Adaptability of skeletal muscle capillary supply and the potential for exercise-induced angiogenesis in cardiovascular disease

Adaptability of skeletal muscle capillary supply and the potential for exercise-induced angiogenesis in cardiovascular disease

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Submitted in accordance with the requirements for the degree of Doctor of Philosophy

The University of Leeds

Faculty of Biological Sciences

School of Biomedical Sciences

November 2021

The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

This thesis is dedicated to my parents, mother in-law, wife and kids for their big sacrifice

Acknowledgements

My sincere gratitude to both of my advisors Stuart Egginton and Graham Askew for their immense support of my Ph.D study and related research, for their patience, motivation, and knowledge. Their guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my Ph.D study.

With a special mention to the Roger and Peter for their generous help in laboratory works and analysis. To the rest of the Egginton lab: Nicole, Natalie and Jack. It was fantastic to work with all of you. What a cracking team to work with!

To my eternal life coaches, my beloved wife (Wan Nor fatihah Wan Hanafi) and parents (Nazir Ab. Rahman and Norma Ismail) and my mother in-law (Zainab Mohamed) who nourished the inquisitive minds in me. I am grateful to all my close relatives for their constant moral and emotional support throughout writing this thesis and my life in general.

Special appreciation to the Government of Malaysia (Ministry of Higher Education), the Universiti Malaysia Kelantan (UMK), the University of Leeds and The Leche Trust for financially assisting my doctoral study financial needs.

And finally, last but not least, also to all Malaysian community in Leeds. Knowing them for the past 4 years makes Leeds feel like home.

Abstract

Exercise intolerance is prominent in diseases resulting in limb ischaemia. One characteristic is microvascular rarefaction that contributes to reduced skeletal muscle performance due to limited capillary perfusion and tissue oxygenation. Exercise training potentially stimulates capillary growth (angiogenesis), thus increasing muscle aerobic capacity, and in humans and rodents also changes fibre type towards a more oxidative phenotype. These physiological changes facilitate long periods of sustained physical work, but patients are often exercise-intolerant. Voluntary wheel running exercise (VWRE) imposes less stress on animals than other approaches, but little information is available about how this affects muscle performance and capillarity. Therefore, this thesis examines the effect of VWRE on muscle adaptation and how it may improve exercise capacity, using a comprehensive analytical approach. The first study showed increased muscle capillarity after 7-week VWRE, but activity peaked around 4 weeks. Next we found significant angiogenesis after 4 weeks VWRE accompanied by fibre hypertrophy. Optimising histological analysis for capillary markers and fibre type allowed aerobic capacity to be determined, and potential influence on muscle work quantified through oxygen transport modelling. A survey of muscles quantified inter-strain differences. The potential of exercise-induced angiogenesis for improving exercise intolerance in models of local (unilateral femoral artery ligation) and systemic (arterial stenosis) ischaemia were examined. While exercise had only a mild effect on capillarisation, it reversed impaired muscle performance in ligated (LRW) and aortic banded (ABRW) animals. The final study assessed peak aerobic capacity ($\dot{V}O_{2\ peak}$) and running economy (RE) following endurance exercise. Both parameters were improved in VWRE rats due to improved musculoskeletal and cardiovascular systems, and that ABRW group enhanced $\dot{V}O_{2\ peak}$ while LRW group only improved RE. In conclusion, these studies provide additional insight into the role of angiogenesis in muscle performance restoration of ischaemic muscle and voluntary wheel running exercise as a therapeutic intervention.

Abbreviations

ABRW	aortic banding/-running wheel exercise
ANOVA	analysis of variance
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
CD	capillary density
CDA	capillary domain area
C:F	capillary to fibre ratio
CO	cardiac output
CS	citrate synthase
EDL	extensor digitorum longus
HR	heart rate
LCD	local capillary density
LCFR	local capillary to fibre ratio
LRW	ligation/-running wheel exercise
MHC	myosin heavy chain
O_2	oxygen
P	statistical significance value
PBS	phosphate buffered saline
pH	acid/base scale
RE	running economy
RER	respiratory exchange ratio
RW	running wheel exercise
SD	standard deviation
Sol	soleus
SV	stroke volume
TA	tibialis anterior
$\dot{V}CO_{2\ peak}$	peak carbon dioxide production rate

$\dot{V}O_{2\ peak}$	peak oxygen consumption rate
VWRE	voluntary wheel running exercise

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Chapter 1 : General Introduction

Exercise intolerance is prominent in cardiovascular disease (CVD) patients that show a significant reduction in exercise capacity. Lower activity levels in thid group would chronically worsen the current disease state and might facilitate other complications e.g. obesity, which reduces their quality of life (QoL) and may contribute to increased morbidity and mortality (Tickle, Hendrickse, Degens, & Egginton, 2020). Exercise training may naturally restore attenuated function of lower limb muscles, as seen in peripheral arterial disease (PAD), and has proven to be able to ameliorate and enhance exercise performance both in human and animal studies (Brown et al., 2003; Hoier, 2013). However, there is limited number of exercise modalities for those with limited physiological function particularly in PAD.

Exercise-induced angiogenesis is a potential alternative approach that causes minimal side effects, compared to many pharmaceutical interventions. Exercise training can stimulate capillary bed expansion (angiogenesis), thus increasing aerobic capacity in a range of skeletal muscles. Increased muscle activity for example by performing endurance exercise such as running, swimming and cycling may result in several physiological adaptations involving the cardiovascular, respiratory and of course musculoskeletal systems. All these adaptations are crucial so that oxygen supply can be efficiently matched with oxygen demand. Endurance exercise has been shown both in human and rodent studies to cause significant fibre morphological changes, increased capillarity, fibre type composition changes towards oxidative fibres and increased oxidative enzymes activity. These physiological changes enable the body to function for long periods of sustained physical work. A common exercise method for exercise intolerant patients is supervised treadmill exercise which has shown significant effects on exercise performance (Hiatt et al., 1994; Larsen & Lassen, 1966; Lundgren et al., 1989; Mannarino et al., 1991) and the effects were even similar to surgical intervention (Creasy et al., 1990; Lundgren et al., 1989).

In rodent studies, there are many types of exercise modalities used, including running wheel or treadmill activity and involving sprint or strength exercise, that have been applied to explore their effects on various parameters. However, relatively few protocols have been studied with respect to their effects on angiogenesis. Most common exercise method is treadmill running, involving recruitment of skeletal muscles and fibre types that

are best adapted to defined exercise intensity (various treadmill speed, duration and inclination) at which the animals were pushed to perform the exercise. This indicates a study design limitation that ignores the effect of stress on biochemical changes in tissue samples when using motorised wheels to force rats to run due to enhanced adrenergic stimulation (Alessio, 2005).

There are many studies performed to look at the effect of exercise intervention in various ischaemic models. It is proposed that measurement of exercise effects on ischaemic muscles would be accurately compared as inter-laboratory variability could be minimised by application of a voluntary form of exercise, which would reduce as far as possible the effect of stress on animals, thus preventing bias which might be lead to overestimation of the normal physiological response to exercise (Leasure & Jones, 2008). This approach would therefore increase data accuracy and quality.

Rat skeletal muscles can increase oxidative capacity as shown by increased oxidative enzyme activity and significant transformation of fast to slow fibre types. For example, the activity rate of citrate synthase and carnitine palmitoyltransferase were increased twofold in the mixed region of quadriceps - after running for 12 weeks on treadmill for 5 days/ week, 2 hours continuously daily at up to 31m/s, from 20.6 and 0.26 μmoles/min/g to 37.5 and 0.44 μmoles/min/g, respectively. In addition, the percentage of Type 1 fibres was increased in trained plantaris muscles stained for DPNH (NADH) diaphorase activity (Baldwin et al., 1972).

Treadmill exercise in animal models often used aversive stimuli such as electrical shock, compressed air and tail tapping to motivate animals to keep running. Stressful stimuli may change rat behaviour and affect physiological adaptation e.g. adrenal hypertrophy (Khataei et al., 2021; Moraska et al., 2000), thymic involution, lowered serum corticosteroid binding globulin, increased lymphocyte nitrite concentration, suppression of lymphocyte proliferation and antigen-specific IgM related to stressful exercise (Moraska et al., 2000). Chronic stress activates the hypothalamic-pituitary adrenal axis, which stimulate pituitary glands to release adrenal corticotropic hormone (ACTH), in turn producing corticosterone that eventually led to adrenal cortisol-induced cell proliferation and hypertrophy (Selye, 1956).

It has been reported that the use of electrical stimulation as a stressor enhanced motor output variability during isometric contraction, impairing locomotor performance in humans (Noteboom et al., 2001a, 2001b; Raglin, 1992). In addition, mice treadmill running that applied electrical shock produced greater intra-animal variability in exercise

performance (Knab, 2009), attributed to increased anxiety-like behaviour (Khataei et al., 2021). It was also noted that additional stress from electrical shock use may develop hypoalgesia (immediately measured post exercise) in rats so that they were relatively more pain resistant than ones without the stimulus (Ferdousi & Finn, 2018; Khataei et al., 2021).

The cardiovascular system (CVS) is important for rapid transport of oxygen, essential substrates (glucose, amino acids, fatty acids), vitamins, hormones and water to tissues during exercise. It is also essential in removing metabolic waste products (e.g. carbon dioxide, urea, creatinine and heat) from working muscle. Acute exercise increases demand on the CVS that is accommodated by increased heart rate and stroke volume, leading to greater cardiac output and increased muscle blood flow provided peripheral resistance is not increased. The barostatic reflex prevents significant hypertension as a result of integrated responses.

Cardiac output (CO) is the product of stroke volume (SV) and heart rate (HR), and clearly is responsive to changes in either one or both. At rest, 20-30% of CO is delivered to skeletal muscle, which increases up to 95% during maximal exercise, and high oxygen extraction leading to low oxygen content in venous return (Laughlin, 1999). Upon exercise the capacity of skeletal muscle to use O_2 exceeds the heart's capacity for blood delivery, so only a portion of muscle can be well perfused at any one time, while maintaining adequate systemic blood pressure (Lamb, 1978).

When CO is increased during activity, blood vessels in nonworking tissue such as kidneys, liver and skin must be constricted in order to ensure sufficient supply to the working muscle; in addition blood from nonworking muscles is diverted to active ones (the 'steal' effect) (Vrbova et al., 2008). During heavy exercise, blood flow may increase up to 15 fold although muscle contraction causes blood flow to fall and rise when it relaxes (Lamb, 1978). This variation in perfusion during the duty cycle will have a major influence on vascular shear stress (the frictional drag of viscous blood on the endothelial lining of blood vessels) and wall tension (determined by perfusion pressure and vessel compliance), which are known angiogenic stimuli (Egginton, 2009; Hudlicka, 1988).

Voluntary wheel running exercise (VWRE) has been showed to reduce pain and stress in rodent studies, indicated by lower plasma cortisone levels (Greenwood et al., 2012; Griesbach et al., 2012; Ke et al., 2011; Pitcher et al., 2017; Pitcher, 2018) and greater heart rate variability (HRV) (Pitcher et al., 2017; Sabharwal et al., 2016) - a decrease in HRV is linked to lower capacity for stress adaptation (Pitcher, 2018). Moreover, increased HRV is associated with decreased in resting heart rate (Sabharwal et al., 2016) likely due to

increased parasympathetic tone (Adlam et al., 2011; Laterza et al., 2007; Martinez et al., 2011; Patel & Zheng, 2012), suggesting VWRE may prevent development of autonomic dysfunction. Interestingly, the greater exercise volume and intensity of VWRE were not associated with a greater degree of pain or stress (Pitcher et al., 2017).

Supervised treadmill exercise offers minimal stress on human subjects, and has been used as one of the interventions in treating patients with peripheral arterial disease (PAD). This exercise modality is similar to VWRE as both modalities provide tolerable exercise intensity to the patients/ animals because they run at their own pace. Studies have shown that PAD patients have increased their functional capacity when performing walking exercise. Therefore, considering advantages of lower stress of VWRE, this thesis used rats to elucidate the mechanism behind how muscles adapt to such kind of exercise of defined period - whether it can elicit significant physiological adaptation both in normal animals and rats with local or systemic-induced ischaemia.

This work involved observations on muscle structure change that was done through immunohistochemistry staining in order to determine the fate of fibre types (number and size) and gross muscle capillarity, as well as local capillary supply to individual fibres. This study further explored muscle oxygen supply and use, muscle oxygen tension and extent of hypoxia was quantified at simulated resting and maximal exercise levels by performing oxygen transport modelling (OTM) *in silico*, and muscle function measured by estimating $\dot{V}O_2$ max (systemic aerobic capacity assessment) and/ or fatigue resistance tests *in vivo*. In addition, the strain on muscle aerobic capacity was studied as an alternative way to examine differences in aerobic capacity involving lower limb skeletal muscles (tibialis anterior, extensor digitorum longus and soleus) and accessory organs (heart and diaphragm). This work answered question such as how does aerobic capacity differ among strain of rats (Wistar and Brown). Results from this study may help guide investigations into potential aerobic capacity differences with ethnicity in humans.

It is suggested that a more tolerable type of exercise with fewer side effects is needed by exercise-intolerant people. Therefore, an animal model was used to explore effects of VWRE exercise on muscle angiogenesis and fibre type shift, as a parallel to required human studies that are currently limited. If the potential of voluntary wheel running exercise in promoting angiogenesis could be elucidated, this work would provide additional information on the benefits of the intervention in treating PAD, which compromises quality of life of elderly patients and is considered as a large burden in both developed and developing countries around the globe.

1.1 Peripheral arterial disease

i) Definition

Peripheral arterial disease (PAD) is a disease of reduced arterial blood flow of lower extremities due to decreased arterial diameter resulting from atherosclerotic plaque formation (Cimminiello, 2002; Regensteiner & Hiatt, 2002; Tran & Anand, 2004). This may reduce blood flow, including nutrient and oxygen supply to and waste products elimination from tissue through circulatory and respiratory systems (Muir, 2009). The main cause of PAD is atherosclerosis which progress slowly. PAD patients may experience painful sensation while walking which known as intermittent claudication that occurred when luminal obstruction is more than 50%, and may increase rest pain as it progresses (Regensteiner et al., 1996).

ii) Epidemiology

Cardiovascular disease (CVD) is the main cause of death globally and PAD is the third leading cause of atherosclerotic cardiovascular morbidity after coronary heart disease and stroke. PAD is considered as a global problem in present world where it affects both high income country (HIC) and middle and low income country (MLIC) people(Fowkes, 2013). It is estimated 17.7 million people died in 2015 due to CVD, representing 31% of total deaths (WHO, 2018). In 2010, the prevalence of PAD was 161.6 million globally, which showed quarterly increments compared to the year 2000 when the Southeast Asia region recorded the highest number (54.8 million) and eastern Mediterranean the least (10.3 million) (Fowkes et al., 2013).

iii) Pathophysiology

Atherosclerosis is the main cause of PAD, initiated by systemic inflammation (Beckman, Creager, & Libby, 2002). The process starts when endothelial cell function deteriorates, which can be due to hyperglycaemia, dyslipidaemia and insulin resistance (Beckman et al., 2002). Normal endothelial cells are able to inhibit atherogenesis and regulate blood vessel function and structure (Beckman et al., 2002). In abnormally functioning endothelial cells, there is low bioavailability of endothelium-derived nitric oxide, which reduces its capacity to control vascular relaxation as seen in hyperglycaemia which allows platelet activation and leads to inflammation by increasing leukocyte adhesion to endothelium and migration into the vessel wall, and thus stimulate vascular smooth muscle cell proliferation and migration (Giugliano, 1997).

PAD is partly mediated by oxidative stress where formation of reactive oxygen species and/ or superoxide anion radicals may oxidise LDL cholesterol (Muir, 2009). As a result, oxidised-LDL may penetrate the endothelium as it is easily recognised by most cells, and that eventually leads to plaque formation in the vessel. Second stage of atherosclerosis is the formation of a fatty streak which consists of smooth muscle cells, monocytes, macrophages, T and B cells. Formation of T and B cell complex initiates a release of lymphokines that attract other cells to the area of interest which leads to inflammation. Both monocytes and leukocytes generate growth factors and cytokines as they bind to endothelial cells, releasing chemoattractant chemokines that increase macrophages migration to intimal lining of blood vessel. Smooth muscle cells then proliferate to develop a larger plaque, known as atheromas. Atheroma then develops into the advanced lesion which is highly cellular and contains endothelial and smooth muscle cells, inflammatory cells and a lipid core covered by a fibrous cap. The exposure of procoagulants, tissues factors and von Willebrand factor to the blood environment due to plaque wall rupture stimulates a thrombus formation. Chronically, diameter of arterial lumen becomes narrow as the thrombus keep on growing, which may affect blood supply to deprived cells (Muir, 2009).

iv) Reactivity

Endothelium plays an important function in modulating smooth muscle tone so that increased oxygen demand can be met. Increased shear stress and/ or agonist binding cognate receptors initiate endothelial nitric oxide synthase (eNOS) activation, which catalyses production of nitric oxide (NO) from L-arginine. NO then triggers cytosolic guanylate cyclase in smooth muscle cells that increases cyclic guanosine monophosphate (cGMP) to cause vascular relaxation (Higashi & Yoshizumi, 2004).

NO bioavailability might partly be affected during hypertension which causes oxidative stress to lead to atherosclerosis pathogenesis (Ross, 1999). Even though specific mechanism remains unclear, it is believed that increased amount of endogenous eNOS inhibitors, asymmetrical dimethylarginine (ADMA), vasoconstrictors, and reactive oxygen species (ROS) may reduce NO production (Higashi & Yoshizumi, 2004).

v) Vessel growth

In lower limb ischaemia rat models, it has been shown that PAD did not increase capillary number in gastrocnemius muscles, despite the pro-angiogenic (hypoxic) environment generated. However treadmill exercise twice a day for 18 days was able to trigger angiogenesis in the muscle, producing an increment in capillary to fibre (C:F) ratio

(Lloyd, Yang, & Terjung, 2001). It is proposed that exercise causes increased shear stress which initiates NO-dependant angiogenic pathways that together with vascular endothelial growth factor (VEGF) lead to capillary growth (Lloyd et al., 2001).

1.2 Skeletal muscle changes in PAD

Exercise intervention has been often used to assess functional limitation in skeletal muscle. Lower limb skeletal muscle activity in PAD patients is partly limited by reduced blood supply to the working muscle. Shortage of metabolic fuel and oxygen may deteriorate normal physiological response to the mismatch of supply and demand during muscle contraction. A study measured walking limitation and quantified resting and exercising blood flow using invasive arterial and venous blood measurements; this showed a poor correlation between leg blood flow and functional ability in PAD patients (Pernow & Zetterquist, 1968). Interestingly, an bypass intervention study with increased blood flow in PAD patients showed a modest increment in skeletal muscle walking distance and peak oxygen consumption (Regensteiner et al., 1993b) indicating that blood flow *per se* is not the sole factor in determining functional limitations.

PAD patient gastrocnemius muscle mass was decreased with lower muscle fibre type I areal density (49%±4% *vs*. 64%±5%, $P<0.05$), higher Type IIa (29%±3% *vs*. 16%±3%, $P<0.05$) but similar in Type IIx (22%±4% *vs*. 19%±4% compared to control subjects (Askew, 2005). Capillarity was also reduced, as capillary to fibre ratio and capillary contacts per fibre (Askew et al., 2005), and lowered muscle force production, dictated by decreased muscle cross sectional area (Regensteiner et al., 1993a) in PAD subjects.

1.3 PAD management and treatment

One third of PAD patients experience intermittent claudication, characterised by pain sensation on calf in one or/ and both legs (Rose, 1968). The usual approach in PAD treatment is to relieve exertional symptoms, enhance walking capacity, and improve quality of life. Normal adults are at risk of having PAD as early as 65 years old, the risk is greater with one of the atherosclerotic risk factors, for instance diabetes mellitus, smoking, hyperlipidaemia, hypertension and/ or elevated plasma homocysteine (Gerhard-Herman, 2017; Graham, 1997; Hiatt, Hoag, & Hamman, 1995; Newman, 1993) or family history of PAD which usually involves adults aged 50-64 years old (Wassel, 2011). The probability is even higher in adults with diabetes mellitus and at least one other atherosclerotic risk factor. Susceptible aged people with risk factors are usually assessed for PAD through detailed evaluation of their condition (Figure 1.1) (Hiatt, 2001). PAD is strongly associated with cardiovascular disease (CVD) morbidity and mortality and the main causes of PAD patients' death are myocardial infarction and stroke (Hirsch, Treat-Jacobson, Lando, & Hatsukami, 1997; Ness & Aronow, 1999). PAD is a manifestation of atherosclerosis and intermittent claudication is a common symptom of PAD (Regensteiner & Hiatt, 2002). Its prevalence increases with age: the Edinburgh Artery Study reported PAD incidence increased from 2.2% (50 to 59 years old) to 7.7% (70 to 74 years old) (Fowkes et al., 1991).

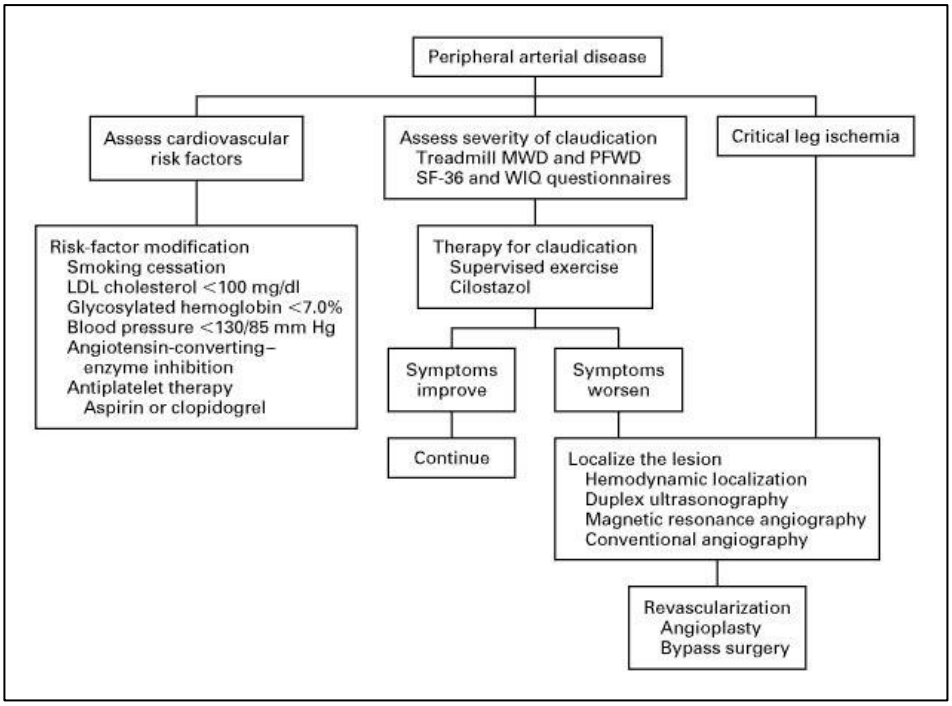

Figure 1.1: Evaluation strategy for patients with suspected Peripheral Arterial Disease (Hiatt, 2001).

