin content with development coinciding with the improvement in respiratory performance noted during development in normoxic culture (Section 4.4.1). In this connection it is interesting that for animals cultured under normoxic conditions, there was no effect on their ability to maintain oxygen uptake during progressive hypoxia, or its improvement during development, of disabling the oxygen transporting properties of hemoglobin in *A. franciscana* (Section 4.4.3). This may mean one of two things. It may mean that the possession of hemoglobin and the changes in affinity associated with development have no importance in the respiratory performance of normoxic cultured brine shrimp. Alternatively, and perhaps more likely, it may mean that although the hemoglobin plays a role in respiratory performance and its development during ontogeny,

it is not an indispensable role, at least in the short term: *A. franciscana* may be able to resort acutely to other respiratory mechanisms (e.g. changes ventilation, perfusion) to maintain oxygen uptake during declining oxygen tensions, 'covering up' for the inoperative respiratory pigment. It is suggested, therefore, that if hemoglobin plays a role in the respiratory biology of developing brine shrimp in normoxia it is not indispensable, at least in the short term. If this were the case, it could be further hypothesised that the change from hemoglobin with a high affinity for oxygen to one with low affinity, is as a consequence of the development of gills and heart allowing the whole organisms more access to dissolved oxygen in the medium. This theory would suggest that before the formation of gills, the outer covering of the brine shrimp is highly impermeable and so the high affinity respiratory pigment would be ideal for working in what was essentially an hypoxic internal environment.

When *Artemia franciscana* were cultured under hypoxia the total hemoglobin content of individuals increased visibly and dramatically compared with normoxic controls (Section 3.3.3). Furthermore, the hemoglobin was not just found in the hemolymph, but also within a large number of the different tissues/organ systems examined (Section 3.3.3). Although rendering the hemoglobin inoperative, via treatment with CO, did not affect the oxygen uptake of hypoxic cultured individuals, examined under normoxia conditions, there was a dramatic effect of CO under conditions of declining oxygen tensions (Section 4.3.2.3). In fact most of the hypoxia-related improvement in respiratory performance, evident under conditions of declining oxygen tensions, was abolished when the oxygen binding properties of the hemoglobin were rendered inoperative. In other words most of the hypoxia-related respiratory improvement could be accounted for by the presence of hemoglobin.

So we can conclude that while we cannot attribute the improvement in respiratory performance (under conditions of declining oxygen tensions) that takes place during

development, to the presence of hemoglobin, we can conclude that hemoglobin is essential in the hypoxia related 'further' improvement.

6.1.4 Aerobic capacity of the tissues

Most studies of the effect of chronic or acute hypoxia on respiratory performance concentrate on alterations in the physiological mechanisms responsible for oxygen uptake, transport and delivery to the tissues, i.e. ventilation, perfusion, oxygen loading and unloading (Section 4.1.1). However, this study has demonstrated the potential for changes in the aerobic capacity of the tissue itself. Although there were interesting subcellular changes catalogued during the development of *Artemia franciscana*, (Section 5.3.1) the most dramatic finding was that there were marked alterations in cytochrome *c* oxidase activity and mitochondria biology (increase in size, cristae area and number) (Section 5.3.1). This means that as well as the options available to improve the amount of oxygen that is taken to the tissues, after it is extracted from hypoxic water, there is also the possibility of more efficient use of the oxygen that is delivered to the tissues, via increased efficiency and scope for aerobic metabolism in the most aerobic tissues. Although in some ways this should not be too surprising, this is not an option which is traditionally considered when examining the response of whole animals to hypoxia.

The apparent relationship between hemoglobin and cytochrome *c* oxidase is an interesting one. In the tissues where there were dramatic increases in hemoglobin (Section 3.3.3) as a result of hypoxic culture, there were also dramatic increases in cytochrome-c-oxidase (Section 5.3.3). There are two plausible explanations for this 'relationship'.

1. The staining techniques are not as highly substrate specific as their advocates have lead us to believe (Sections 3.2.4 and 5.2.3). Thus we could have stained for either hemoglobin or cytochrome *c* oxidase but probably not both independently. While this

possibility cannot be totally discounted, the fact that the stains employed seem to rely on specific catalytic abilities means that we can be fairly confident that the relationship we observe is real.