There is a need to monitor PAD risk factors listed above, as well as decreased estrogen levels, as any deviation of the factor(s) may progress the condition (Figure 1.2). Intermittent claudication was 30-40% among smokers and that habit increased 16-fold the probability of developing the symptom at 45 years old and above. More than 60% of smoking PAD patients showed a 5-year mortality mostly due to myocardial infarction (Hirsch et al., 1997). Continue smoking accounted for 80-90% of patients requiring angioplasty surgery (Kannel & Shurfleff, 1973), potentially leading to amputation (Kannel & Shurfleff, 1973). Studies proved that smoking cessation reduced PAD patient's mortality rate and increased amputation-free survival (Álvarez, 2013; Armstrong et al., 2014). Due to microvascular complication in diabetic patients with PAD, intensive treatment needs to be applied as they are at high risk of CVD morbidity and mortality(Regensteiner & Hiatt, 2002), leading to a 3- to 5- fold increase in intermittent claudication in women and men, respectively (Kannel & McGee, 1985) (Kannel & McGee, 1979).

Management Issue	*Target*
Smoking	Complete cessation
Diabetes	HbA_{1c} <7.0%
Dyslipidemia	LDL-C <100 mg/dL
Hypertension	Blood pressure <130/80 mm Hg
Antiplatelet therapy	Clopidogrel (or aspirin)
ACE inhibition	Ramipril

ACE = angiotensin-converting enzyme; HbA_{1c} = hemoglobin A_{1c}; LDL-C = low-density lipoprotein cholesterol.

Figure 1.2: PAD treatment through monitoring key risk factors (Hiatt, 2001)

1.4 Drugs and their side effects

Pharmacological intervention using drugs with defined mechanisms showed promising effects in treating the PAD symptom of intermittent claudication. Some of the drugs exhibited vasodilator and anti-platelet effects, which improve mobility and quality of life. Cilostazol is the current drug approved by FDA to be used for treating claudication among PAD patients, principally by inhibiting phosphodiesterase 3 activation that increases cAMP. Activated protein kinase A then deactivates myosin light chain kinase resulting in smooth muscle cell relaxation (Gallagher, Herring, & Stull, 1997). It has been showed that cilostazol inhibits platelet aggregation reducing atherosclerotic formation and prevents vascular smooth muscle proliferation thus improving vasodilatation (Igawa, 1990; Kohda, 1999).

Four randomised, placebo-controlled trials of cilostazol showed the drug (50 or 100 mg twice daily) improved pain free and maximal treadmill walking distance (Beebe, 1999; Dawson et al., 1998, 2000; Money, 1998). However, the ankle-brachial blood pressure index (ABI) was not improved, as only one study demonstrated a small increase out of four clinical trials, showing the weakness of this approach. In addition, this drug leads to side effects such as headache, transient diarrhoea, palpitations, and dizziness (Hiatt, 2001).

1.5 Exercise as an alternative to surgical intervention

As PAD patients have exercise intolerance, running is another approach to treat the disease since voluntary running (within their tolerable intensity) imposes less stress to PAD patients to initiate appropriate local responses. From a human perspective, supervised treadmill exercise parallels animal wheel running exercise, and has potential for restoring muscle function. In a clinical setting, exercise is supervised by an assistant or sometimes performed by patients alone at private premises. Many studies have used a range of exercise mode and periods, and treadmill exercise is the most common intervention in studying effect of exercise on ischaemic leg of PAD patients, with different degree of treadmill slopes and speeds. All apparently lead to significant beneficial effects, and eventually improves quality of life. Treadmill training at 3hrs/week for 12 weeks improved functional status, assessed through questionnaires, in patients with intermittent claudication (IC) (Regensteiner, Steiner, & Hiatt, 1996). Physical activity levels (*via* physical activity recall questionnaire, PAR scores) increased by 48 metabolic equivalent hr/wk, the functioning, well-being, and overall health (medical outcome study SF-20, MOS) score increased by 24%, and the number of bouts of walking activity measured by the Vitalog by 4.5 bouts/hr. Effects increased when the exercise period was extended for 24 weeks with greater walk distances (Regensteiner et al., 1996). Treadmill exercise was able to increase pain-free walking in patients in whom duration of intermittent claudication was negatively correlated with increased walking performance, and improved total walking distance (Hiatt et al., 1994; Larsen & Lassen, 1966; Lundgren et al., 1989; Mannarino et al., 1991). Studies also showed that defined exercise training among PAD patients could produce similar effect as surgical reconstruction and angioplasty (Creasy et al., 1990; Lundgren et al., 1989) and extended maximal walking distance compared to surgery alone (Lundgren et al., 1989).

Natural running behaviour of nocturnal animals such as rodents would therefore likely benefit from running wheel exercise, particularly in disease models e.g. PAD. This produces less bias on studied parameters than treadmill exercise due to less intervention, and reduced factors such as increased adrenaline levels during chronic stress on blood vessels that may lead to stroke and/or heart attacks (McEwen, 1998), thus potentially masking the beneficial effects of exercise. Voluntary wheel running is an exercise model that uses free access to a running wheel to chronically exercise rodents. This exercise modality has been applied successfully in rats and mice studies where the animals exhibit a natural running behaviour when access is given (Allen, 2001; Goodrick, 1980; Kariya et al., 2004; Waters et al., 2004). Animals run in the wheels without any external stimuli, e.g. use

of electric shock or compressed air, thus minimising unwanted animal behaviours (Kregel, 2006).

Forced exercise influences animals' brain and behaviours as treadmill exercise showed emotional defecation and anxiety-like behaviours (Leasure & Jones, 2008), the forced exercising rats also demonstrated reduced average running speed reflecting the animals not exhibiting their natural exercise performance which may reduce its effect (Leasure & Jones, 2008; Rodnick et al., 1989). Forced and voluntary exercises yielded different rodent body mass, which is an important element in determining trained muscle capillarity and hence endurance (Narath et al., 2001).

Maximal oxygen consumption is the gold standard for determining aerobic capacity, in which animals need to perform a ramp exercise test on a treadmill. It was reported that 10% of sample population showed reluctance in running when placed on the treadmill (Bedford et al., 1979; Dudley et al., 1982). One of the advantages of wheel exercise is that it improves rats' running capacity on the treadmill (compared to ones performing treadmill running without wheel running exercise) where the animals are unlikely to run at speed of 32cm/s (the highest speed achieved without a running stimulus) (Leasure & Jones, 2008). It increases rats' running capacity when assessed by maximal oxygen consumption or peak oxygen consumption measurements. However, in measuring oxygen consumption in running wheel rats, weekly treadmill familiarisation is required (Copp et al., 2009) to maintain animal proficiency with treadmill running, thus reducing foot injury (Kregel et al., 2006). In addition, studies have showed that wheel exercise is able to increase maximal aerobic capacity in both normal and diseased rats (Allen et al., 2001; Halseth et al., 1995; Kinnick et al., 2000; Lambert & Noakes, 1989; Overton et al., 1986; Yano et al., 1997), showing its potential in restoring or improving aerobic capacity in an ischaemic rat model. Wheel exercise was also associated with increased longevity in both male and female Wistar rats, with lower body weight compared to a control group (Goodrick, 1980).

Carbohydrate is commonly used as an effective ergogenic aid to support a prolong exercise activities (Jeukendrup, 2004), ingested as carbohydrate solution, shown to be effective as an energy supply compared to non-caloric liquids (Jeukendrup, 2010; Valeriani, 1991; Vandenbogaerde & Hopkins, 2011). Sport drink supplementation during endurance exercise is partly used to extend endogenous carbohydrate stores so that fuel supply meet the metabolic demand for continuous muscle contraction (Coyle et al., 1986). Many studies have shown that carbohydrate intake has increased endurance performance (Currell &

Jeukendrup, 2008; Gisolfi & Duchman, 1992; Jeukendrup, 2014; O'Brien & Rowlands, 2011; O'Brien et al., 2013; Rowlands et al., 2012; Valeriani, 1991; Vandenbogaerde & Hopkins, 2011) and contributed to increased muscle energy (Adopo et al., 1994; Jentjens et al., 2004a), which growing evidence showed a combination of fructose and glucose (composite carbohydrate) intake resulted in higher carbohydrate oxidation, improved exercise performance and reduced stomach discomfort compared to glucose or galactose use alone (Murray et al., 1989; Murray, 1987). There was no consensus on the most effective ratio of mixture, concentration and dose for endurance improvement. However, several studies using a range of different composite ratio 0.5:1, 0.6:1 and 1:1 of fructose:glucose (15% concentrated) have shown relatively higher endurance performance than isocaloric glucose group (Currell & Jeukendrup, 2008; Roberts et al., 2014; Tarpey et al., 2013; Triplett et al., 2010). Similar endurance enhancement was observed in fructose:maltodextrin composite compared to a single isocaloric fructose/ maltodextrin at 0.5:1 and 0.8:1 ratio (Baur, 2014; Rowlands et al., 2012; Wilson & Ingraham, 2015). This indicated that composite carbohydrate drinks provides a more powerful effect on exercise capacity in endurance sport.

Composite carbohydrate use may benefit from an increase in the rate of gastric emptying and unilateral fluid absorption compared to isocaloric carbohydrate e.g. fructose:glucose *vs.* glucose (Currell, 2008; Jeukendrup & Moseley, 2010; Neufer, 1986) and composite carbohydrate 1:1 fructose:glucose recorded highest rate of fructose absorption with lowest gastrointestinal discomfort compared with lower ratio (Rumessen & Gudmand-Høyer, 1986; Shi, 1995). It is also associated with a higher exogenous carbohydrate oxidation rates (Jentjens et al., 2004b; Jentjens, 2006) and improved gut discomfort (Currell & Jeukendrup, 2008; Jentjens et al., 2004c; Triplett et al., 2010). Gut discomfort was reported in concentrated isocaloric glucose that was due to gut distention because of lower rate of gastric emptying and water secretion into the gastrointestinal lumen (Rehrer, 1992).

Therefore, fructose intake may be used to enhance exercise capacity in rodents, where the combination of glucose from food and fluid intake would result in excess metabolic fuel intake. Dual substrates absorption provides additional source of energy, as fructose is specifically absorbed through GLUT5 into gastrointestinal enterocytes but is also, together with glucose and galactose, absorbed through GLUT2 (Leturque et al., 2005; Wright et al., 2003). In muscle, fructose and glucose are absorbed specifically through GLUT5 and GLUT4 (Hundal et al., 1998), respectively that resulted in muscle and liver glycogen sparing effect for performance benefit (Ahlborg & Bjorkman, 1990). So, fructose application in drinking water would be used in combination with voluntary wheel running

exercise, particularly in a pilot study to determine effect of increased exercise volume on angiogenic response.

Stress is one of risk factors of chronic disease, metabolic disorder (Hammar et al., 1994; Theorell, 1998) and burn out (Maslach & Jackson, 1981; Pruessner et al., 1999; Schulz et al., 1998) in work place. Burn out due to chronic stress is linked to fatigability that leads to attenuated cognitive function and lowered empathy to others (Cherniss, 1980; Freudenberger, 1983; Maslach & Jackson, 1981). Long term cortisol availability in circulatory system during stress caused harmful effect to body, indicating stress may upregulate the hypothalamo-pituitary-adrenal (HPA) axis (McEwen, 1998). Cortisol may suppress testosterone that attenuates normal growth of skeletal muscles that reduced testosterone level lead to muscle protein synthesis deterioration thus lowering skeletal muscle mass (Griggs, 1989). It is an important modulator of muscle mass that promotes muscle growth and hypertrophy (Vingren, 2010). It has been shown in a rodent study that testosterone is critical for normal physiological growth as elevated testosterone level in mice improved muscle weight, muscle ultrastructure, muscle fiber cross-sectional area in gastrocnemius (Sinha et al., 2014). In addition, stress would also reduce immune system levels, thus expose people or animals more to illness (Chrousos, 1997; Martin, 2009; Shavit & Martin, 1987). This suggested that stress effects should be minimized so that it would not affect normal muscle adaptation during endurance exercise training.

In addition to increased catecholamine secretion stress also can lead to greater fatigue, which overtime work showed a concurrent increase in work overload showing fatigue-reduced work productivity (Rissler & Elgerot, 1978). Stress is also linked to sleep problems (Åkerstedt et al., 2002a; Marquie et al., 1999; Urponen et al., 1988) as prolong stress would result in primary insomnia (Åkerstedt et al., 2002a; Dahlgren et al., 2005; Kecklund & Åkerstedt, 2004; Marquie et al., 1999), characterized with problems falling asleep and a shortened sleep length (Steptoe et al., 1999). It has been reported that lack of sleep can cause ill health and mortality (Kripke et al., 2002) and it was reported that cortisol levels and insulin intolerance are increased in insomnia (Spiegel et al., 1999), showing metabolic disturbance due to sleep deprivation. Studies showed a positive significant relationship between burn out and sleepiness, particularly the morning of an off-day, indicating the presence of fatigue after a stressful work period (Åkerstedt et al., 2002b; Dahlgren et al., 2005; Horne & Minard, 1985; Söderström et al., 2004) that could provide a bodily limit towards recovery and maintain homeostasis (Snyder, 1987).

Chronic stress should therefore be a matter of concern in introducing exercise intervention in animal studies. Much evidence showed that stress impairs the quality of recovery from exercise, which determines exercise performance on the subsequent day after an exercise period. This definitely may reduce actual exercise performance that indirectly impairs for instance muscle physiological adaptation. Therefore, voluntary exercise may be an alternative, beneficial approach to minimise the stress effect on body adaptation, particularly in the muscle remodelling response so that the real effects of defined exercise could be determined accurately.

1.6 Wheel exercise

In addition to treadmill and swimming, voluntary wheel running exercise is one of the aerobic exercise models used for studying the effect of muscle activity on various pathological conditions (Copp et al., 2009; Emter & Baines, 2010; Ke et al., 2011; Radovits et al., 2013). This exercise model uses vertical running wheels fabricated with metal or plastic, or ones with an angled rotating running track with a range of wheel circumference depending on the species used (Allen et al., 2001; De Bono et al., 2006; Ke et al., 2011; Lerman, 2002; Natali et al., 2001). It has been used to determine chronic exercise capacity in rodent species of various strains. A major advantage of using this exercise modality is volition, in which animals can perform exercise with minimal intervention by investigators and so induce minimal stress to the animals since they run freely without external aversive stimulation. Natural running behaviour is vital to assess normal physiological adaptations that a stress response could minimise, possibly through food and water consumption alteration, and even lead to a disease state (McEwen, 2008). However, neither exercise intensity nor duration can be controlled by the researchers, and so it is inappropriate for studying fatigue or maximal oxygen consumption. It also may injure the animals, particularly catching toenails and causing abrasions on hind paws (Seo, 2014b), although we have not observed such adverse effects.

Wheel exercise is rarely used in animal studies to examine its effect on muscle physiology, as the magnitude of exercise cannot be controlled by investigators, particularly in pathological conditions where a graded protocol may be desirable. Therefore, treadmill exercise was considered a better model as both intensity and duration of exercise can be accurately monitored (Clarkson & Hubal, 2002; Dudley et al., 1982; Wang et al., 2010). Genetic background plays an important role in influencing rats' circadian rhythms, thus determining their running behaviour, which is partly influenced by environmental factors such as light and temperature (Aschoff, 1979), internal factors such as hormone levels (Turek & Gwinner, 1982), and drug intake (Joy, Losee-Olson, & Turek, 1989; Turek & Van Reeth, 1988). Different strains display different food and water intake (Possidente & Hegmann, 1980), body temperature (Connolly & Lynch, 1981, 1983) and running activity pattern (Ebihara et al., 1978; Wollnik, 1991) that will affect the physiological adaptation to exercise training, e.g. muscle metabolism, ventilation and fat loss were different among rat strains (Gordon, Phillips, & Johnstone, 2016). Muscle changes in structure and function in response to chronic exercise is influenced by exercise intensity and volumes (duration) of exercise (Dudley et al., 1982), which also varies across strains (Figure 1.8).

1.7 Muscle fibre type composition

Rodent skeletal muscles are composed of three types of muscle fibres with specific functional and metabolic properties, usually containing a mixture of slow and fast fibres: Type I to maintain posture, and Type IIa and IIb for phasic activity (Simoneau & Bouchard, 1995). A variety of genetic factors and physical activity status can contribute to the observed biological variability in fibre type composition of individual muscles, both among animals of the same strain or between different strains. Such variability is likely to be higher in outbred than in inbred strains (Soukup et al., 2002). Many studies have quantified muscle fibre composition in mammals such as rat, mice, rabbit, guinea pig and cat; also in a range of muscles across rat strains (Ariano et al., 1973; Armstrong & Phelps, 1984; Soukup et al., 2002; Staron, 1999). For example, the content of slow oxidative fibres in soleus muscle of female Lewis rats (96.1±2.9%) was significantly greater than Wistar (93.5±3.7%) and Sprague-Dawley (83.7±2.3) rats, respectively.

However, the proportion of slow muscle fibres of EDL muscle was similar across strains (5.5±1.0%), suggesting muscle activity patterns influence such differences (Staron et al., 1999). The body mass of animals should be considered in sampling so that allometric bias is avoided, e.g. where studies have only compared composition among strains based on comparable developmental age of 4 to 6 months. Figure 1.3 shows the presence of three different fibre types of a cross section of rat extensor digitorum longus (EDL) muscle (Vrbova et al., 2008) based on myosin ATPase, the enzyme responsible for energy transduction at cross bridges, where the intensity of stain is inversely proportional to enzyme activity following acid pre-incubation (Type I and Type IIB have the highest and lowest activity, respectively) (Vrbova et al., 2008) and Table 1.1 showed the percentage of SO (=Type I), FOG (=Type IIa) and FG (=Type IIb) fibres from a range of hindlimb skeletal muscles of Sprague-Dawley rat. Note that regional variability in composition is recognised in some muscles, but in others such as EDL and TA are ignored.

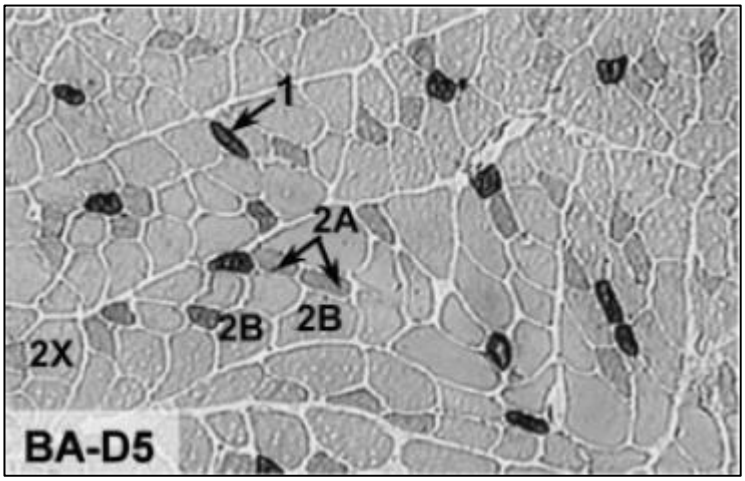

Figure 1.3: Cross section of extensor digitorum longus of Lewis rat shows the presence of different fibre types: Type I of slow motor unit (darkly stained), Type IIA and Type IIB of fast motor units (light dark stained and white fibre respectively on the basis of mATPase activity (Vrbova et al., 2008).

Table 1.1: The composition of muscle fibres in the major hindlimb muscle of Sprague-Dawley rat (Armstrong & Phelps, 1984).

Muscles	Slow oxidative (SO) %	Fast oxidative glycolytic (FOG) %	Fast glycolytic (FG) %
Extensor digitorum longus (EDL)	2	42	56
Soleus	89	11	0
Plantaris	7	40	53
Gastrocnemius, red	35	56	9
Gastrocnemius, white	0	16	84
Gastrocnemius, middle	5	17	78
Vastus lateralis, red	6	43	51
Vastus lateralis, middle	1	20	79
Vastus lateralis, white	0	1	99
Tibialis anterior	3	42	55

1.8 Extensor digitorum longus (EDL)

1.8.1 **Anatomy and physiology**

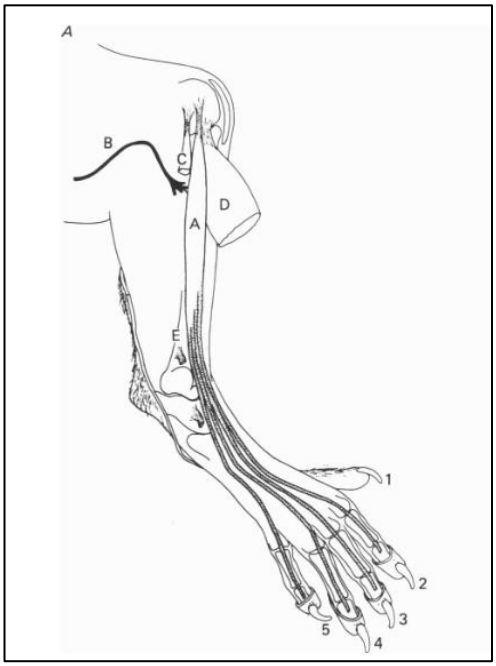

Figure 1.4: Anatomical position of rat EDL (Balice-Gordon & Thompson, 1988); numbers and letters are explained in the text.

The EDL muscle originates from one tendon attached to the lateral epicondyle of the distal femur and is located in the lateral portion of the hindlimb, Figure 1.4A. It is lateral to the tibia (E), anterior to the peroneus longus muscle (C) and is posterior to the tibialis anterior muscle (D). Towards the ankle, the four tendons of insertion pass under the annular and calcaneal ligaments, extending over the surface of the foot and muscle fibres divide into four groups, each of which attaches to a separate tendon and attach to the distal phalanx of digits 2, 3, 4 and 5 of the foot (Balice-Gordon & Thompson, 1988). Fibre-type composition of EDL is heterogeneous, and spatially distributed with gradient fibre size and composition across the muscle cross section (Deveci et al., 2001). Fibre type percentage of EDL can be divided into oxidative medial (areal composition 24.3% Type I/IIa) and glycolytic lateral (92.4% Type IIx/IIb) compartments (Kissane et al., 2018).

1.8.2 **Cross section and systematic region sampling of EDL**

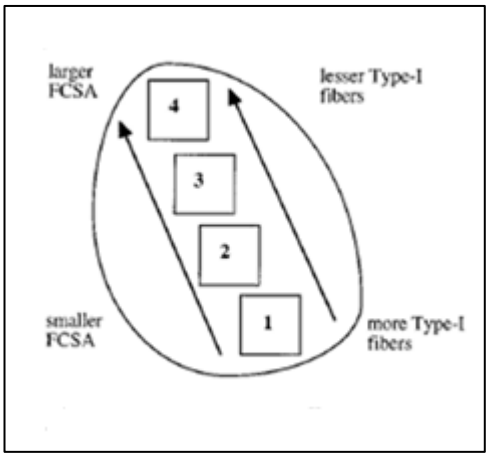

Figure 1.5: Cross section of EDL muscle. The muscle has more oxidative fibres with smaller fibre cross sectional area in the medial part (Deveci et al., 2001).

An EDL transverse section is oval in shape with a gradation of fibre type composition, with larger fibre size and more glycolytic fibres from medial to lateral compartments of the muscle section (Deveci et al., 2001). Due to the heterogeneous composition of EDL, unbiased and systematic region sampling is crucial in obtaining reliable and accurate values. In this work, all EDL samples were processed and analysed based on 4 regions; R1, R2, R3, R4 and mean values were the average of these.

1.9 Adaptive response to muscle activity

1.9.1 **Muscle fibre type composition changes**

Slow twitch (Type I) and fast twitch (Type II) fibres contraction times are about 110 ms and 50 ms, respectively, while the former are smaller in size and usually produce lower force than the latter (Lamb, 1978). Type I fibres are well adapted for prolonged contraction such as posture and long distance (low speed) running, due to higher local capillary supply and oxidative enzyme activity(Hudlicka et al., 1992), indicating that they are more energy efficient than those fibres relying on glycolysis. In contrast, fast twitch fibres generate large forces but are prone to fatigue (Vrbová, 1979). Adaptability of skeletal muscle initially involves enhancing capacity within the normal cellular phenotypic plasticity, and only with prolonged and/or intense stimuli causing fibre type transformation (Lamb, 1978).

Muscle fibre types

Fibre Type	Type I	Type IIa	Type IIx	Type IIb
Contraction time	Slow	Moderately Fast	Fast	Very fast
Size of motor neuron	Small	Medium	Large	Very large
Resistance to fatigue	High	Fairly high	Intermediate	Low
Activity Used for	Aerobic	Long-term aerobic/anaerobic	Short-term anaerobic	Short-term anaerobic
Max duration of use	Hours	<30 minutes	<5 minutes	<1 minute
Force production	Low	Medium	High	Very high
Mitochondrial density	High	High	Medium	Low
Capillary density	High	Intermediate	Low	Low
Oxidative capacity	High	High	Intermediate	Low
Glycolytic capacity	Low	Intermediate	High	High
Major storage fuel	Triglycerides	PCr, glycogen, triglycerides	PCr, glycogen	PCr, glycogen
Myosin heavy chain (human genes)	MYH7	MYH2	MYH1	MYH4

Figure 1.6: General characteristic of mammalian muscle fibre types. For glycolytic fibres, Type IIx is dominant in humans and Type IIb in rodents (Lamb, 1978).

Increased physical activity by endurance exercise training may elicit skeletal muscle changes in terms of fibre type composition, fibre area, and levels of oxidative enzymes to adapt to the increased metabolic demand, which has been demonstrated in both human and rodent muscles (Demirel et al., 1999; Harber et al., 2002). In humans, endurance sport altered muscle fibre composition to give a higher proportion of oxidative fibres, involving a shift from Type IIb to Type IIa and Type I fibres (Andersen & Henriksson, 1977; Jaschinski et al., 1998; Kariya et al., 2004) as seen in for instance swimming, running, cycling and cross country ski which involve different skeletal muscles - vastus lateralis and deltoid (Figure 1.7) (Armstron et al., 1972; Blomstrand et al., 1986; Gollnick et al., 1972). A longitudinal study of long term cycling exercise for 5 months resulted in 20% increase in Type I fibre area in vastus lateralis muscle (Gollnick et al., 1973), while strength exercise i.e. sprint exercise training increases the proportion of Type IIb fibres (Jansson et al., 1978). Rodent studies have demonstrated a significant increase of Type IIa fibres in plantaris muscle after 28 days of voluntary wheel running, from 15.3 $\pm$ 1.1% to 31.7 $\pm$ 1.9%, showing a transformation of Type IIb fibres which decreased from 83.0 $\pm$ 1.0% to 66.5$\pm$ 1.8% (Waters et al., 2004). This consistent with a study of 35 weeks endurance training on Sprague-Dawley rats which showed a significant decrease in Type IIb content from 35% to 15% in plantaris muscle. (Kariya et al., 2004).

Muscle fibre adaptation to exercise may be influenced by type, intensity and duration of exercise (Dudley et al., 1982). The effect of rat strain on fibre type composition transformation has been less well characterised, but this may lead to differential running activity pattern. Three inbred strains of ACI/Ztm, BH/Ztm and LEW/Ztm revealed different values in these parameters (Figure 1.8) (Wollnik, 1991). There were significant associations between running distance, magnitude of exercise intensity and fibre types changes in rodent after 4 weeks (Allen et al., 2001) and 15 weeks (Overton et al., 1986) of voluntary wheel running and ramp test at 10% incline at 55m/min of 4 bouts of increasing exercise period up to 2.5 minutes for seven weeks (Luginbuhl et al., 1984). In humans, 6 weeks endurance training on a bicycle ergometer elicited significant changes in Type I (+12%) and Type IIb (-24%) fibres of vastus lateralis muscle obtained by needle biopsy (Howald et al., 1985).

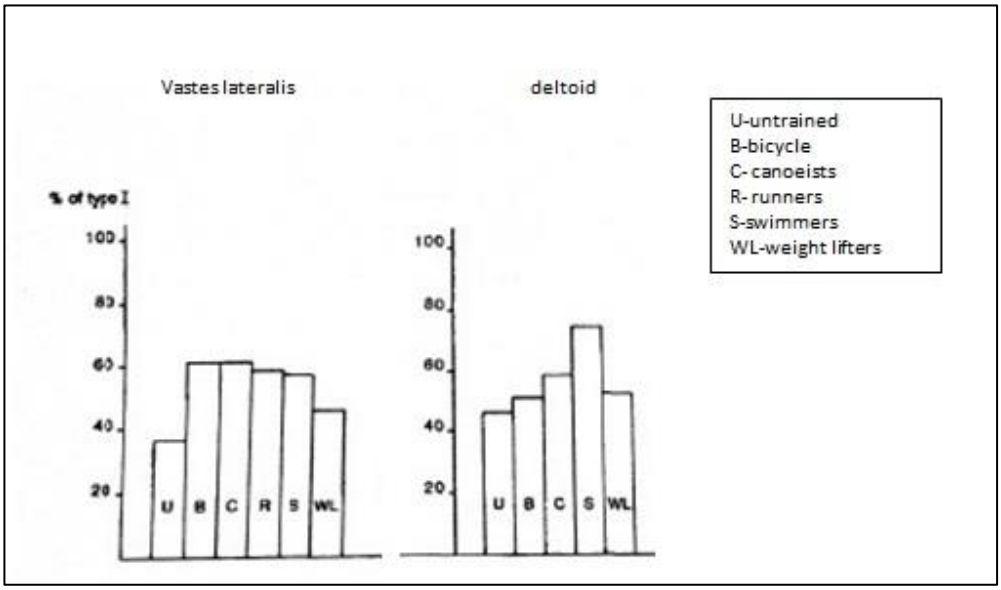

Figure 1.7: Type I fibre composition changes with different types of training relative to untrained individuals (Gollnick et al., 1972).

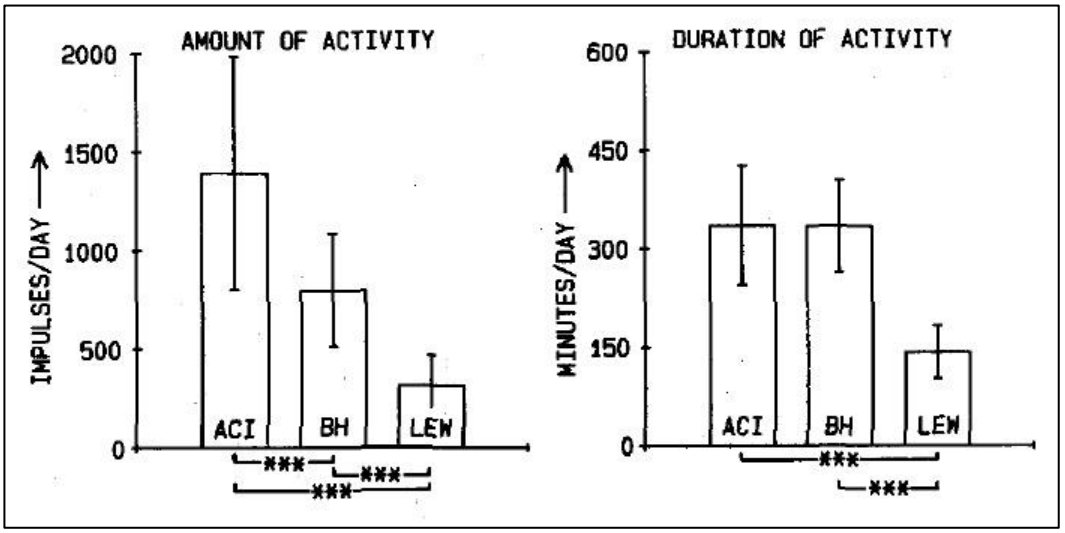

Figure 1.8: Amount and duration of activity for strains of ACI/Ztm, BH/Ztm and LEW/Ztm. *** indicates significant different ($P \leq 0.01$) (Wollnik, 1991).

1.9.2 **Microvascular supply**

In general, capillary supply of skeletal muscle varies across vertebrates, being generally higher in more aerobic species, and within muscles, usually higher in oxidative compared to glycolytic regions. It is also different across muscles as shown in chicken where a greater capillary density (CD) in leg than flight muscles but opposite in the case of doves, reflecting the influence of the main mode of locomotion (Ivanova, 1973). In rat, CD and CF in locomotor muscles is correlated with the proportion of oxidative fibres (Sol, TA and EDL; C:F = 2.3±0.03, 1.47±0.04 and 1.15±0.02, respectively (Hudlicka, 1985) and volume density of mitochondria (Conley et al., 1987; Hoppeler et al., 1981), suggesting oxygen supply is matched with oxygen demand. Species strain may also affect the C:F as outbred animals showed higher values than domesticated ones in the same muscles (soleus and gastrocnemius), while a similar pattern was observed in cardiac muscles (Wachtlova & Parizkova, 1972), reflecting the difference in habitual levels of activity. Capillary density of skeletal muscle is influenced by mean fibre area, as demonstrated in a study where rats of various age with restricted diet showed higher CD but low C:F in both slow and fast muscles, Sol and EDL, and the values were similar when the animals aged more than 1 year old - indicating muscles shrinkage affecting fibre area increased the density but not number of capillaries (Wachtlova & Parizkova, 1972).

Endurance exercise training is usually linked with increased capillarity, preceded by or simultaneously with increased aerobic capacity, determined by oxidative enzyme activity (Andersen & Henriksson, 1977) or mitochondrial volume density (Hoppeler et al., 1985;

Rösler et al., 1985). Thus, elements of aerobic capacity may be linked by chemotransduction mechanisms, i.e. demand determines supply. Capillarity is also influenced by mean fibre area in humans, as demonstrated by an endurance study that showed increased CD with reduced fibre area (Kayar, 1986; Oelz, 1986). However, some studies demonstrated an increased CD with unaltered fibre area (Brodal et al., 1977; Hoppeler et al., 1985; Ingjer & Brodal, 1978) showing true capillary expansion, which was greater around slow and fast oxidative than fast glycolytic fibres (Ingjer, 1979). Increased C:F was also reported in men performing endurance sports linked with fibre hypertrophy (Hermansen & Wachtlova, 1971), suggesting a potential co-regulation involving mechanotransduction, i.e. strain determines supply (Hudlicka, 1988).

Animal studies showed similar findings as in human humans. Treadmill exercise increased capillary number around oxidative fibres in rats (Ogawa, 1977). A greater increase in CD was observed in the oxidative than glycolytic region of biceps brachii (Zika et al., 1973), and was higher in mixed than purely glycolytic regions of EDL, 1000±52 *vs.* 762±86 mm^{-2} (Kissane et al., 2018). Similar findings were reported in other species e.g. guinea pigs (Mai et al., 1970), duck (Butler & Turner, 1988) and pigs (Michel & Salomon, 1977).

Muscle blood flow capacity is increased by exercise training, presumably due to structural and functional vascular adaptation. Structural adaptation involves formation of new exchange vessels from pre-existing capillaries (angiogenesis) to increase diffusive capacity and remodeling of the arterial tree to increase flow capacity; functional vascular changes might be due to increased shear stress causing enhanced smooth muscle reactivity to vasodilator substances like nitric oxide (NO), leading to increased vasodilatation and/ or endothelial cell proliferation to increase capillary surface area (Laughlin & Roseguini, 2008). The number of capillaries is affected by the size and metabolic properties of fibres as large numbers of capillaries are usually located near highly oxidative fibre types. Hence, the capillary to fibre ratio (C:F) is higher in the region of muscle composed of oxidative fibres (Hudlicka, 2011) to enhance supply along the fibre length (Emerson & Segal, 1997). Expansion of microvascular units (arteriole-capillaries-venule) of rat heart increases its capacity to accommodate oxygen delivery, following swim training for 1h, 6 day a week for 4 weeks (Ljungqvist & Unge, 1977), similarly as demonstrated in skeletal muscle (Egginton, 2010).

Muscles adapt to chronic exercise by increasing their capacity for extracting oxygen from blood, but endurance and sprint training preferentially increased perfusion around

slow oxidative and fast glycolytic muscle fibres, respectively (Hudlicka, 2008). Angiogenesis can be observed in the glycolytic region of muscles by elongation of the existing capillaries, probably *via* capillary splitting, and in the more oxidative regions by capillary sprouting (Egginton, 2009; Ljungqvist & Unge, 1977). Training also stimulates growth both in number and diameter of terminal arterioles (Lash & Bohlen, 1992). Generally it has been understood that there will be an angiogenic response in order to fulfill increased demand for oxygen, i.e. by increasing capillary density (CD) and capillary to fibre ratio (C:F), thus decreasing oxygen diffusion distances in exercising muscles. Voluntary wheel running exercise induces angiogenesis in skeletal muscle, as 4 weeks long term running among C57BL/6J mice doubled CD (466$\pm$ 16 to 909 ±55 mm^{-2}) and C:F (0.95$\pm$0.04 to 1.70 $\pm$0.08) in plantaris muscle (Waters et al., 2004). However, insufficient exercise intensity and duration was unable to increase CD and C:F in soleus and gastrocnemius of young rats that were trained for 3 weeks (Banchero et al., 1979; Wachtlova & Parizkova, 1972). Increased fast muscle activity by chronic electrical stimulation may also induce angiogenesis; increased C:F (from 1.25 to 1.54) and CD (20% higher than control) was observed in EDL muscles following 4 days of chronic electrical stimulation at 10 Hz, while the value almost doubled after 28 days of stimulation (Brown et al., 1976).

The physical environment is thought to influence capillary growth where endothelial cells utilise mechanotransduction in controlling *in vivo* angiogenesis. Increased capillary growth by internal splitting can be initiated by elevated shear stress, following activation of endothelial nitric oxide synthase, generation of nitric oxide and increased vascular endothelial growth factor receptor 2 (VEGFR2) proteins due to chronic vasodilatation. Angiogenesis can also be stimulated by muscle overload, with chronic stretch activating metalloproteinases (MMPs), but also VEGF leading to capillary sprouting (Egginton et al., 2001).

Capillaries form a complex network with multiple intercapillary connections (anastomoses) and sprouts (Figure 1.9). Angiogenesis is initiated by growth factors secreted from active muscle or by mechanical factors: VEGF, other angiogenic stimulators like MMPs and NO, and anti-angiogenic factors such as angiostatin and endostatin, are involved in regulating (initiating and maintaining) capillary growth. Angiogenesis can occur by sprouting or longitudinal splitting due to stretch and shear stress, respectively (Egginton, 2009; Gustafsson & Kraus, 2001; Hansen-Smith et al., 1996; Olfert & Birot, 2011) and can be activated or deactivated, depending on the net result of interplays between stimulators and inhibitors of angiogenesis (Egginton, 2009).

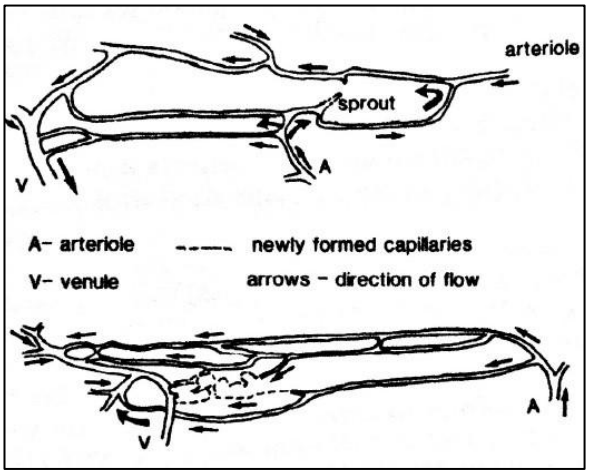

Figure 1.9: Expansion of capillary bed in rat extensor hallucis proprius (top) and rabbit tenuissimus (bottom) after being stimulated for 7 days at 10Hz for 8h/day. Source: (Hudlicka et al., 1992).

1.10 Aims and objectives

Exercise-induced angiogenesis may provide an alternative therapeutic approach in treating cardiovascular disease, particularly peripheral arterial disease (PAD), and involve fewer side effects compared with pharmacological treatments. Exercise training potentially stimulates capillary bed expansion by angiogenesis, thus improving aerobic capacity of skeletal muscles. But not all exercise training is suitable for the elderly which are the most affected group with PAD. Studies have shown that patients with PAD were able to increase their functional capacity when performing supervised treadmill exercise, but that might not be the case with unsupervised exercise due to opting for less stressful activity that provides insufficient stimulus intensity. Animal studies that use running wheels may closely mimic unsupervised treadmill exercise and may be used to explore how a less stressful exercise may influence muscle functional and metabolic changes, including capillarity.

The aim of this work is to determine running characteristics and optimal duration of voluntary wheel running exercise of Wistar rats so that an angiogenic response could be elicited before capillary rarefaction occurs during ischaemia or with age. So, a pilot study of 7 week voluntary wheel running exercise in which a group with fructose consumption (to increase exercise volume) would be performed. It was hypothesized that running distance performed by Wistar rats increased over the period before it dropped and elicited angiogenic responses and fibre type shift.

In addition, we reasoned that a less aerobic strain, Wistar rats would optimise skeletal muscle remodelling due to relatively lower physiological limit that stimulates an angiogenic response, which effects could be observed earlier than if using more a more aerobic strain. Little information is available to characterise the animal strain effect on muscle composition, which could serve as an alternative way to examine differences in aerobic capacity. So, we also performed muscle survey in a range of rat striated muscles - cardiac, TA, EDL, Sol and diaphragm (comparing high and low aerobic capacity strains, Brown *vs*. Wistar) would better characterise those outbred and inbred strains, thus determining the influence of level of fitness on muscle functional capacity and capillarity. Estimation on muscle function through intramuscular modelling oxygen tension would be determined to examine differences between those two strains at simulated resting and maximal exercise levels, thus determining the influence of level of fitness on likely muscle functional capacity and capillarity. It was hypothesized that Brown rat EDL muscle would exhibit better oxygen saturation and lower presence of hypoxia, particularly during maximal exercise level.

Modern techniques (immunohistochemical staining of biopsies and a more comprehensive analytical approach) would be used to better characterise muscle phenotype, which includes important local angiogenic indices to provide a more comprehensive data on adaptation responses towards running wheel exercise. Then, further analysis in examining potential benefit of the exercise in oxygen diffusion capacity and adaptive remodelling was performed by modelling oxygen tension that provide estimated muscle aerobic capacity at resting and maximal exercise level, and followed by actual muscle function (fatigue index) measurement. $\dot{V}O_2$ max is a systemic oxidative capacity index which quantifies whole body aerobic capacity with improved functional status of accessory organs e.g. cardiorespiratory systems to support increased metabolic demand.

To determine mechanism of therapeutic benefit of angiogenesis from the exercise regime (optimal duration from pilot study), two defined ischaemic models have been applied to mimic PAD disease, a mild local (femoral arterial ligation) and a severe systemic form of ischaemia (aortic banding), where the magnitude of angiogenesis and differences in muscle fibre composition and function would be determined. Data for angiogenic indices and fibre type morphological changes and composition would be correlated with running parameters to characterise running behaviour changes during adaptive remodelling following local and systemic ischaemia.

We hypothesized that voluntary wheel running exercise may induce angiogenic response and fibre type transformation to a more oxidative phenotype in hindlimb skeletal muscle of Wistar rats. Voluntary running mode by application wheel exercise may minimise stress effect on running performance, which partly may enhance running desire to maximise angiogenic stimuli. With information provided from capillary domain area analysis, many useful outputs can be used to better explain skeletal muscle adaptive response - capillary distribution and oxygen supply area. It is promising that less stressful exercise intervention may result in capillary growth and functional enhancement benefit in pathological rats. Therefore, we hypothesize that ischaemic tissue could be alleviated by expansion of capillary bed to enhance oxygen delivery at different magnitudes depending on ischaemia severity. In conclusion, if the potential of VWRE in promoting angiogenesis could be elucidated and further characterised, then this intervention could be the preferred therapy to be applied in treating PAD.

Chapter 2 : General methods

2.1 Ethical approval

All experimental work complied with the UK Animals (Scientific Procedures) Act 1986, and local approval was granted by the University of Leeds Animal Welfare and Ethical Review Committee under project licence PPL 70/8674. An induction training course on good animal handing and welfare was attended by the student, and permission granted to perform all the experiments under PIL number I35F036F3.

2.2 Voluntary running wheel apparatus

A purpose-built apparatus was necessary as commercially available systems do not provide the level of analytical detail required for the following experiments. Wheel diameter was 33 cm with 4 magnets equally spaced around its perimeter, and capable of bidirectional rotation (diameter 112 cm; Lafayette Instruments, West Lafayette, IN, USA). A pulse with a velocity measurement is generated (represents a quarter wheel revolution) as the magnet passes a sensor which is attached to the wheel.