2. That tissues with a high aerobic capacity have been provisioned with a respiratory pigment. So in this case, not only would hemoglobin be involved in transporting oxygen to the tissues extracellularly, but intracellular concentrations would also be involved in facilitating the diffusion of oxygen into the tissues and then unloading that oxygen to the mitochondria as the oxygen tension falls. In other words brine shrimp have in their tissues a respiratory pigment similar in its function to the myoglobin found in the tissues of vertebrates (Schmidt-Neilson, 1990). Intracellular hemoglobins have been recorded before for annelids and molluscs but their occurrence in the Crustacea is not so well documented (Vinogradov *et al.*, 1993).

So we conclude that culture under chronic hypoxia not only results in an increase in the aerobic capacity of critical highly aerobic tissues, but the possibility exists that those tissues are provisioned with an intracellular respiratory pigment which facilitates the diffusion of oxygen into the cell and then unloads that oxygen to the mitochondria in response to decreases in intracellular oxygen tension. This possibility deserves the attention of subsequent researchers.

6.2 Hypoxia, reproduction and maternal provisioning with hemoglobin

Culture under chronic hypoxia altered the life history strategy of the brine shrimp *Artemia franciscana*. Fewer offspring were produced by hypoxic cultured females (Section 3.3.4). This could be interpreted in terms of a trade-off between either growth

and reproduction or maintenance (the cost of physiological responses to maintain an animal in its environment) and reproduction (Sibly & Calow, 1986). Under hypoxic conditions, the costs of bringing the respiratory improvement 'forward' in time (this includes costs associated with early accelerated growth (Section 3.3.1) as well as respiratory physiology costs, e.g. production of large concentrations of hemoglobin (Section 3.3.3)) are met by reducing the costs associated with later growth and/or reproduction (Section 3.3.4).

For female brine shrimp the costs may be higher than for males, as the females seem to have a greater hemoglobin concentration (Section 3.3.3). In her case hemoglobin is used to supply oxygen to the tissues (as in the male) but also to provision the young (see below). In fact the trade-offs described above may be very simplistic as, for example, it does appear that the cost of early growth in hypoxia cultured animals could be off-set by a slowing down of growth up to and after sexual maturity, and also 'putting off' reproduction, at least in some cases (Section 3.3.4).

Not only were the number of offspring produced under hypoxic culture conditions reduced compared with normoxic controls, but there was also a switch in the type of offspring produced (Section 3.3.4). Instead of producing a mixture of live young and dormant cysts, hypoxia cultured mothers produced cysts alone. The interesting feature was that the mechanism underlying the switch appears to be the suppression of ovoviviparity, as there was no difference in the number of dormant cysts produced by hypoxia and normoxia cultured females. It is difficult to say this for certain given the small amount of data presented, but those data, as they are, look convincing. Certainly further studies to support or refute this new hypothesis are urgently required.

The dormant cysts produced by hypoxia cultured mothers were produced and kept in very hemoglobin rich environments (the uterus and ovisacs) (Section 3.3.4). This could have two non-mutually exclusive purposes. Firstly, it means that the developing egg, up

until it encysts as an arrested gastrula, develops in an oxygen 'rich' environment. It may be that early developmental processes are strongly aerobic and require this type of provisioning. Secondly the surrounding hemoglobin may actually be transferred into the developing egg/gastrula. This theory is supported by the fact that as the hemoglobin 'disappeared' from cysts,environment with time, the cysts became much more red in colour (Section 3.3.4). However, this observation is also consistent with the view that the mother reabsorbs the hemoglobin after encystment of eggs is accomplished. What makes this unlikely is the fact that if eggs were not fertilized, firstly the cysts did not go red in colour and secondly the hemoglobin concentration in the ovisac kept on increasing, right up until the female died (Section 3.3.4). As well as supporting the hypothesis that mothers are provisioning their young with hemoglobin, this last observation also indicates how important fertilization is in maternal provisioning. There is no maternal provisioning until the egg is fertilized - this suggests that the uptake of hemoglobin by the developing embryo or resultant cyst is either only possible across the cell membrane of a fertilized egg, or is under control of the egg/cyst one the 'developmental machinery' has been 'switched on' by the fertilization event.