2.3 Running wheel recording and data management

Running activities were recorded using a logger system connected to a computer. Revolutions were recorded in 1min intervals and stored as a mean value of the 4 readings, looped continuously to produce 1440 files each day. Velocity threshold was set to at 80rpm (1.3823 m/s) such that any pulses with higher velocity than that are filtered from data, and activity threshold was set at 1 where a loop containing at least 1 pulse is assumed active. Raw data were saved in a specified folder cumulatively using software to sort data into individual animals (Post Processor v12). Data were later collated and sorted using an Excel spreadsheet for mean value determination.

2.3.1 **Running wheel parameters**

A set of running wheel parameters (split between dark and light cycles recorded each 24 hours) were tabulated and compared between studied groups:

a) Running distance (km)
b) Dark cycle running distance (km)
c) Light cycle running distance (km)
d) Running velocity (m/s)
e) Mode running velocity (m/s)
f) Median running velocity (m/s)
g) Dark running velocity (m/s)
h) Light running velocity (m/s)
i) Percentage time active (%)
j) Bouts of running activity
k) Maximal running duration (min)
l) Mean of running duration (min)
m) Mode of running duration (min)
n) Dark percentage time active (%)
o) Dark bouts of running activity
p) Dark maximal running duration (min)
q) Dark mean of running duration (min)
r) Dark mode of running duration (min)
s) Light percentage time active (%)
t) Light bouts of running activity
u) Light maximal running duration (min)
v) Light mean of running duration (min)
w) Light mode of running duration (min)

2.4 Schedule 1 killing and muscle dissection

The experimental and sedentary control animals were killed according to a Schedule 1 procedure. Rats were taken out from carrying boxes by holding their tail base, with the other hand used to support the rat's body. Immediately they were stunned by striking the cranium against a solid object. Animal death was then confirmed by neck dislocation. Extensor digitorum longus (EDL), soleus (Sol), tibialis anterior (TA), diaphragm, and cardiac (left ventricle free wall) muscles were dissected. Adipose and connective tissue were removed from muscles which were then weighed.

2.5 Freezing and cryosections

The middle part of muscles were sampled, mounted in OCT embedding medium (Thermo Scientific), and quickly frozen in isopentane cooled in liquid nitrogen (Dubowitz, 1973) and

stored at -80°C in a freezer until processing. Serial sections of each muscle (10 µm thick) were cut using a cryostat at -20°C, air dried on poly-L-lysine coated slides (VWR International) and then stored frozen (-20°C) until staining.

2.6 Monoclonal-myosin heavy chain antibodies, lectin and laminin staining

This staining is based on specific binding between antibodies to surface antigens. Briefly, Type I and Type IIa fibres were stained using BA-D5 and SC-71 antibodies (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, Iowa), respectively, leaving Type IIb/IIx unstained. Fluorescent probes Alexa Fluor 555 goat anti-mouse immunoglobulin G (IgG; A21422; Life Technologies, Carlsbad, California) and Alexa Fluor 488 rabbit anti-mouse IgG (A11059; Life Technologies), respectively, were used as secondary antibodies to bind on BA-D5 and SC-71 ligands, visualised as red and green fluorescent images. An anti-laminin antibody (L9393; Sigma, St Louis, Missouri) biotinylated with anti-rabbit IgG (BA1000; Vector Laboratories, Burlingame, California) and labelled with streptavidin (S11222; Pacific Blue Conjugate; Life Technologies) was used to label fibre boundaries. Simultaneously, *Griffonia simplicifolia* lectin-1 (Vector Laboratories) that specifically binds to basement membrane and galactose residues of proteoglycans in the glycocalyx of endothelial cells, identified capillary locations.

2.6.1 Immunohistological staining protocol

Preparation:

Slides were air dried by allowing them to stand at room temperature for 30 minutes. All tubes were labelled according to antibody mixes 1, 2, 3 and 4 for antibody mix of antibody BA-D5 and laminin, anti-mouse 555 and biotinylated – anti-rabbit, antibody SC-71 and Pacific Blue and anti-mouse 488 and *Griffonia simplicifolia* lectin, respectively. All substances were diluted with PBS solution according to following dilution ratio:

	Antibody mixes
A	Antibody BA-D5 (1:500) and laminin (1:250)
B	Anti-mouse 555 (1:1000) and biotinylated-anti-rabbit (1:250)
C	Antibody SC-71 (1:500) and Pacific Blue (1:250)
D	Anti-mouse 488 (1:1000) and *Griffonia simplicifolia* lectin (1:200)

Blocking solution was prepared by diluting 0.01g of bovine serum albumin (BSA) with 1ml phosphate buffered saline (PBS) solution in a labelled tube. Next, 2% fixation

solution was prepared made by diluting 500µl of 4% PFA stock solution with 500µl of PBS in a 1:1 ratio. They were then vortexed to mix well and labelled 'BS and 'FS', respectively. Slides were taken out of the freezer for at least 30 minutes to warm up, avoiding direct condensation. A wax well then was created around each muscle section. Fix solution were applied to all wells by fully covering the sections for 2 minutes. The fix solution was then vacuumed off and washed 10 times with PBS. Block solution was applied to the wells and left for 10 minutes. They were then vacuumed off.

The first antibody (mixture A) was applied. For each, newly prepared antibody mixture was mixed 10 minutes prior to application. Vortex was used to mix well. The antibody was incubated for 1 hour followed with 10 times PBS washes. This step was repeated for the other 3 antibody mixtures (B, C and D). All solutions were thoroughly removed by using vacuum suction. 10ul of mounting medium was slowly applied in the middle of each section to prevent bubble formation. Then, a glass coverslip was placed and some pressure was applied. Lastly, nail varnish was applied at all cover slip edges to prevent them from moving and avoid desiccation (method optimised by Roger Kissane).

2.7 Immunohistochemistry optimisation: Influence of lectin staining and image magnification on capillary counting.

2.7.1 Introduction

Early work on capillary staining used fluorescein-conjugated *Griffonia (Bandeiraea) simplicifolia* lectin I for capillarity determination. However, it was observed that green capillary staining image quality was less sharp with presence of noise, possibly due to some autofluorescence, potentially lead to error in differentiating two neighbouring capillaries. Therefore, it was compared with rhodamine-conjugated lectin to determine if there was better image quality of the latter leading to less noise and avoiding underestimation of capillarity.

Sufficient fibre number is an important element in determining capillarity, minimising the 'edge effect', and should be within an acceptable range to reduce quantitation error in analysis and allow for repetition within shorter duration. Therefore, capturing images under defined magnification should be considered so that reasonable fibre number should be included.

2.7.1.1 **Objective**

To determine influence of type of capillary staining and magnification on quantification of capillary supply.

2.7.2 **Methods**

TA muscles of control Wistar rats were frozen in isopentane cooled in liquid nitrogen, cryosectioned at 10 µm thick and stained for capillaries using fluorescein- or rhodamine-conjugated *Griffonia (Bandeiraea) simplicifolia* lectin I. Stained capillaries were visualised using a fluorescent microscope (Nikon E600) and images were captured from core and cortex region at x20 magnification. They were then counted manually using ImageJ software (NIH) covering 75% of total sample area, and capillary supply quantified using an unbiased sampling frame, which included two inclusion (dotted) and exclusion (solid) edges.

2.7.3 **Results**

2.7.3.1 **Influence of type of capillary staining on image quality**

Lectin stain for marking capillaries using either rhodamine or fluorescein showed similar counts for capillary and fibre numbers, thus generating a similar C:F (Table 2.1), and the quality of capillary staining with these stains was relatively similar (Figure 2.1). Therefore, using either of those staining approaches (rhodamine or fluorescein conjugation) would result in similar fibre and capillary counting.

Table 2.1: Capillary, fibre number and capillary to fibre ratio between rhodamine and fluorescein staining. Data presented as mean±SD, N=5.

Staining	Rhodamine	Fluorescein	Significance
Capillaries	104±31	104±32	*P*>0.05
Fibres	55±10	55±10	*P*>0.05
C:F	1.73±0.23	1.75±0.24	*P*>0.05

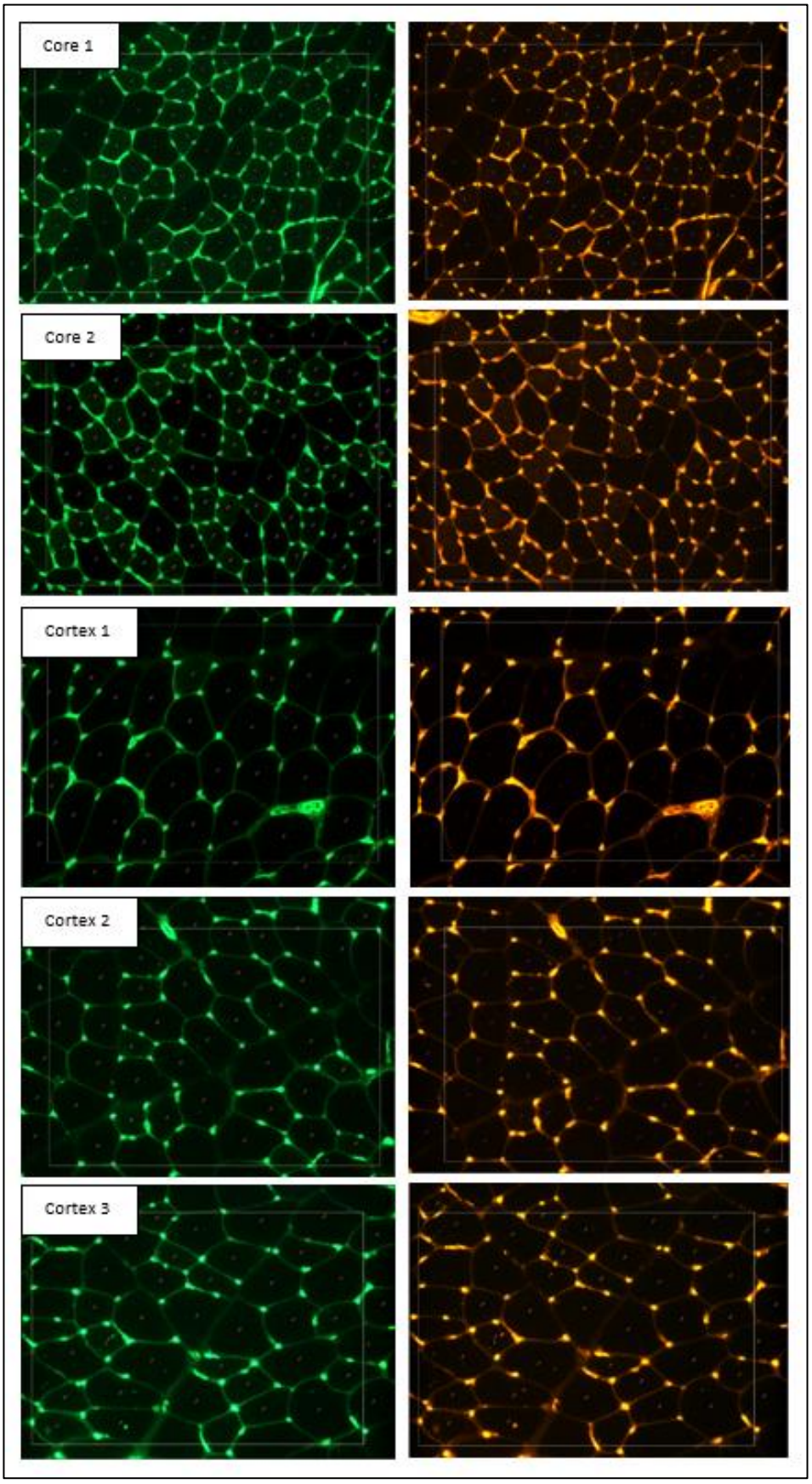

Figure 2.1: Fluorescein (left) and rhodamine (right) capillary staining of TA muscle (x20). Systematic sampling areas, bright spots denote capillary location.

2.7.3.2 **Influence of magnification on fibre and capillary number inclusion.**

Angiogenic indices of core and cortex of TA muscles with images captured at x10 and x20 magnification yielded similar values (*P*>0.05) (Table 2.2).

Table 2.2: Similar C:F and capillary density (CD) values at x10 and x20 in core and cortex region.

Core				
Capillarity	Staining	Magnification	Mean±SD	Significance
C:F	Rhodamine	x10	2.0±0.07	*P*>0.05
		x20	2.1±0.09	
CD(mm^{-2})	Fluorescein	x10	1262±66	*P*>0.05
		x20	1463±78	

Cortex				
Capillarity	Staining	Magnification	Mean±SD	Significance
C:F	Rhodamine	x10	1.21±0.03	*P*>0.05
		x20	1.23±0.03	
CD(mm^{-2})	Fluorescein	x10	559±44	*P*>0.05
		x20	594±40	

2.7.3.3 **Appropriate magnification to include sufficient fibre number in fields of view containing predominantly fast oxidative and fast glycolytic fibre types of large animals.**

For muscle samples of large animals (pilot study of 7 week study), number of fibre included in core region of TA under x20 magnification was sufficient for angiogenic indices determination (Table 2.3). However, there were fewer fibres included in cortex region of TA (Table 2.3 and Figure 2.2B). This showed that a smaller magnification should be applied when dealing with samples containing large fibres so that an ample number would be included for capillarity determination.

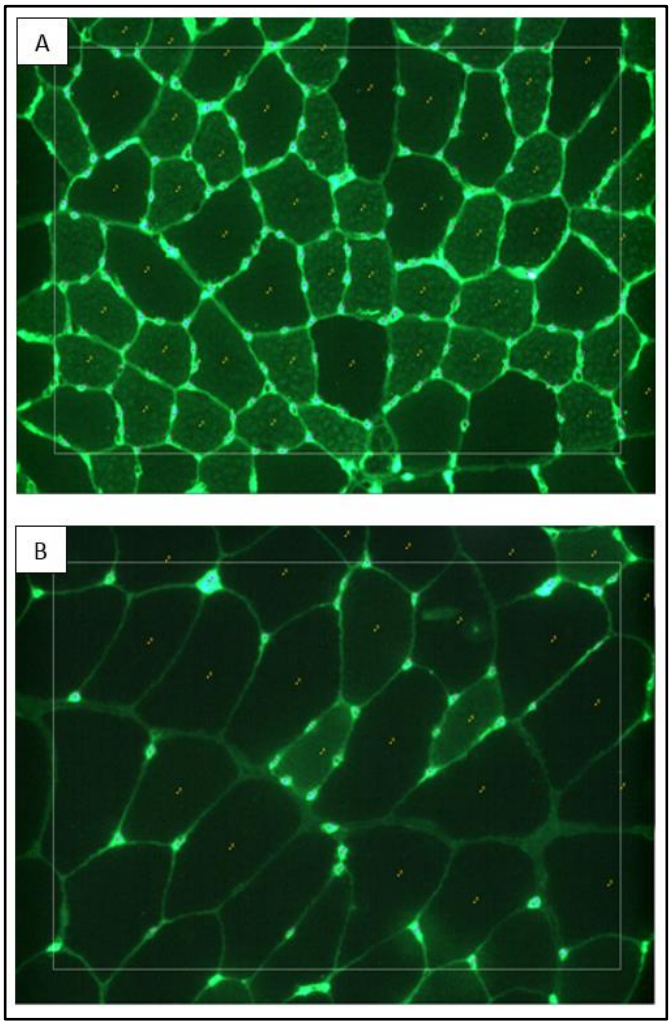

Figure 2.2: Capillary staining on TA section; core (A) and cortex (B) at x20 magnification. Fewer fibres are included in cortex region (predominantly with large fibres) of large animals (~360g). A lower magnification should be used when animal size is large to include sufficient fibre number.

Table 2.3: Fibre number of TA muscle at X20 magnification, rat body mass (360g), core (n=2), cortex (n=3).

Region	Mean±SD
Core	52±2
Cortex	27±1

2.7.4 **Discussion**

Manual counting of capillary and fibre numbers showed similar values between the two different staining protocols. C:F was not significantly different (P>0.05), indicating either marker could be used for reliable capillary staining. However, rhodamine conjugated lectin produced better quality capillary images than fluorescein staining, in particular at higher exposure times with reduced noise, as this conjugate binds less avidly to fibre boundary, thus improving resolution of individual capillaries.

The influence of image magnification on C:F and CD was determined (at x10 and x20) where the values showed no significant difference in either core or cortex regions of TA muscle (Table 2.2). This suggested that x20 is optimal magnification to include sufficient fibre number for any fibre related counting thus reducing counting error (less percentage error in case of fibres exclusion). However, magnification had to be reduced from x20 to x16 when bigger animals were used as x20 magnification would potentially lead to either overestimation or underestimation of C:F due to reduced fibre number included in the unbiased counting frame. This was applicable for larger fibre types, particularly Type IIb in cortex and Type I in soleus muscles that required lower magnification to include sufficient fibres. Therefore, for larger animals (i.e. body mass >360g, including rats from 7 week voluntary wheel running exercise), X16 magnification was used for immunohistochemical analysis to determine C:F, CD and other local angiogenic indices.

2.7.5 **Conclusion**

In conclusion, rhodamine-conjugated *Griffonia (Bandeiraea) simplicifolia* lectin I produced better contrast images than fluorescein-conjugated *Griffonia (Bandeiraea) simplicifolia* lectin I and x20 magnification is optimal magnification for histological work and analysis.

2.8 Systematic morphometric analysis

2.8.1 Area of sampling region and unbiased counting rule

Specific areas of muscle sections were sampled with systematic-random sampling (Figure 2.3) (Deveci et al., 2001) and data collection based on an unbiased counting rule, which included only 2 edges of square region of interest under x20 magnifications (EDL and core of TA) and X16 (Sol and glycolytic of TA)(Egginton, 1990b).

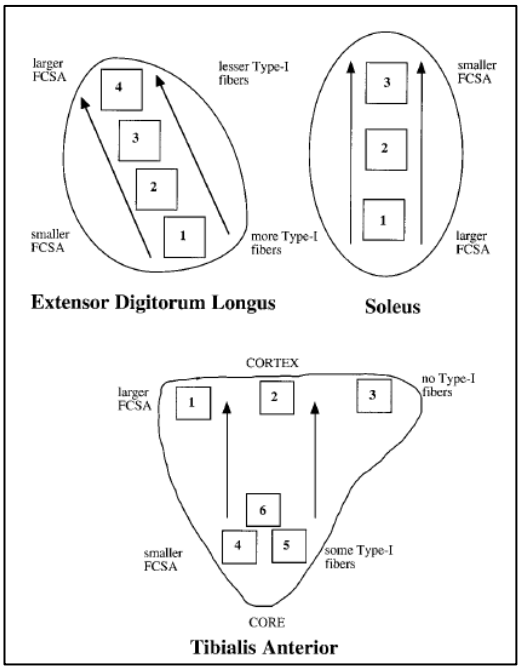

Figure 2.3: Localisation of sampled regions of cross section of TA, EDL and Sol with arrows denoting the gradient of fibre size, composition and oxidative capacity (Deveci et al., 2001).

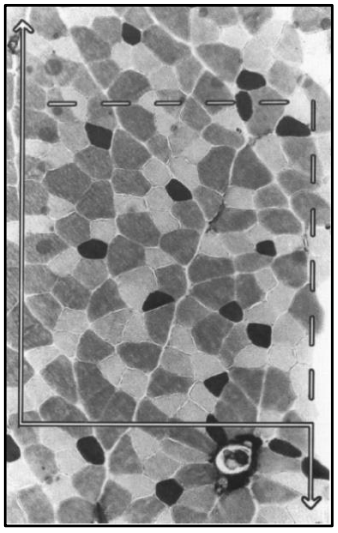

Figure 2.4: Determination of region of interest and inclusion (dashed line) and exclusion (solid line) zones zone (Egginton, 1990b).

2.8.2 **Global angiogenic indices and fibre type composition**

There are many indices used in the literature to describe the characteristics of the microvasculature, either in control or experimental animals. Global measures generally involve simple counting procedures that provide estimates of gross changes across a whole muscle section, e.g. capillary to fibre ratio (C:F) and capillary density (CD) used in this thesis. CD and C:F are scale-dependent, i.e. both are influenced by fibre cross sectional area showing a linear increase and a curvilinear decrease, respectively (Egginton, 1990a).

C:F is one of the most common angiogenic indices used to estimate oxygen supply reported in previous studies. It is the numerical ratio of capillary and muscle fibres: in stereological notation given as $N_N(c,f) = N(c)/N(f)$. It is relatively independent of fibre area over a small size range, allowing for comparing different muscles samples, and shows little variation among mammalian muscles (Plyley & Groom, 1975). C:F is usually greater in more oxidative muscle phenotypes (soleus) or in oxidative region of mixed muscle (EDL and TA)), and naturally higher in wild type animals compared to inbred counterparts. C:F is a robust angiogenic index as capillary bed expansion occurring with or without hypertrophy shows an increase in C:F, revealing a true angiogenesis (Egginton, 1990a).

CD is defined as capillaries per unit area of tissue section (mm^{-2}): $N_A(c,f) = N(c)/n.a(f)$, where a(f) is mean fibre area. It is considered as a good index of the functional capacity for oxygen supply that reflects tissue oxygen consumption (Egginton, 1990a). It is useful index in case of capillary expansion with little change in muscle fibre area as it is greatly sensitive to muscle fibre size change, so could be underestimated or overestimated in case of oblique section and fibre shrinkage during sectioning and immunohistological works, respectively.

2.8.2.1 **Dtect and Capillar histological analysis: Global capillary density (CD), capillary to fibre ratio (C:F), mean fibre area (MFA) determination.**

Capillaries and fibres of EDL, Sol and TA images were visualised through a fluorescent microscope (Nikon Eclipse E600). EDL images were captured based on region: R1, R2, R3, and R4 at x20 magnification which produced images with dimension of 439.86 x 329.90 µm, giving a total area of 145,108.62 μm^2. Quality of the captured images was improved using Image J, by adjusting colour balance and threshold so that fibre boundary and fibres type were clearly distinguishable. In-house generated morphometric analysis programmes, Dtect and Capillar, were used to determine angiogenic indices (Al-Shammari, 2019).

Initially, the captured images were loaded into Dtect to sort fibre types into respective groups; Type I, Type IIA and Type IIB. Image scale was set at 5820 pixels/mm, derived from the length of image (2560pixels/0.439.86 mm). The region of interest (ROI) was set to 75% of total area, which included two dotted edges (Figure 2.5). Fibre boundary (basal lamina) visualisation was processed until fibre types were individually separated (Figure 2.6). In the next stage, fibre types were categorised by assigning them to respective boxes (Figure 2.7). Output MAT file from Dtect analysis was then loaded and run in Capillar to quantify capillary number (Figure 2.8). Any capillary or fibre fully included in the ROI, or that uniquely intersected the dotted (inclusion) lines, were included in the analysis.

Gross angiogenic indices; CD, C:F, mean fibre area (MFA) as well as numerical density (N_N) and areal density (A_A) of those particular fibre types were automatically derived from histological analysis.

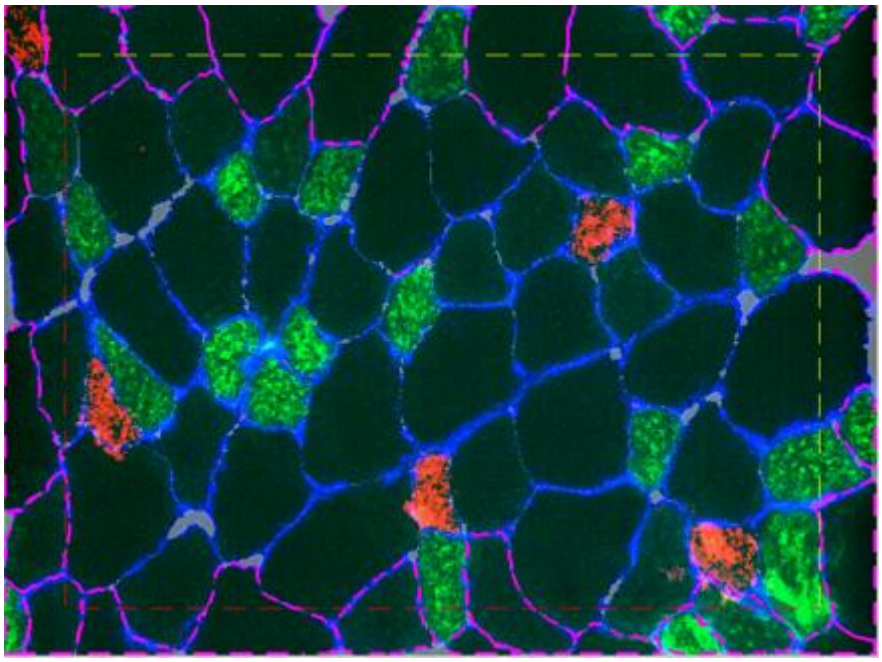

Figure 2.5: Setting of region of interest that covers about 75% of total area in Dtect analysis, EDL image at x20 magnification. Type I=red, Type IIa=green, Type IIb-unstained (black), laminin=blue. Excluded fibres outlined by dotted lines.

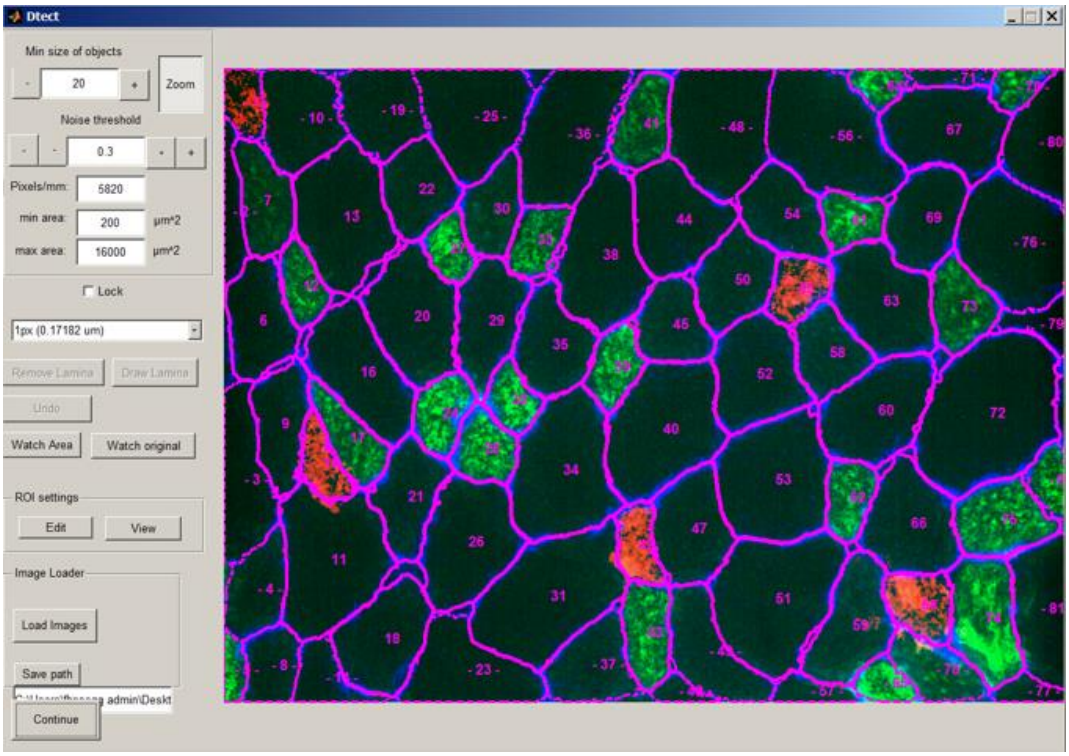

Figure 2.6: Drawing of fibre boundaries as part of Dtect histological analysis process, with numbering of individual fibres.

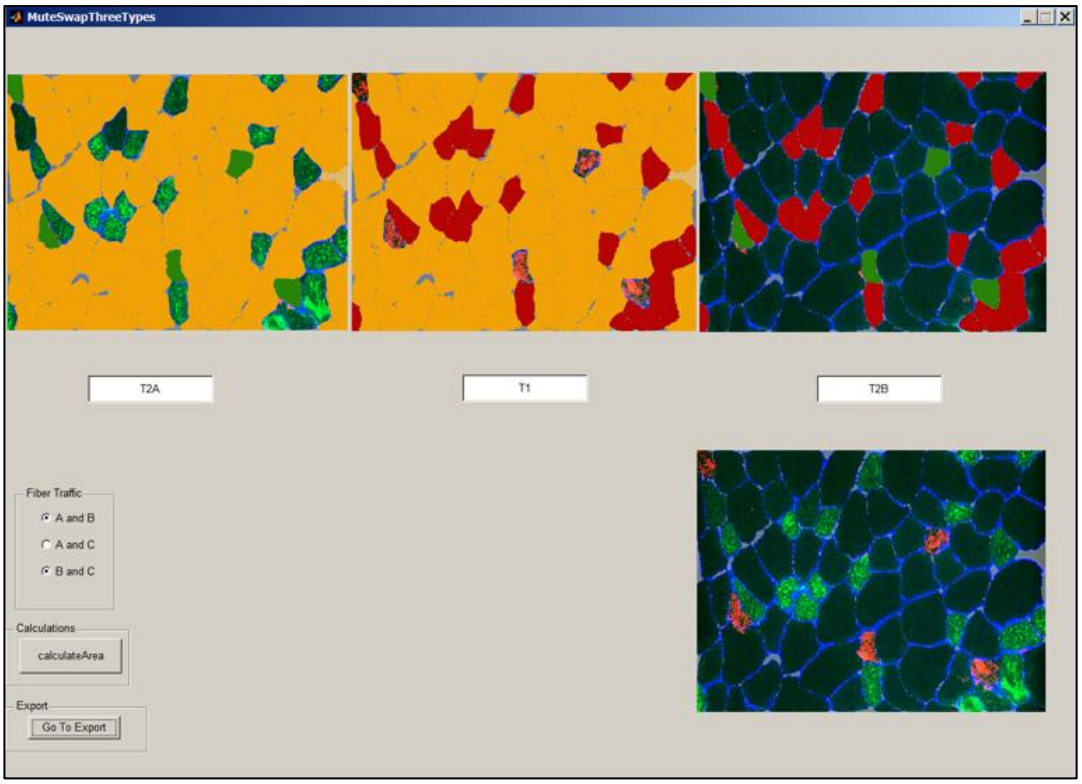

Figure 2.7: Categorising fibre types into respective groups in Dtect histological analysis process; any detection errors can be corrected at this stage.

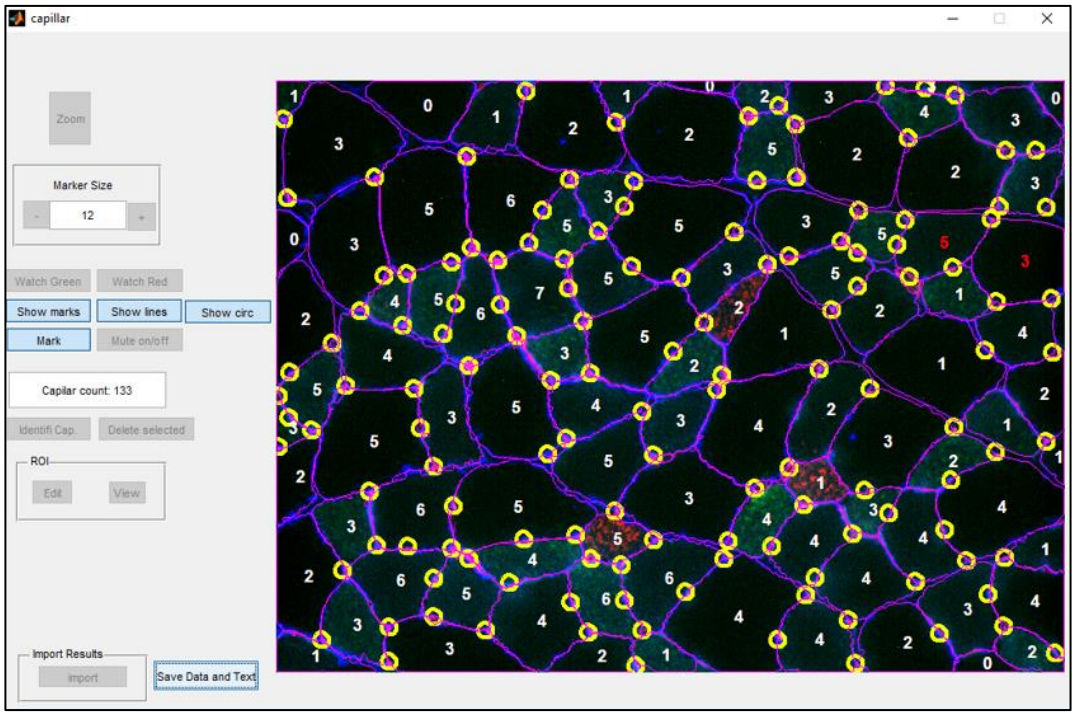

Figure 2.8: Determination of capillary number in Capillar histological analysis programme, with capillary location (circles) added manually with reference to lectin stain.

2.9 Local capillarity and capillary domain area determination

Local capillarity provides information on capillary distribution in particular muscles that inform capacity of microvascular in supplying oxygen to muscle fibres (Al-Shammari et al., 2014; Egginton & Gaffney, 2010), and its indices are scale independent. Capillary location with fibre boundaries lead to the generation of local non-integer based indices, an input for modelling capillary supply regions assuming homogeneous oxygen consumption. Integrating additional heterogeneities in oxygen uptake *via* fibre type allocation permits the generation of fibre type specific, local capillary indices and then tissue PO_2 modelling (Al-Shammari et al., 2014). Equation for each local capillary index is as follows:

Capillary domain area (CDA):

Let N_c represent the number of capillaries, and X_i represent the position of the centroid of the ith capillary with Ω representing the global domain. The Voronoi polygon (VP), also referred to as the capillary domain, that is associated with the ith capillary is a 2-dimensional region surrounding it with boundaries that are equidistant from adjacent capillaries (Al-Shammari et al., 2014).

$$V_i = \{\mathbf{x} | \mathbf{x} \in \Omega; \| \mathbf{x} - \mathbf{x}_i \| \leq \| \mathbf{x} - \mathbf{x}_k \|, k \neq i\}$$

Local capillary to fibre ratio (LCFR):

It is a cumulative fraction of capillary domain areas that overlap a fibre. It represents the fractional number of capillaries supplying the jth muscle fibre with oxygen that is the number of capillary equivalents of supply at maximum capacity. Ω denotes the region of the ith fibre and A the area (Al-Shammari et al., 2014).

$$LCFR_j^{VP} = \sum_i \frac{A(\Omega_j \cap V_i)}{A(V_i)}$$

Local capillary density (LCD):

It is derived from division of LCFR with individual fibre areas, producing a normalised value of capillary density for allometric scalling. It represents the number of capillaries supplying the jth muscle fibre per unit fibre area. . Ω denotes the region of the ith fibre and A the area (Al-Shammari et al., 2014).

$$LCD_j^{VP} = \frac{1}{A(\Omega_j)} \sum_i \frac{A(\Omega_j \cap V_i)}{A(V_i)}$$

Using the MatLab programme (Mathworks.com), MAT files from Capillar outputs were loaded to determine local capillary to fibre ratio (LCFR), local capillary density (LCD) and capillary domain areas in which boundaries mid-way between adjacent capillaries were used (constructing Voronoi tessellations) to delineate individual regions of capillary supply (Figure 2.9). Frequency distribution of capillary domain areas was analysed and displayed as histograms. Spatial heterogeneity of capillary supply was derived from the logarithmic normal distribution by calculating the standard deviation of log-transformed capillary domain area (logSD) (Al-Shammari et al., 2019). LCFR is calculated from sum of the fraction of individual capillary domain area overlapping a fibre, which provides an estimate of average capillary supply to a fibre (in terms of capillary equivalents) including the influence of neighbouring capillaries rather than just those adjacent to the fibre of interest (Egginton & Ross, 1992). Dividing LCFR by mean fibre area normalises LCFR to provide a local scale-independent measure of capillarity, local capillary density, which is specific to individual fibres (Al-Shammari et al., 2019).

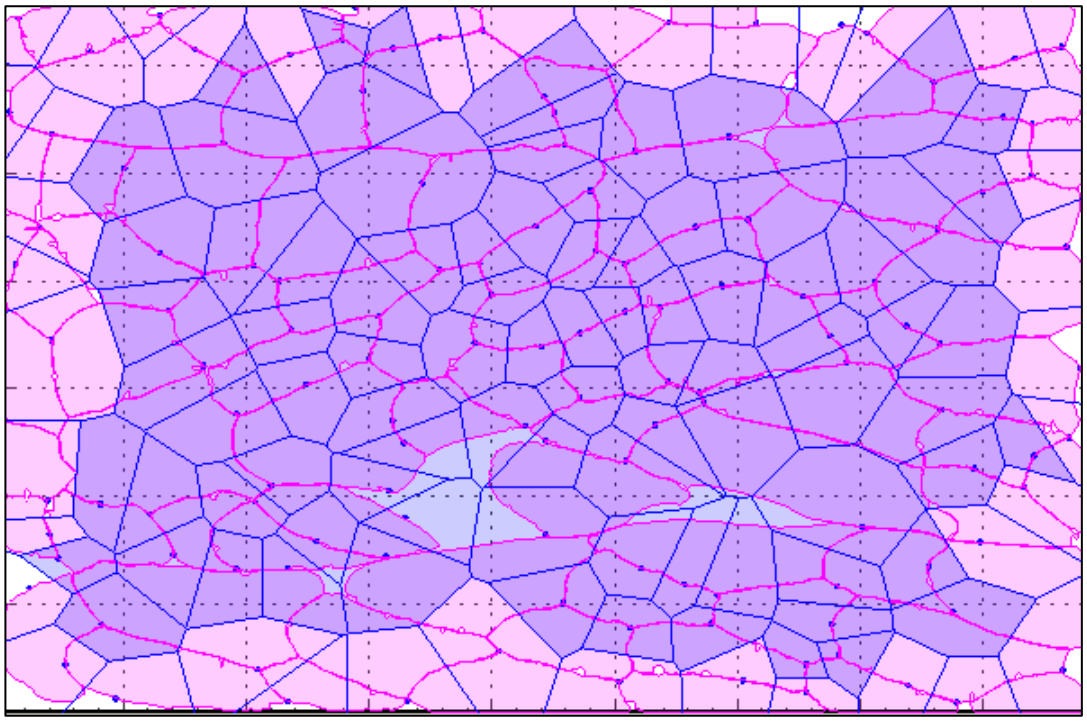

Figure 2.9: Region of interest (purple region) of capillary domain areas (blue lines) overlaid on digitised fibre boundaries (pink lines).

2.10 Oxygen transport modelling

Model of oxygen diffusion in tissue is performed under conditions of compartment-specific diffusion and uptake, myoglobin-facilitated diffusion, and oxygen consumption rate according to Michaelis–Menten kinetics. All values and assumptions accord with published studies of relevant skeletal muscle investigations. Intravascular transport is considered homogeneous i.e under conditions of microvascular perfusion when all elements are open to flow, and incorporated via a boundary condition at capillary walls as the primary assumption is that the tissue is under maximal sustainable (aerobic) activity. Therefore, oxygen transport in each tissue compartment, β is explained by the local free oxygen partial pressure (PO_2), p, where Dβ and aβ are the molecular diffusivity and the solubility of free oxygen respectively, C^{Mb} β and D^{Mb} β are the bulk myoglobin (Mb) concentration and diffusivity, and M_β is the rate of O_2 consumption in muscle tissue compartment β. Here β denotes the following tissue compartments: interstitial spaces (β=IS) and fibre types I (β=I), IIa (β=IIa), and IIb (β=IIb), with a_β is constant and thus $\nabla(a_\beta p) = a_\beta \nabla p$.

$$\nabla \cdot \left[D_\beta \nabla (a_\beta p) + C_\beta^{Mb} D_\beta^{Mb} \left(\frac{dS_{Mb}}{dp} \nabla p \right) \right] - M_\beta(p) = 0$$

With an assumption of homogeneous distribution of myoglobin molecules within muscle fibres and rapid local kinetics of Mb-O_2 dissociation, the equilibrium O_2-saturation of Mb is governed by:

$$S_{Mb}(p) = \frac{p}{p + p_{50,Mb}}$$

where $P_{50,Mb}$ is the tissue oxygen partial pressure at half Mb-saturation. In addition, the rate of oxygen consumption within muscle fibres by a tissue compartment is assumed to follow Michaelis–Menten kinetics:

$$M_\beta(p) = \frac{M_0^\beta p}{p + p_c}$$

where M_0^β is the maximal consumption rate volume-averaged over tissue compartment β, and P_c is the tissue PO_2 value which reflects the partial pressure scale where fibre mitochondria are no longer able to extract oxygen at maximal rate (Al-Shammari et al., 2014).

Representative histological images were selected from each study groups, for which the C:F was close to the respective group mean value, to estimate EDL muscle oxygenation (an index of functional aerobic capacity). In the final stage of immunohistological analysis, the loaded image was used to define the physical characteristics needed to accurately model oxygen partial pressure distribution, in order to estimate tissue oxygen tension at rest and simulated maximal oxygen consumption using a finite element approach incorporating published values for O_2 demand, solubility, diffusivity etc. (Al-Shammari et al., 2019).

2.11 Data analysis

Data were sorted and collated using a spread sheet (Excel 2010) and analysed using IBM SPSS statistical package v.20. Normality test was performed on the data prior to independent- samples T-Test or one-way ANOVA to compare mean values among the rat groups. Two-way ANOVA was further performed to determine interaction effect of independent variables. Tukey post hoc analyses were further applied to determine pairwise significance. Pearson correlation test was performed to measure strength of association between variables. *P* value ≤ 0.05 was considered as significant. All results were presented in table and graph forms.

2.12 : Pilot study on muscle survey of inbred and wild type rat strains

2.12.1 **Introduction**

This section identifies characteristic of skeletal muscle fibre capillarity and fibre type composition of inbred and wild type rat strains in which difference between strains is discussed within the context of muscle aerobic capacity (capillarity and oxidative fibre composition). Specific rat strains may perform exercise at different intensity/frequency/duration, thus producing a different degree of angiogenesis dependent on the physiological challenge exerted. Therefore, aerobic capacity is assumed to vary across strains, and is determined by initial fibre type content and muscle capillarity.

Different rat strains would have defined muscle phenotype that result in different exercise capacity and response. Previous rat studies have used different animal size or different strain; domestic vs. wild animals (Lyons, Mathieu-Costello, & Moyes, 2006; Odile Mathieu-Costello, 1987; O Mathieu-Costello, Agey, Wu, Hang, & Adair, 1996; O Mathieu-Costello, Ju, Trejo-Morales, & Cui, 2005; Poole & Mathieu-costello, 1996). While looking at aerobic capacity indices in a particular strain, there is a tendency to extrapolate those data to all rodents which is bias.

Baseline fitness level is useful information for any researcher in choosing a suitable rat strain for particular exercise protocols so that adequate responses could be expected within a specified time period. The initial aerobic capacity of a particular strain may determine the degree of muscle adaptation (less aerobic strains are more responsive to physiological challenges) associated with exercise volume or intensity, such that a more aerobic strain requires a greater stimulus for the change (capillarity and/or fibre shift in this context) compared to the least aerobic ones. In addition, there is also little information on comparison of muscle baseline aerobic capacity between inbred and wild type strains that may further characterise similarities and differences in muscle phenotype, particularly for hind limb muscles. Therefore rat strain may play an important factor in determining the degree of skeletal muscle adaptation upon exercise intervention, and adaptation responses are generally muscle- and fibre specific that depends on type of exercise interventions (particular exercise recruits particular muscles and fibre types interest) and initial condition of muscle aerobic capacity.

This information would be beneficial for future studies in choosing an appropriate rat strain and skeletal muscle of defined baseline values and suitable exercise regime so that specific effects could be compared and observed. Exploring different strains of rat (e.g.

Brown and Wistar) is useful to characterise the influence of natural variation in aerobic capacity on relative fibre composition and capillarity of a range of striated muscles: TA, EDL, Sol, heart and diaphragm. Difference in phenotype could be used to assess the relative importance of inherent muscle function in supporting locomotor muscle aerobic capacity.

There is evidence that Brown rats are relatively higher in aerobic capacity strain than Wistar rats (Bauer, 1990; Johnson & Mitchell, 2003). Therefore, this study aimed to determine muscle phenotype and capillary density in a range of lower limb and cardiorespiratory muscles in wild type and inbred rats, respectively. Then we performed oxygen partial pressure modelling to estimate muscle function, and compared oxygen saturation and presence of hypoxia between those two strains, at simulated resting and maximal exercise levels thus determining the influence of level of inherent fitness on muscle functional capacity and capillarity. It was hypothesised that Brown rat hind limb muscles would show higher capillarity and greater oxidative fibre content, and oxygen transport modelling of the former strain would exhibit better oxygen saturation and lower levels of hypoxia, particularly during maximal exercise level, than the latter.

2.12.2 **Methods**

2.12.2.1 **Animals**

Six male rats of Wistar and Brown with 209.0±2.3 g and 208.6±1.7 g (about 6-7 week old), respectively, were sacrificed according to a Schedule 1 method (above). Lower limb muscles (TA, EDL and Sol), and accessory organs (heart and diaphragm) were dissected, weighed and frozen in isopentane cooled in liquid nitrogen. The tissue blocks were cryosectioned at -20°C (10µm thick).