If we assume that maternal provisioning of the cysts with hemoglobin occurs, the question arises as to the function of the hemoglobin in the cyst. There are two possibilities.

1. The hemoglobin is retained by the cyst as a prospective respiratory pigment for newly hatched nauplii after hatching. However, this is unlikely for two reasons. Firstly, for this to happen we would have to assume that the mother is provisioning her young for hatching in a future hypoxic environment. But as the reason for the production of cysts is exposure to chronic hypoxia (Section 3.3.4), and as the cysts will not hatch unless favourable conditions 'return' (Sections 2.3.2.1 and 3.3.4), a respiratory purpose for maternal provisioning seems superfluous. Secondly, we know that hemoglobin-provisioned young

when they hatch have no respiratory advantage over non-hemoglobin provisioned young (Section 4.3.2.3).

2. Secondly, maternal provisioning of hemoglobin may be a post-egg production way of 'feeding' the newly fertilized egg. Why such provision is not given at the egg production stage, seems to be a weakness in this theory. However, as provisioning only happens for fertilized eggs, post egg production 'feeding' may be a way of ensuring that maximum maternal investment only goes to eggs with a chance of survival, i.e. fertilized as opposed to unfertilized eggs. It is possible that constituents of hemoglobin could be used for building/reinforcing the cyst wall, which is constructed in part from haem or haematin (Dutrieu, 1960) as well as acting as a source of protein amino acids for the egg itself up until it arrests development. Certainly a non-respiratory role for maternal hemoglobin provisioning would be consistent with an early study by Gilchrist & Green (1959) who found that while dark brown eggs of *Artemia* contained as much as four times as much haem as pale cream coloured eggs, the total haem content was concentrated in the egg shell. the information we have on a similar process in the cladoceran *Daphnia* (Fox, 1948; Green, 1956). However, we are then faced with the question, why does such provisioning not take place in normoxic brine shrimp which also can produce cysts? There are at least possible solutions to this.

1. Provisioning does take place in normoxic gravid females but it is more obvious in hypoxia-cultured gravid females. This could be tested experimentally if it were possible in some way to label (radioactive or heavy elements) either the amino acids or iron of maternal hemoglobin and see if any of the label was transferred to the young.

2. There is a difference in the biology of cysts produced in normoxic and hypoxic culture conditions. This could be tested by examining the chemical constituents of the different cysts (e.g. total protein) or by measuring the thickness of the shell of the cyst - perhaps hypoxic cultured cysts have thicker shells? Similarly hatching success and subsequent

growth and reproduction could be measured. Viability of cysts stored for different periods of time could also be a factor.

In conclusion, culture under chronic hypoxia results in the production of cysts rather than cysts and live young. These cysts are provisioned with hemoglobin by the mother, which is then used for non-respiratory purposes.

6.3 Significance of response to periodic hypoxia and physiological 'telescoping' for life in brine pools.

Artemia franciscana, like many other brine shrimp species inhabit, hypersaline environments that can vary dramatically in their temperature and oxygenation status (Section 2.3.2). While hypoxia itself is liable to be a serious environmental 'problem', due to nocturnal animal respiration or due to the increased respiratory demands made on a water body by crowding, either increasing salinity or temperature will also result in a reduction in oxygen concentration (Section 3.2.2). Consequently hypoxia in the environment of *A. franciscana* will be both periodic and chronic, and most likely the oxygenation status at any one time is a product of both.

A criticism that could be potentially levelled against many studies of physiological responses to hypoxia is that not only do they examine acute (rather than chronic) responses, but as hypoxia in many natural environments, including brine pools and rock pools (Sections 1.1 and 2.3.2.3) is both periodic and chronic in nature, such studies have little environmental relevance. However, the effect of periodic hypoxia on growth and respiration (Sections 3.3.1 and 4.3.1) in *A. franciscana* was shown to be intermediate to that of the normoxic control and the chronic hypoxic experimental treatment. In other words, it could be suggested that it was not the pattern of hypoxic/normoxic exposure that

mattered quite so much as the duration of the hypoxia. If this is true then it means that many of the acclimation effects elicited as a response to either periodic or chronic hypoxia, are only quantitatively, not qualitatively different. It also means that we could meaningfully extrapolate from physiological studies which have examined exposure to chronic hypoxia to what would happen under conditions of periodic hypoxia. The generality of this hypothesis remains to be tested, and as the hypothesis has important implications for our understanding of the effects of environmental hypoxia, it should be tested as a matter of urgency.