2.12.2.2 **Immunohistochemistry**

The same immunohistochemistry method was used as described previously, however it was adjusted as staining quality varied. The potential crossover binding between secondary antibody used for laminin (anti-rabbit IgG (H+L), CF™ 405M) and anti-mouse SC-71 was investigated. Then, incubation period was increased (to 1½ hours for both Type IIa and Type I secondary antibodies) to increase specificity of antibody-antigen reaction. It was observed that the current technique produced pale green staining and high levels of noise in Type IIa and I fibres, respectively.

Images of TA, EDL and Sol were visualised under fluorescent illumination (Nikon E600) using systematic-random sampling based regions (Figure 2.10) at x20 magnification.

Diaphragm muscle was imaged at 4 regions due to its homogenous composition at x20 magnification, and heart muscle was imaged at 3 regions at x40 magnification.

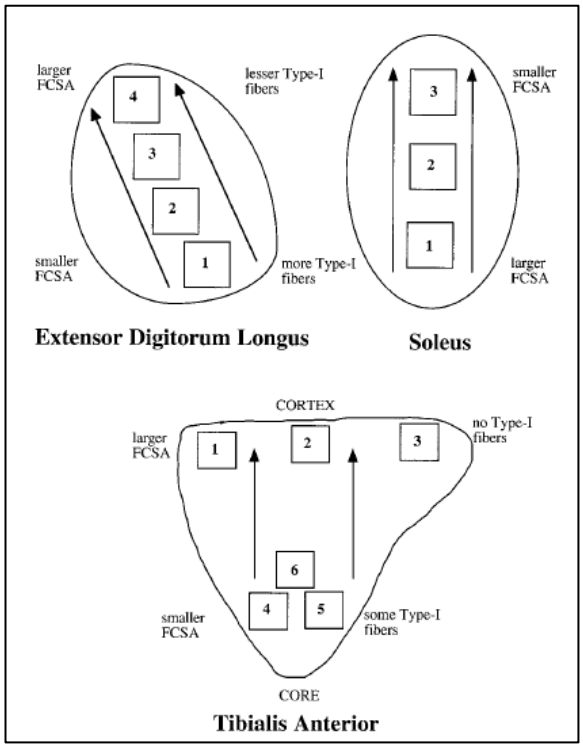

Figure 2.10: Systematic random sampling of TA, EDL and Sol with arrows showing gradient of fibre composition and size (Deveci et al., 2001).

2.12.2.3 **Troubleshooting poor quality staining**

A. Potential antibody cross-reaction

It was suspected that there was inadequate tonal separation to distinguish between the new laminin secondary antibody and Type IIa antibody, leading to pale green staining of Type IIa fibres. This gives potential problems in robust distinguishing between Type IIa and IIb (unstained) fibres. Therefore, two TA sections were stained to determine the extent of cross reaction. The immunohistochemically staining protocols used different antibody mixtures based on the following tables.

Table A (TA 1)

	Antibody mixes
A	Antibody BA-D5 (1:500) and anti-laminin (1:250)
B	Anti-mouse 555 (1:1000) and antibody SC-71 (1:500)
C	Anti-mouse 488 (1:1000)
D	Anti-rabbit IgG (H+L), CF™ 405M (1:250) and *Griffonia simplicifolia* lectin (1:200)

Table B (TA 2)

	Antibody mixes
A	Antibody BA-D5 (1:500) and anti-laminin (1:250)
B	Anti-mouse 555 (1:1000) and antibody SC-71 (1:500)
C	Anti-mouse 488 1:1000), anti-rabbit IgG (H+L), CF™ 405M (1:250) and *Griffonia simplicifolia* lectin (1:200)

B. Determining incubation period during fixation

The previous investigation did not determine the causal factor in pale staining in fast fibres. It was realised that staining quality decreased with increasing number of samples, and that all incubation periods in the protocol exceeded the recommended 2 minutes when longer a time spent during washing step. As fixation duration might change an epitope's conformation, a study was performed comparing different incubation periods based on the standard method used previously. TA and Sol muscle sections were stained and staining quality was assessed.

The same immunohistochemistry protocol was used to stain fibre types in 2 TA and 2 Sol sections which incubated for 4 and 2 (current protocol) minutes during fixation phase. Core regions of TA and Sol were imaged and captured at x20 and x10 magnification.

2.12.2.4 **Myosin ATPase staining (alkaline preincubation)**

For each muscle type, three slides were placed in each labelled staining jar for three different durations, as follows:

Glass jar	pH	Duration (minutes)
1	10.2	10, 15 and 20
2	10.3	10, 15 and 20
3	10.4	10, 15 and 20

Following pre-incubation, slides were rinsed once with distilled water, and ATP solution was added to each staining jar for 15 minutes. Then slides were rinsed 3 times with 1% $CaCl_2$ solution (3 minutes each), and incubated with 2% cobalt chloride ($CoCl_2$) for 10 minutes. The slides were washed five times with 0.005M sodium barbital, followed by five washes with distilled water. 2% ammonium sulphide was added to the jars in the fume hood and left for 30 seconds to develop the dark stain. The slides were then rinsed with five changes of distilled water. The slides were transferred to slide holders, and back side cleaned with tissue. A drop of tissue mounting medium (Fluoromount-G) was placed on middle of sections and covered with a coverslip. Images were captured using a light microscope at X20 magnification.

2.12.2.5 **Global angiogenic indices**

A region of interest (ROI) was set to 75% of total sample area based on an unbiased sampling rule, which included two inclusion (dotted) and exclusion (solid) edges (Figure 2.4). Global indices - capillary to fibre ratio (C:F), capillary density (CD, mm^{-2}), and mean fibre area (MFA, μm^2) - were determined using in-house Dtect and Capillar morphometric analysis programmes (coded in MatLab; (Al-Shammari et al., 2014) .

2.12.2.6 **Fibre type composition**

Fibre type compositions of skeletal and respiratory muscles were determined using Dtect and Capillar programmes which based on the unbiased sampling rule.

2.12.2.7 **Local capillarity**

Local capillarity indices (LCFR and LCD) were determined by placing supply area boundaries mid-way between adjacent capillaries (Voronoi tessellations) to delineate individual regions of capillary supply which known as capillary domain areas (Figure 2.11); (Al-Shammari et al., 2014).

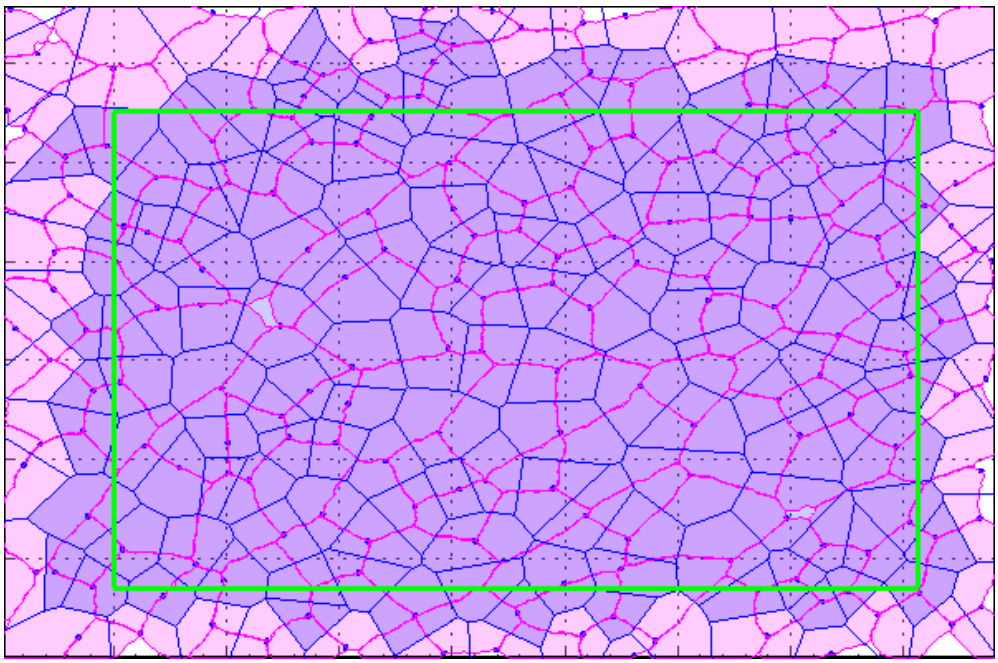

Figure 2.11: Region of interest (green box) of capillary domains (blue lines) and fibre boundaries (pink lines). The purple coloured domains define these capillaries whose boundaries are delineated by adjacent capillaries. Sample taken from Brown rat EDL region 1.

2.12.2.8 **CDA frequency and heterogeneity**

Frequency distribution was determined and displayed as histograms. LogSD of log CDA was determined and compared between the two strains.

2.12.2.9 **Estimated muscle function**

Data were then loaded into an oxygen modelling program (OTM; (Al-Shammari et al., 2019)) to estimate tissue oxygen tension at rest and during simulated maximal oxygen consumption.

2.12.3 **Results**

2.12.3.1 **Rat body and relative muscle mass**

Table 2.4: At similar body mass, TA, EDL, Sol and heart muscle mass of Brown rats were greater than Wistar. *P<0.05 *vs*. Wistar.

	Rat body mass (g) and relative muscle mass (mg)			
Strain/ muscles	Strain	N	Mean±SD	Significance
Strain	Wistar	8	209.1±6.5	
	Brown	8	208.6±4.8	n.s.
TA	Wistar	6	1.70±0.07	
	Brown	6	1.91±0.05	*
EDL	Wistar	8	0.407±0.03	
	Brown	8	0.484±0.03	*
Sol	Wistar	6	0.349±0.02	
	Brown	6	0.502±0.02	*
Heart	Wistar	6	3.01±0.15	
	Brown	6	3.29±0.19	*

2.12.3.2 **Immunohistochemistry**

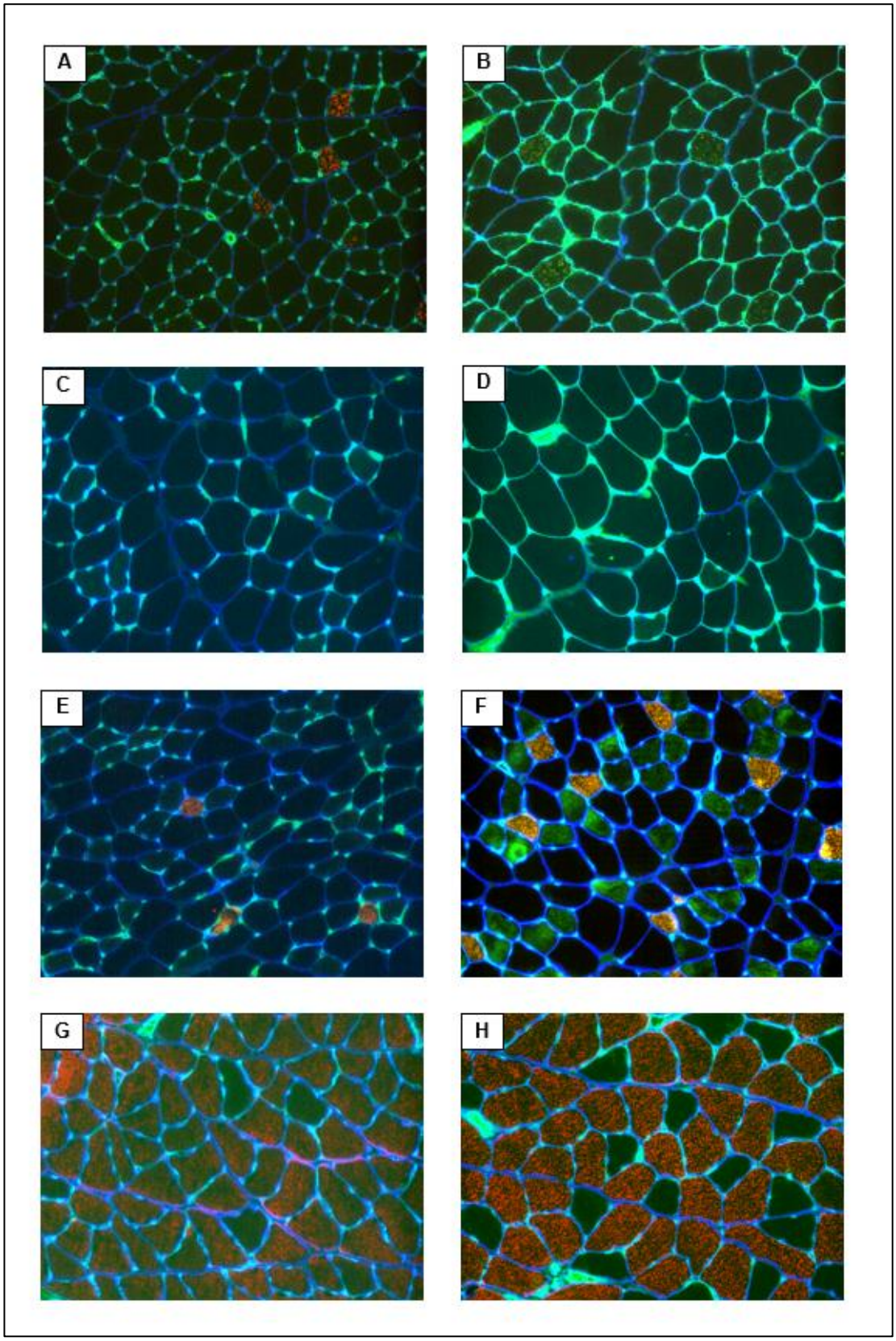

Figure 2.12: Representative immunohistochemically stained sections from Wistar (left panel) and Brown (right panel) rats. Fibre type I and IIa were red and green stained, respectively, with IIb unstained (black). TA core (A and B), TA cortex (C and D), EDL (E and F) and Sol (G and H), images captured at x20 (ABEFG and H) and x16 (C and D).

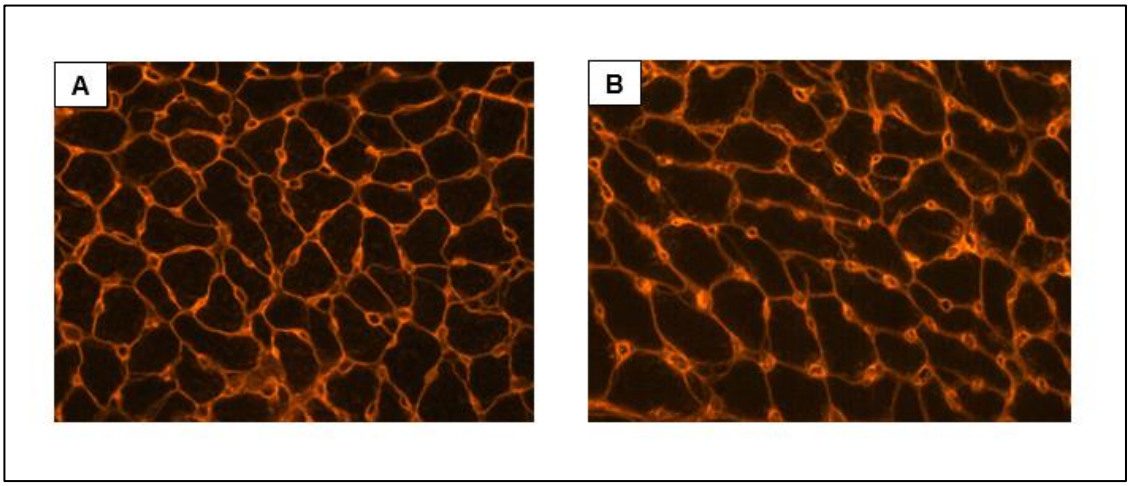

Figure 2.13: Capillary staining of heart muscle; Wistar (A) and Brown (B), image captured at x40.

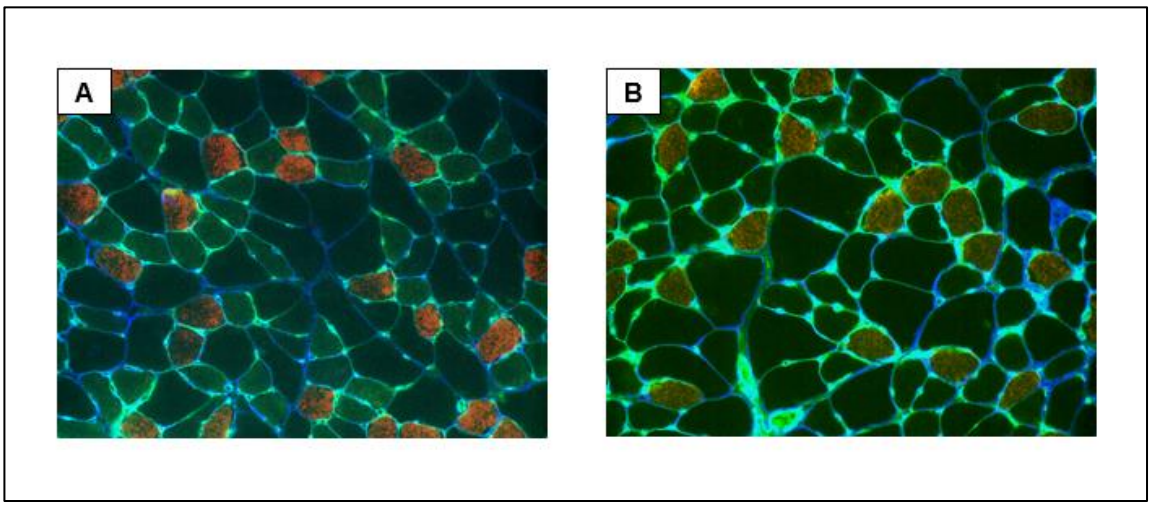

Figure 2.14: Immunohistochemically stained section of diaphragm of Wistar (left panel) and Brown (right panel) rats. Fibre type I and IIa were red and green stained, respectively and type IIb was unstained (black). Images captured at x20.

2.12.3.3 **Staining protocol troubleshooting**

2.12.3.3.1 Potential of antibody cross reaction

Both TA sections which stained based on different protocols produced relatively similar green TIIa staining quality. Presence of pale green stains in both samples indicated no cross reaction existed.

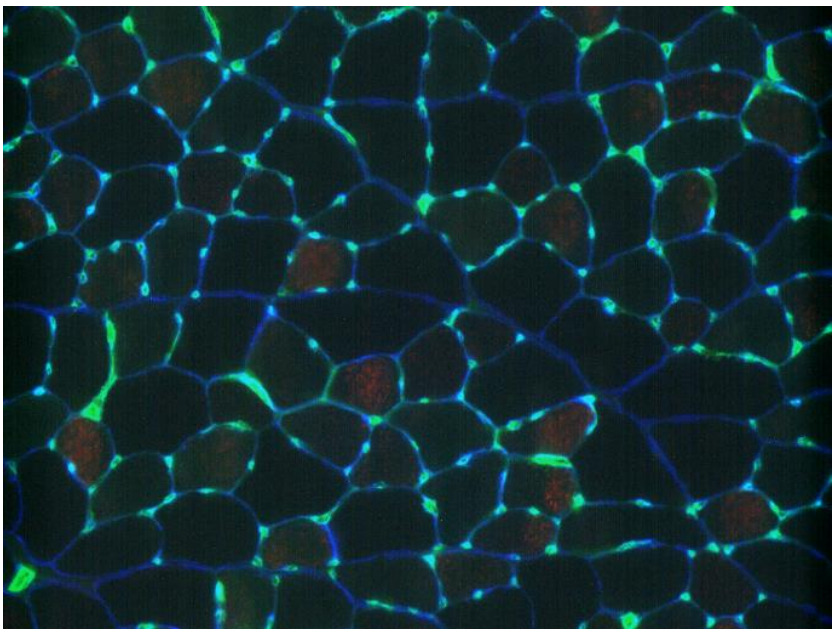

Figure 2.15: Immunohistochemically stained TA fibre types: TIIa (green) and TI (red) (TA 1).

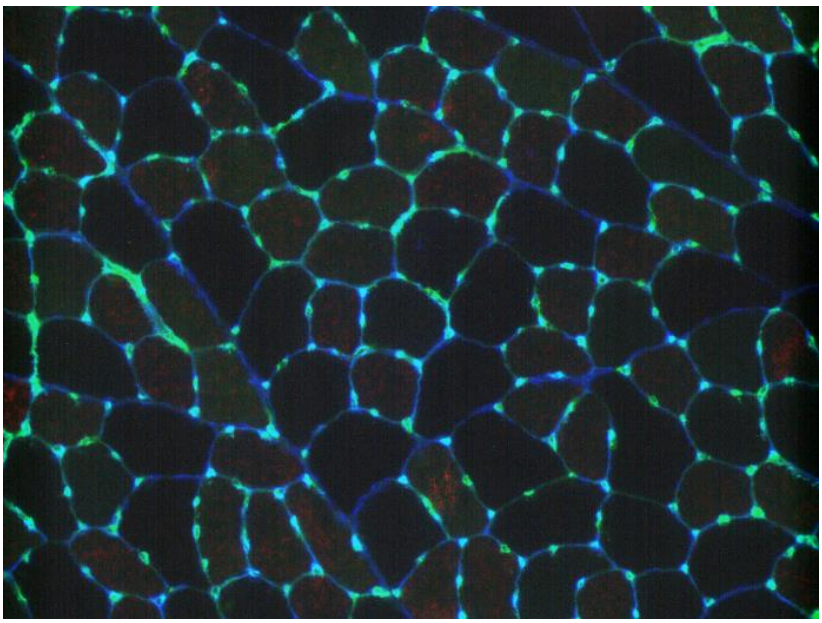

Figure 2.16: Immunohistochemically stained TA fibre types: TIIa (green) and TI (red) (TA 2).

2.12.3.3.2 Fixation incubation period

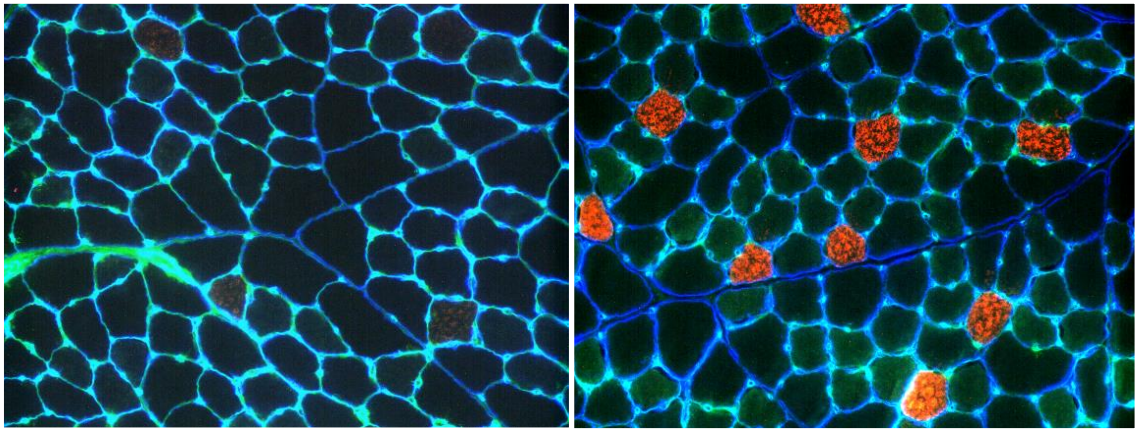

Figure 2.17: Immunohistochemically staining of core region of TA muscle: Type IIa were not strongly stained green, left: 4 minute fixation incubation period, right: 2 minutes period (produced good staining quality with Type IIa and Type I fibres stained green and red, respectively), images captured at x20.

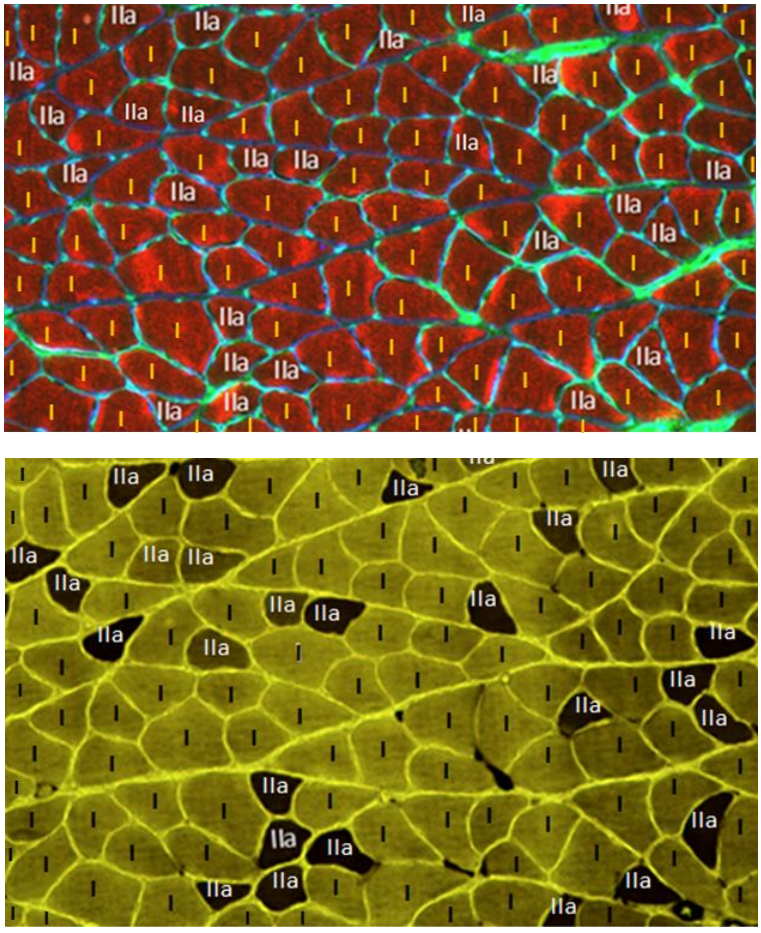

Figure 2.18: Immunohistochemically staining of Sol showed Type I and IIa were stained red (top) and mATPase staining was used to confirm fibre types (bottom) indicated by IIa (Type IIa) and I (Type I), images captured x10.

2.12.3.4 **mATPase staining**

It was observed that myosin ATPase (mATPase) staining on TA muscle sections was able to distinguish three main fibre types at pH 10.3 and 10.4 after 20 minutes pre-incubation while preincubation at pH 10.4 for 20 minutes produced the best quality results (i.e. consistent fibre type differentiation).

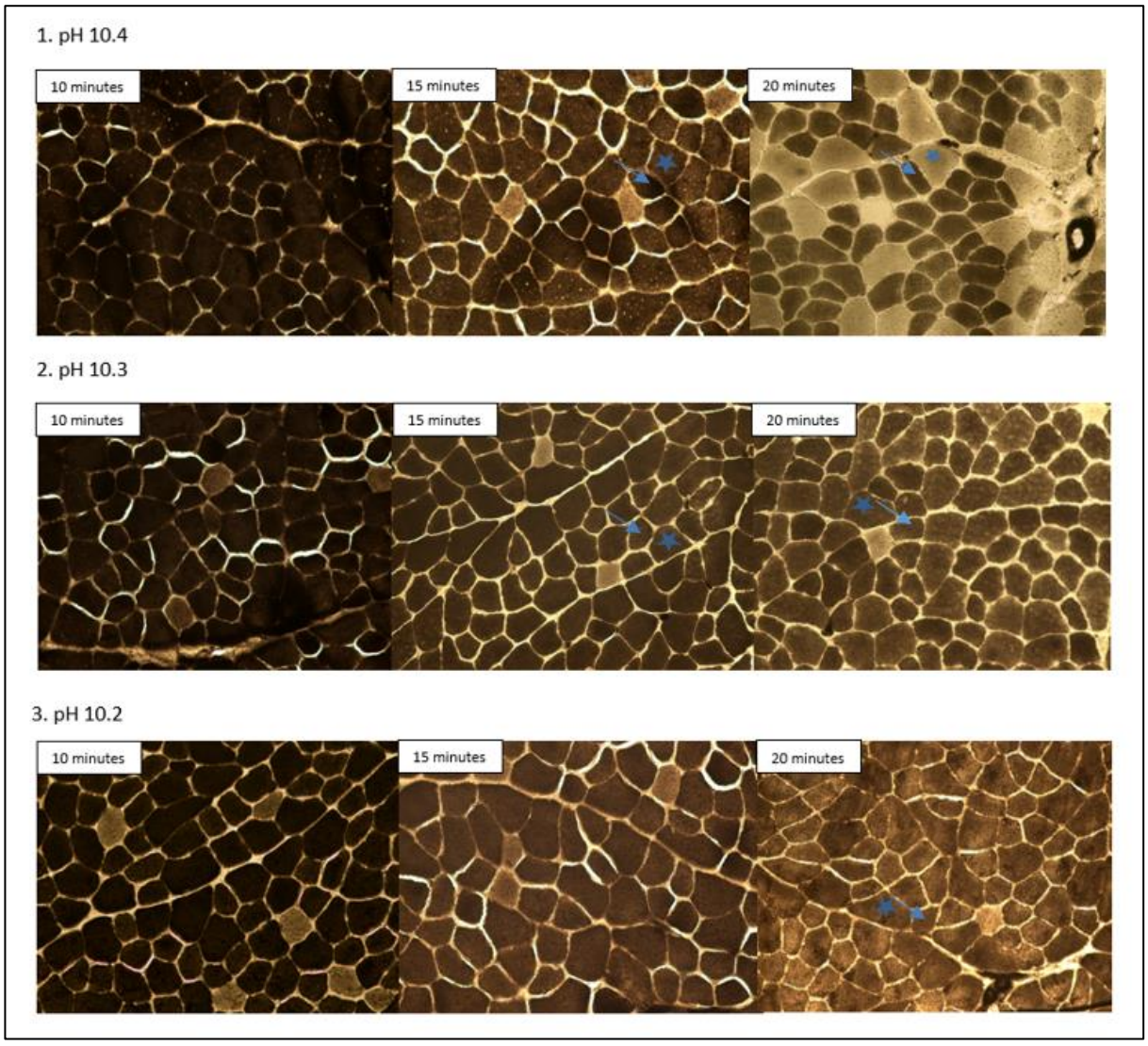

Figure 2.19: Alkaline pre-incubation at different pH and duration of mATPase staining on TA muscle of Wistar rats. Arrows denote Type IIa, stars denotes Type IIb fibres.

2.12.3.5 **Angiogenic indices of lower limb muscles, diaphragm and heart**

Example of data output of morphological analysis of angiogenic indices:

Table 2.5: Data output of morphological analysis of Wistar TA core muscle for determining angiogenic indices and fibre type composition.

Total number of fibres in image	130
Total number of capillaries in image	240
Lamina dimensions 1px	0.17182 um
Image x scale	439.86 um
Image y scale	329.9 um
ROI Area	110024.2 um^2
ROI x scale	385.05 um
ROI y scale	285.74 um
Total number of fibres in ROI	120
Total number of capillaries in ROI	185
Capillary density in ROI area	0.001681
Capillary density in ROI fibre	1.541667
Number of fibres in ROI	
Type I in ROI	6
Type IIa in ROI	53
Type IIb in ROI	61
Numerical fibre ratio in ROI	
Type I in ROI	0.05
Type IIa in ROI	0.442
Type IIb in ROI	0.508
Average Area of fibres in ROI	
Type I in ROI	739 um^2
Type IIa in ROI	759 um^2
Type IIb in ROI	1331 um^2
Average area ratio in ROI	
Type I in ROI	0.035 um^2
Type IIa in ROI	0.32 um^2
Type IIb in ROI	0.645 um^2
Capillary to fibre ratio in ROI	
Type I in ROI	3.833
Type IIa in ROI	4.34
Type IIb in ROI	1
Ideal Mean Fibre Analysis	
Type I in ROI	5
Type IIa in ROI	43
Type IIb in ROI	46
Type I in ROI	715 um^2
Type IIa in ROI	772 um^2
Type IIb in ROI	1363 um^2

Type I in ROI	0.053
Type IIa in ROI	0.457
Type IIb in ROI	0.489
Type I in ROI	3.4
Type IIa in ROI	4.488
Type IIb in ROI	1

Ideal Percentage Fibre Analysis	
Average fibre percentage area ideal percentage	
Type I in ROI	0.032 um^2
Type IIa in ROI	0.257 um^2
Type IIb in ROI	0.399 um^2
Type I in ROI	3.4
Type IIa in ROI	4.568
Type IIb in ROI	1

1. Tibialis anterior (TA)

Table 2.6: C:F, CD and MFA of TA core region of Wistar and Brown rats were not significantly different, N=6 per group.

Angiogenic indices	Strain	Mean±SD	Significance
C:F	Wistar	1.60±0.03	
	Brown	1.59±0.06	n.s.
CD	Wistar	1431±96	
	Brown	1347±39	n.s.
MFA	Wistar	1152±82	
	Brown	1173±57	n.s.

Table 2.7: C:F, CD and MFA of TA cortex region of Wistar and Brown rats were not significantly different, N=6 per group.

Angiogenic indices	Strain	Mean±SD	Significance
C:F	Wistar	0.98±0.06	
	Brown	0.97±0.04	n.s.
CD	Wistar	556±35	
	Brown	531±102	n.s.
MFA	Wistar	1797±111	
	Brown	2038±204	n.s.

2. Extensor digitorum longus (EDL)

Table 2.8: C:F and MFA of EDL of Brown were significantly higher than Wistar rats, N=6 per group, *P*<0.05. CD was similar between the strains.

Angiogenic indices	Strain	Mean±SD	Significance
C:F	Wistar	1.14±0.03	
	Brown	1.29±0.04	*
CD	Wistar	1002±45	
	Brown	984±35	n.s.
MFA	Wistar	1187±52	
	Brown	1356±32	*

3. Soleus (Sol)

Table 2.9: C:F and CD of Sol of Brown were greater than Wistar rats, *P*<0.05. Similar MFA of Sol of those strain showed a true higher capillarity in Brown rats, (N=6 per group).

Angiogenic indices	Strain	Mean±SD	Significance
C:F	Wistar	1.48±0.08	
	Brown	1.81±0.03	*
CD	Wistar	910±50	
	Brown	1098±30	*
MFA	Wistar	1665±96	
	Brown	1683±63	n.s.

4. Diaphragm

Table 2.10: C:F, CD and MFA of diaphragm of Wistar and Brown rats were not significantly different, N=6 per group.

Angiogenic indices	Strain	Mean±SD	Significance
C:F	Wistar	1.47±0.12	
	Brown	1.50±0.05	n.s.
CD	Wistar	1179±42	
	Brown	1325±111	n.s.
MFA	Wistar	1263±131	
	Brown	1196±85	n.s.

5. Heart muscle

Table 2.11: C:F and MFA of left ventricle of Brown rats were significantly higher than Wistar rats, *P*<0.05. CD was greater in Wistar than Brown rats, *P*<0.05. N=6 per group.

Parameters	Rat strain	Mean±SD	Significance
C:F	Wistar	1.25±0.01	
	Brown	1.39±0.02	*
CD	Wistar	3258±62	
	Brown	2858±108	*
MFA	Wistar	385±5	
	Brown	490±21	*

2.12.3.6 **Fibre type composition**

Table 2.12: Numerical and areal density of all fibre types of TA core region of Wistar and Brown rats were similar, *P*>0.05, N=6 per group.

Numerical/ areal density	Rat	Mean±SD	Significance
N_NTIIa	Wistar	0.501±0.03	
	Brown	0.471±0.03	n.s.
N_NTIIb	Wistar	0.453±0.03	
	Brown	0.477±0.03	n.s.
N_NTI	Wistar	0.047±0.01	
	Brown	0.051±0.01	n.s.
A_ATIIa	Wistar	0.406±0.03	
	Brown	0.377±0.03	n.s.
A_ATIIb	Wistar	0.561±0.03	
	Brown	0.584±0.03	n.s.
A_ATI	Wistar	0.033±0.01	
	Brown	0.043±0.01	n.s.

Table 2.13: Numerical and areal density of all fibre types of TA cortex region of Wistar and Brown rats were similar, *P*>0.05, N=6 per group.

Numerical/ areal density	Rat	Mean±SD	Significance
N_NTIIa	Wistar	0.155±0.015	
	Brown	0.203±0.046	n.s.
N_NTIIb	Wistar	0.845±0.015	
	Brown	0.798±0.046	n.s.
A_ATIIa	Wistar	0.088±0.011	
	Brown	0.128±0.030	n.s.
A_ATIIb	Wistar	0.912±0.011	
	Brown	0.872±0.030	n.s.

Table 2.14: Numerical density of TI and TIIb of EDL of Brown rats were higher and lower than Wistar rats with similar density in TIIa fibre. Areal density of TI and TIIa were greater than Wistar strain with lower density in TIIb. * *P*<0.05, N=8 per group.

Numerical/ areal density	Rat	Mean±SD	Significance
N_NTIIa	Wistar	0.214±0.019	n.s.
	Brown	0.264±0.014	
N_NTIIb	Wistar	0.749±0.019	*
	Brown	0.684±0.013	
N_NTI	Wistar	0.05±0.004	*
	Brown	0.064±0.005	
A_ATIIa	Wistar	0.133±0.013	*
	Brown	0.175±0.009	
A_ATIIb	Wistar	0.846±0.013	*
	Brown	0.792±0.007	
A_ATI	Wistar	0.029±0.003	*
	Brown	0.042±0.004	

Table 2.15: Numerical density of TIIa and TI of soleus were higher in Brown and Wistar rats respectively. Areal density of TIIa was greater in Brown with similar degree in TI. *P<0.05 *vs.* Wistar. N=6 per group.

Parameters	Rat strain	Mean±SD	Significance
N_NTIIa	Wistar	0.101±0.019	
	Brown	0.272±0.027	*
N_NTI	Wistar	0.905±0.022	
	Brown	0.728±0.027	*
A_ATIIa	Wistar	0.081±0.014	
	Brown	0.238±0.024	*
A_ATI	Wistar	0.924±0.017	
	Brown	0.762±0.024	n.s.

Table 2.16: Numerical density and areal density of all fibre types of diaphragm were similar in both strains. N=6 per group.

Parameters	Rat strain	Mean±SEM	Significance
N_NTIIa	Wistar	0.385±0.024	
	Brown	0.404±0.024	n.s.
N_NTIIb	Wistar	0.353±0.027	
	Brown	0.325±0.026	n.s.
N_NTI	Wistar	0.262±0.032	
	Brown	0.271±0.017	n.s.
A_ATIIa	Wistar	0.319±0.021	
	Brown	0.313±0.030	n.s.
A_ATIIb	Wistar	0.462±0.042	
	Brown	0.481±0.038	n.s.
A_ATI	Wistar	0.219±0.027	
	Brown	0.206±0.016	n.s.

2.12.3.7 **Local capillarity**

Example of data output of morphological analysis of local angiogenic indices:

Table 2.17: Data output of morphological analysis of Wistar TA core muscle for determining local capillarity.

Number of Capillaries	240
Capillary Density	1653.94/mm^2
X Length Scale	439.86 um
Y Length Scale	329.90 um
Total number of fibres	130
Number of included fibres	85
C:F	2.36
Mean fibres area	
All fibres	1032.25 um^2
Type I	733.44 um^2
Type IIA	762.82 um^2
Type IIB	1339.76 um^2
Fibre numerical density	
Type I	0.07
Type IIA	0.46
Type IIB	0.47
Fibre areal density	
Type I	0.05
Type IIA	0.34
Type IIB	0.61
NCAF	
All fibres	5.94
Type I	4.83
Type IIA	5.41
Type IIB	6.63
NCAF/fibre perimeter	
All fibres	15.35/mm
Type I	15.42/mm
Type IIA	15.56/mm
Type IIB	15.14/mm
LCFR	
All fibres	
Type I	1.31
Type IIA	1.42
Type IIB	2.11
LCD	
All fibres	

Type I	1731.58/mm^2
Type IIA	1873.65/mm^2
Type IIB	1608.31/mm^2
Number of Voronoi Cells	240
Included Voronoi Cells	167
Mean[Voronoi Cell Area]	596.57 um^2
STD[Voronoi Cell Area]	218.18 um^2
SEM[Voronoi Cell Area]	16.88 um^2
LogSD[Voronoi Cell Area]	0.14
Mean[Domain Eq. Diameter]	27.16 um
STD[Domain Eq. Diameter]	4.67 um
SEM[Domain Eq. Diameter]	0.36 um
LogSD[Domain Eq. Diameter]	0.07
Mean[Mean NN Distance]	26.98 um
STD[Mean NN Distance]	4.79 um
SEM[Mean NN Distance]	0.37 um
Mean[STD NN Distance]	8.22 um
Mean[SEM NN Distance]	3.40 um

Domain Areas (um^2)	Min NN (um)	Unique Min NN (um)	Mean NN (um)	Rand NN (um)	FDR
393.19	14.94	14.94	20.37	14.94	3
452.76	18.06	18.06	24.34	18.06	3
500.61	16.11	16.11	24.29	27.29	3
864.92	22.26	22.26	33.18	37.98	3
574.83	19.89	22.26	26.75	31.31	3
457.24	9.69	9.69	23.17	37.98	3
367.12	9.69	14.34	23.3	14.17	3
763.11	19.44	19.44	30.45	27.22	4
625.68	14.21	14.21	27.16	28.72	2
1285.66	14.21	14.21	40.1	26.86	5
426.91	13.59	13.59	23.58	19.4	2
403.38	14.34	16.5	20.29	19.4	4
502.21	12.95	16.25	25.84	35.88	3
572.92	12.95	12.95	29.24	24.07	2
279.05	12.22	15.99	14.59	14.22	3
482.31	17.32	22.12	25.55	22.12	2
474.77	14.22	14.22	21.93	14.22	3
721.49	17.13	18.39	33.57	43	2
460.14	14.34	14.34	26.39	28.72	2
569.25	15	31.05	27.34	27.29	3
775.73	18.39	14.22	31.08	22.98	4
385.09	12.77	18.1	23.4	31.31	3
487.5	12.77	31.05	24.77	22.98	3
732.09	19.86	24.61	30.36	28.07	3
746.26	17.2	24.94	32.92	41	3
217.85	7.46	7.46	15.43	22.05	3

1032.36	25.15	25.15	36.37	47.96	3
774.53	20.29	20.29	29.41	24.05	3
413.42	14.58	20.14	22.36	28.29	3
641.67	14.91	31.05	27.91	28.29	2
884.48	21.22	21.22	33.8	21.22	4
585.32	21.15	21.22	25.67	21.22	3
981.31	24.94	24.94	34.83	34.66	4
706.27	18.1	33.43	27.64	41	3
622.72	15.77	16.47	28.08	14.22	2
378.91	15.77	16.84	22.15	69	3
669.75	16.5	70	27.4	16.5	3
579.57	16.5	16.5	25.22	16.5	3
542.84	15.58	15.58	27.29	72	3
468.31	16.84	16.84	23.55	37.98	3
419.71	15.99	15.99	22.3	16.27	2
351.58	8.08	15.99	20.69	28.07	3
320.6	8.08	24.61	17.3	16.63	3
505.9	14.88	13.47	28.34	27.5	3
589.55	16.25	16.25	27.82	16.25	3
459.36	14.02	14.02	25.44	14.02	2
707.42	14.02	14.02	31.33	14.02	3
549.51	16.25	19.95	25.86	16.25	3
574.7	19.95	15.68	28.19	27.5	3
557.38	18.03	18.03	26.64	35.87	3
664.75	18.03	18.03	28.56	26.76	3
387.39	15.68	15.68	22.85	15.68	3
350.1	12.77	12.77	19.47	15.68	4
754.27	12.77	12.77	33.05	44.17	3
673.21	18.39	18.39	29.6	26.76	4
525.46	14.17	18.1	25.46	14.17	2
597.78	21.87	21.99	25.54	21.99	3
419.94	16.5	16.72	23.35	30.4	2
665.25	20.66	21.08	28.8	44.17	3
713.72	20.17	14.62	29.69	20.17	3
670.21	18.6	21.08	32.26	43.24	4
600.85	7.91	7.91	25.05	32.64	3
795.58	13.47	13.47	34.5	38.92	3
436.99	13.47	13.47	25.83	27.82	2
424.75	14.88	103	21.93	27.1	4
464.26	16.47	16.47	25.04	21.3	3
500.69	15.58	16.36	26.06	21.3	3
647.14	18.97	14.02	28.59	47.02	3
397.6	18.78	17.28	21.1	28.29	3
409.99	14.95	16.47	25.81	18.78	2
357.45	16.72	16.72	18.74	22.31	4
493.11	14.95	20.97	24.4	30.4	4
410.62	16.25	16.25	23.12	16.25	2