In Chapter 4 we saw that for most of the life cycle of *Artemia franciscana*, this species displays a marked physiological capacity to maintain oxygen uptake under conditions of declining oxygen tensions. Furthermore if this species is bred under conditions of chronic oxygen shortage, the period in which those physiological regulations, essential for surviving hypoxia, development is considerably abbreviated compared with normoxic controls. Also the ability to regulate oxygen uptake in the face of declining oxygen tensions is better developed in individuals cultured under chronic hypoxia. If we consider the example of a pool that is drying out, it is likely to be getting smaller, becoming more saline and increasing in ambient temperature. All of these features will result in a progressive chronic hypoxia, for as long as the pool remains. As long as all of the individuals of *A. franciscana* in a particular brine pool that has become hypoxic (or is subject to periodic hypoxic events) are not at the same developmental stage (which is unlikely based on the few ecological studies that have been carried out on brine shrimp, see Section 2.3), and as long as the hypoxia is not too severe, there is no reason to believe that many of the individuals will make it through to reproductive condition. This assumes a time course of drying out of the pool for, based on the studies presented here, of most probably less than 11 days. Whether the pool dries up, or becomes chronically hypoxic, the offspring are produced in the form of arrested cysts. These cysts will then remain

arrested until the oxygenation status of the water changes, or in the case of a dried out pool, the water returns again, and so the cycle will begin again. It is unlikely that cysts will hatch in oxygen poor water, meaning that even if the pool they hatch into in the future becomes hypoxic, because of the rapidity with which the respiratory system of brine shrimp can react to hypoxia, most individuals will have past through the very early developmental stages which are particularly sensitive to hypoxia. To recap, in response to chronic hypoxic exposure, *A. franciscana* quickly develop a marked physiological capability to maintain oxygen uptake which allows individuals to produce cysts that will remain dormant until favourable conditions are resumed.

It has been mentioned that brine shrimp are the subject of aquaculture in the form of cyst harvesting but also culturing in small isolated ponds or salt pans (Section 2.4.2). The results of the physiological studies as recorded in the preceding chapters have serious implications for the culture of these organisms in small pools. Firstly, if the pools become overcrowded, or hypoxic for any other reason (large algal respiratory component at night for example), as long as the hypoxia is not too severe *A. franciscana* should be able to cope physiologically with these conditions and individuals will grow to sexual maturity. Secondly, however, such hypoxia will reduce the total reproductive output of individual females as cysts only will be produced. Certainly the oxygenation status of pools for the culture of brine shrimp should be monitored, for the respiratory adaptations are such that while hypoxia does not seem to compromise aerobic metabolism in these animals, it does profoundly alter the mode of reproduction in a way that will 'stall' the brine shrimp population in a way that may be costly to restart.

6.4 Hypoxia and animal life

Animals that are exposed to hypoxia in their natural environments often have recourse to physiological mechanisms that enable them either to maintain oxygen uptake as external oxygen tensions decline or resort to anaerobic metabolism. The present body of work has confirmed this for the brine shrimp *Artemia franciscana*. However, it has enhanced our broader understanding of hypoxia and animal life in the following ways. It has shown that respiratory performance, either at the level of the whole organism or at the level of the tissues, may improve and/or alter during ontogeny, and the timing and nature of these events can be altered by external factors, in this case hypoxia. Secondly, hypoxic stress may not be as 'insurmountable obstacle' for an individual throughout its life cycle as might be envisaged - indeed it could be argued that culture under hypoxia tightened up and improved respiratory performance - but the real effect on the population would be mediated through the effect of hypoxia on reproduction.

Despite the conclusions reached above it is still true that our understanding of the effects of long term hypoxia (periodic or chronic) on respiratory performance, at every level, and on the biology of aquatic animals generally, is grossly inadequate. What this study has demonstrated, however, is that it is an area which has its own intrinsic interest as well as its own very practical applications.

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