401.59	14.62	28.09	23.63	29.7	2
371.31	14.62	14.62	18.64	16.27	4
357.23	16.25	16.25	20.35	16.25	3
478.08	14.94	14.94	26.15	37.28	3
527.88	16.47	16.47	26.04	14.02	3
402.66	17.04	17.04	22.05	117	2
533.07	16.47	16.47	25.43	21.99	2
315.36	7.72	21.08	18.45	27.82	3
272.4	7.72	17.04	17.47	17.04	4
1097.4	18.73	18.73	31.79	18.73	4
554.49	15.73	15.73	27.59	37.28	2
438.28	14.73	15.73	25.92	23.15	3
489.65	14.98	14.98	24.85	29.7	3
871.22	23.73	29.35	35.37	47.02	2
903.01	16.58	16.58	34.78	43.85	5
532.55	16.58	16.58	24.82	21.3	2
642.91	19.32	29.35	28.96	128	4
866.91	20.11	22.26	33.53	28.72	2
955.14	27.72	24.61	35.7	47.96	3
1820.06	21.01	21.01	38.36	31.72	3
781.04	21.01	21.01	32	15.23	3
406.19	15.23	15.23	20.5	15.23	4
484.19	16.36	16.36	26.46	31.72	2
392.12	16.36	16.36	22.29	17.8	3
560.88	14.86	14.86	27.23	14.86	3
466.56	13.3	33.43	24.85	33.43	3
457.76	17.97	17.97	23.93	32.82	3
508.65	18.53	33.43	26.58	33.43	3
486.93	10.5	10.5	27.56	10.5	3
373.12	10.5	15.09	20.57	10.5	2
680.69	24.35	27.72	27.65	30.93	4
660.35	15.94	15.94	26.34	36.53	4
555.12	15.94	14.21	24.99	15.94	3
1223.64	29.35	57.83	39.55	57.83	4
1163.21	23.41	37.11	40.54	37.41	3
943.06	20	20	34.62	36.53	3
543.7	19.76	20	26.99	32.87	3
690.24	21.16	23.56	27.73	17.04	3
891.62	22.41	23.56	33.63	19.4	4
934.69	22.41	40.3	34.76	27.1	5
558.12	14.95	14.95	25.52	21.36	4
912.35	24.17	40.3	31.12	24.05	4
741.66	25.71	25.71	28.62	28.08	2
751.65	13.25	167	31.38	31.39	2
748.84	13.53	13.53	33.48	168	3
536.14	21.37	21.37	24.36	21.37	5
540.21	16.84	28.4	26.82	32.74	3

567.8	15.19	15.19	26.95	27.54	3
426.77	15.19	15.19	21.01	30.93	3
660.31	22.07	28.4	27.04	32.63	3
590.65	16.36	56.83	33.29	56.83	3
375.94	12.92	12.92	22.58	14.84	2
461.93	15.23	15.23	23.59	31.27	2
301.18	13.3	14.94	18.35	14.84	3
672.81	19.21	19.21	31.91	35.87	2
577.97	15.78	15.78	31.07	43.44	3
638.38	19.21	19.21	28.41	43.44	3
423.13	14.05	17.99	25.71	17.99	3
568.78	14.05	184	30.78	184	2
564.74	19.51	19.51	25.16	25.44	4
615.55	17.73	186	28.98	15.68	2
592.15	17.99	17.99	25.69	17.99	3
729.8	17.73	31.97	29.88	28.07	3
1252.64	15.74	15.74	28.84	194	2
496.18	13.27	196	26.91	15.09	3
326.47	12.92	40.3	21.52	13.99	3
474.47	13.27	13.27	22.64	22.1	4
517.62	20.06	23.56	24.67	21.37	2
650.59	22.1	25.3	27.83	22.1	2
589.47	22.69	25.3	26.87	32.63	2
753.92	22.5	22.5	28.11	31.76	2
649.34	20.06	23.02	26.93	32.74	2
401.68	15.06	23.02	21.2	24.54	3
447.64	16.84	16.84	23.82	28.58	4
776.24	16.84	16.84	32.27	24.54	3
562.75	13.25	12.95	28.95	16.5	4
542.06	15.98	15.98	26.5	16.63	2
915.53	18.79	9.69	33.53	25.44	4
801.92	18.32	18.32	32.56	24.24	4
510.34	15.98	15.98	24.82	28.58	4
701.98	6.68	25.77	29.95	31.39	4
359.68	14.91	14.91	22.64	34.66	3
735.48	14.98	14.98	30.68	31.76	4
708.04	18.01	18.73	29.79	18.73	3
489.43	15.09	15.09	24.65	15.09	2
830.95	16.36	16.36	31.12	32.82	3
572.02	20.11	20.29	27.09	23.15	4
482.69	14.86	14.86	22.29	14.86	3
412.64	11.04	11.04	23.68	17.8	3
442.37	11.04	11.04	23.14	27.5	3
518.67	13.99	42.4	30.96	22.31	3
509.73	13.53	22.5	26.6	32.87	3
573.65	17.28	17.28	26.88	22.98	3
829.38	20.62	56.83	34.82	56.83	3

Table 2.18: LCFR and LCD of skeletal muscles of Wistar and Brown rats. LCFR was significantly higher in EDL and Sol muscles of Brown rats. *P<0.05 *vs.* Wistar N=6 per group.

Muscle	Parameter	Strain	Mean±SD	Significance
TA core	LCFR	Wistar	1.77±0.06	
		Brown	1.89±0.08	n.s.
	LCD	Wistar	1501±94	
		Brown	1343±56	n.s.
TA cortex	LCFR	Wistar	1.06±0.04	
		Brown	1.11±0.04	n.s.
	LCD	Wistar	651±43	
		Brown	649±96	n.s.
EDL	LCFR	Wistar	1.08±0.03	
		Brown	1.39±0.04	*
	LCD	Wistar	1175±71	
		Brown	1144±50	n.s.
Sol	LCFR	Wistar	1.50±0.16	
		Brown	2.17±0.05	*
	LCD	Wistar	944±47	
		Brown	1056±26	n.s.
Diaphragm	LCFR	Wistar	1.75±0.37	
		Brown	1.76±0.04	n.s
	LCD	Wistar	1223±86	
		Brown	1330±98	n.s

2.12.3.8 **Frequency of capillary domain area**

1. TA core

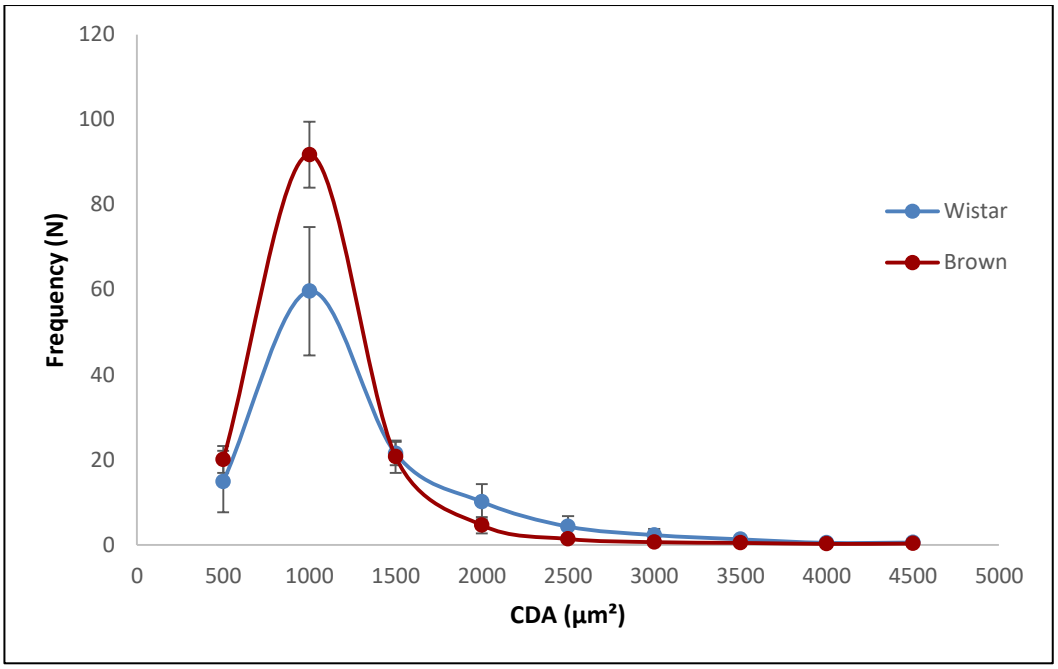

Figure 2.20: Frequency of capillary domain area of TA core of Brown rat was similar to Wistar rat, *P*>0.05.

2. TA cortex

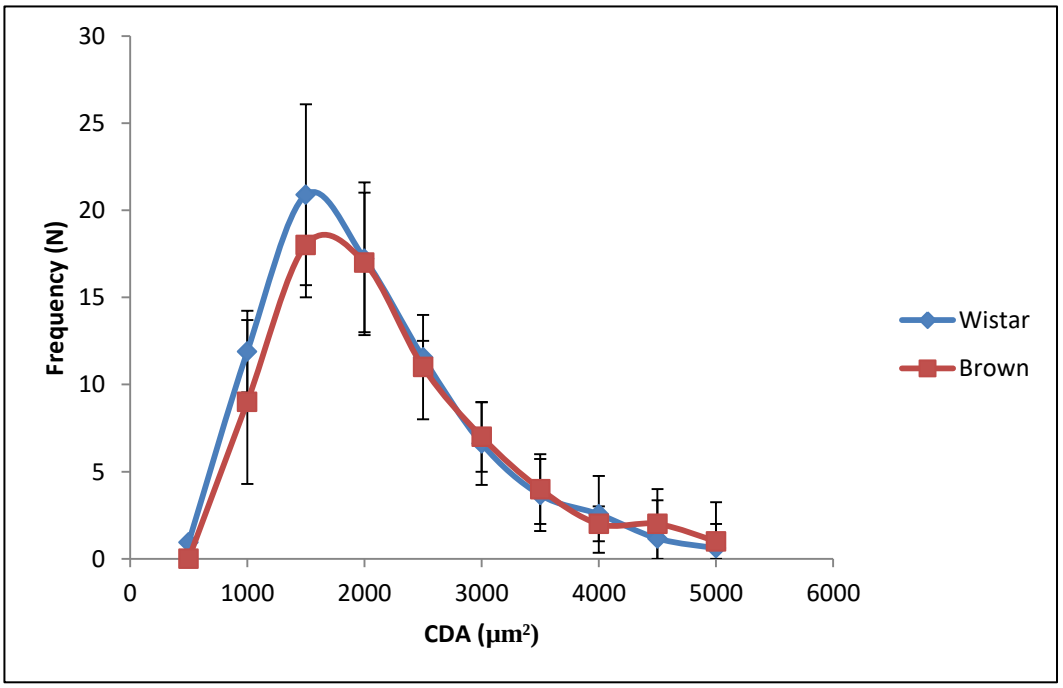

Figure 2.21: Frequency of capillary domain area of TA cortex of Brown rat was similar to Wistar rat, *P*>0.05.

3. EDL

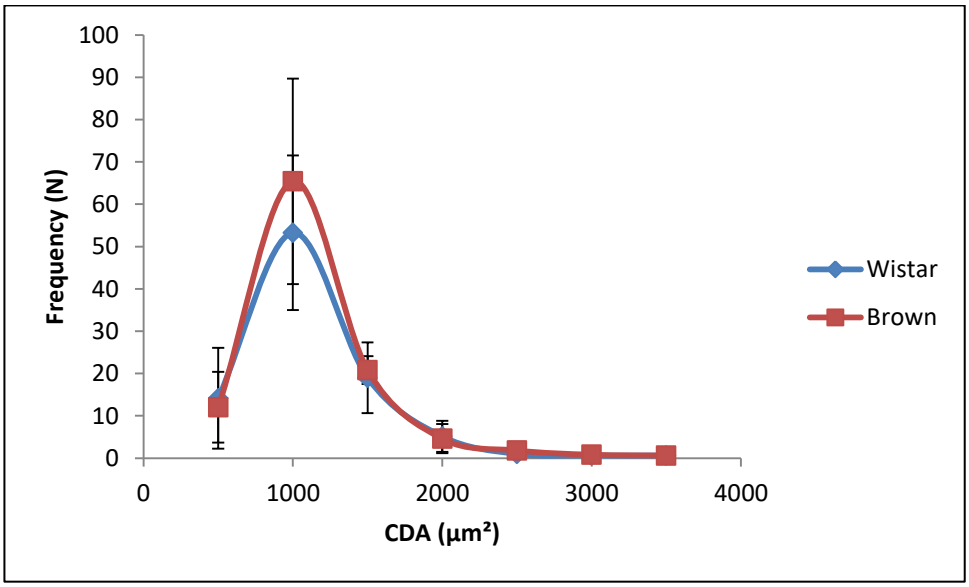

Figure 2.22: Frequency of capillary domain area of EDL of Brown rat was similar to Wistar rat, *P*>0.05.

4. Sol

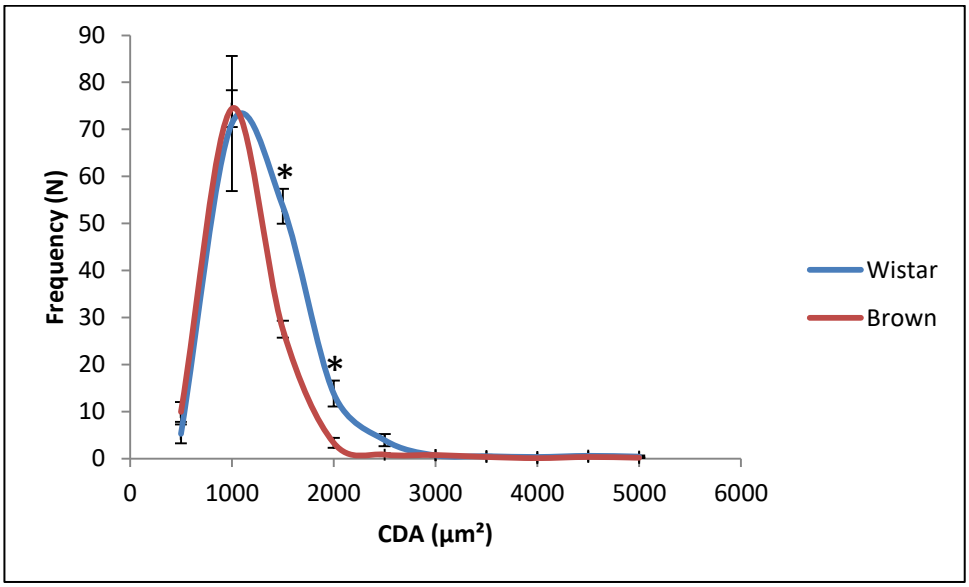

Figure 2.23: Frequency of capillary domain area of Sol of Brown rat was lower in class of 1500 and 2000 µm^2 compared to Wistar rat, **P*<0.05.

5. Diaphragm

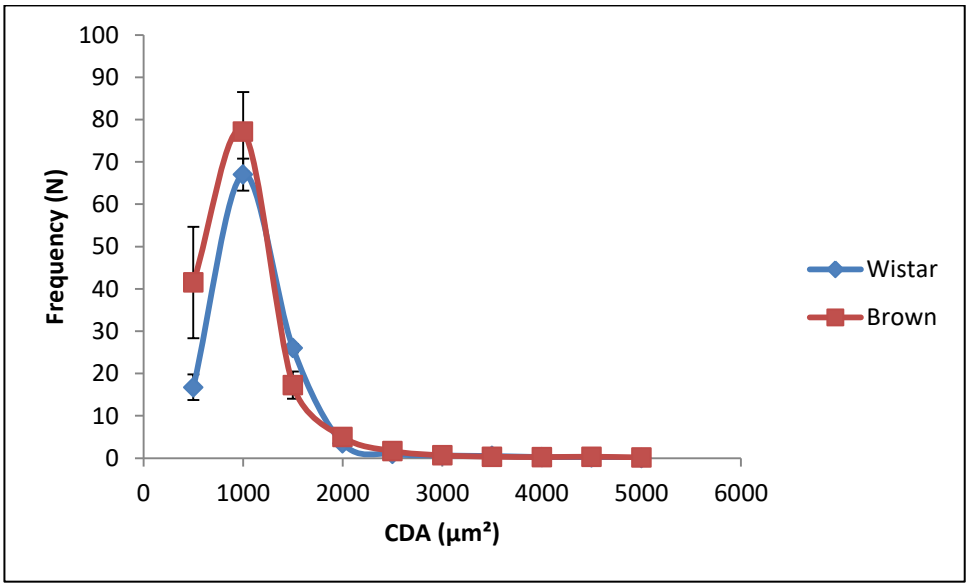

Figure 2.24: Frequency of capillary domain area of diaphragm of Brown rat was similar to Wistar rat for all classes. *P*>0.05.

2.12.3.9 **Heterogeneity of capillary distribution**

Table 2.19: Distribution of capillary in TA, EDL, Sol and diaphragm muscles. Capillary spacing was similar between those two strains in all skeletal muscles but it was more homogenous in TA cortex of Brown rats.**P*<0.05.

Muscle	Strain	N	SD log (Mean±SD)	Significance
TA core	Wistar	6	0.17±0.01	
	Brown	6	0.16±0.01	0.198
TA cortex	Wistar	6	0.19±0.01	
	Brown	6	0.15±0.02	*0.001
EDL	Wistar	8	0.19±0.02	
	Brown	5	0.19±0.03	0.681
Sol	Wistar	6	0.16±0.01	
	Brown	6	0.16±0.02	0.879
Dia	Wistar	5	0.18±0.02	
	Brown	6	0.19±0.01	0.843

2.12.3.10 Fibre specific local capillary to fibre ratio (LCFR)

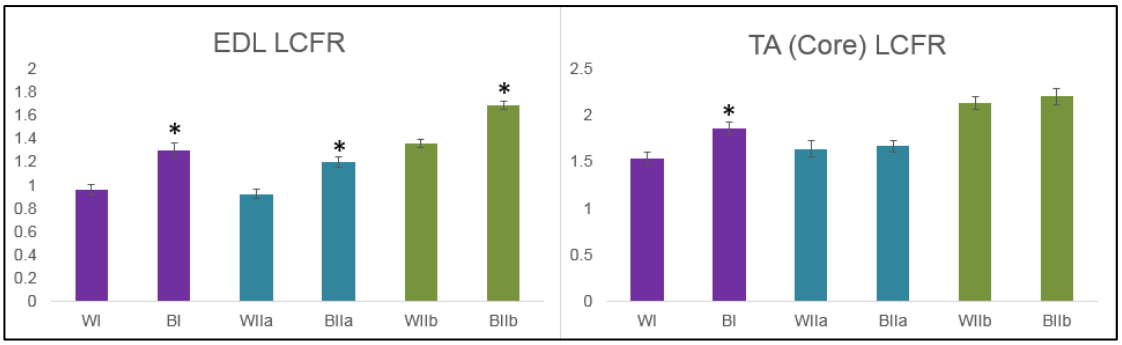

Figure 2.25: LCFR of fibre types in EDL and TA Core (W=Wistar, B=Brown). Fibre type I, IIa and IIb of Brown EDL, and fibre type TI of Brown TA core were higher in capillary supply than Wistar rats.* *P*<0.05, N= 8 (EDL) and 6 (TA core).

2.12.3.11 Estimated muscle function

2.12.3.11.1 Estimated oxygen partial pressure (PO_2) and relative area of muscle hypoxia

Table 2.20: Estimated PO_2 and percentage hypoxia at resting state of skeletal muscles of Wistar and Brown strains.

Muscles	Strains	PO_2 (mmHg)	% Hypoxia
TA core	Wistar	28.7±7.9	0
	Brown	29.1±4.3	0
TA cortex	Wistar	25.7±1.7	0
	Brown	23.6±2.2	0
EDL	Wistar	28.0±2.5	0
	Brown	29.0±1.9	0
Sol	Wistar	29.1±2.7	0
	Brown	28.9±3.1	0
Dia	Wistar	28.7+5.6	0
	Brown	29.1+3.7	0

Table 2.21: PO_2 and percentage hypoxia at exercise level. TA cortex and EDL muscles of Wistar showed relatively higher extent of hypoxia than Brown strain.

Muscles	Strains	PO_2 (mmHg)	% Hypoxia
TA core	Wistar	24.1±4.0	0
	Brown	26.2±2.1	0
TA cortex	Wistar	12.3±6.3	3.66
	Brown	14.2±5.7	1.10
EDL	Wistar	21.3±1.6	1.23
	Brown	25.6+2.2	0
Sol	Wistar	23.8+2.3	0
	Brown	25.4+1.6	0
Dia	Wistar	24.4+2.8	0
	Brown	26.4+1.8	0

2.12.3.11.2 Oxygen transport modelling at resting state and maximal exercise level

1. EDL

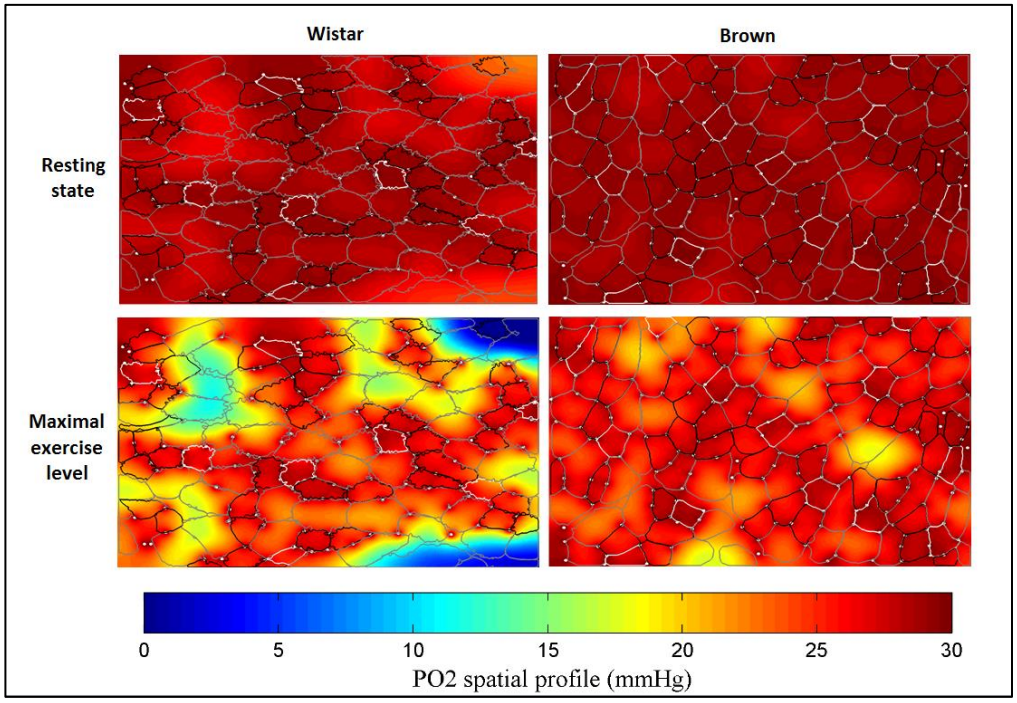

Figure 2.26: Estimated response of EDL through mathematical modelling of muscle O_2 partial pressure, with O_2 consumption modelled at rest and during maximal exercise level. Red areas represent well oxygenated regions, blue areas those regions where PO_2 may be sufficiently low to impair oxidative metabolism and hence impact muscle endurance.

2. TA Core

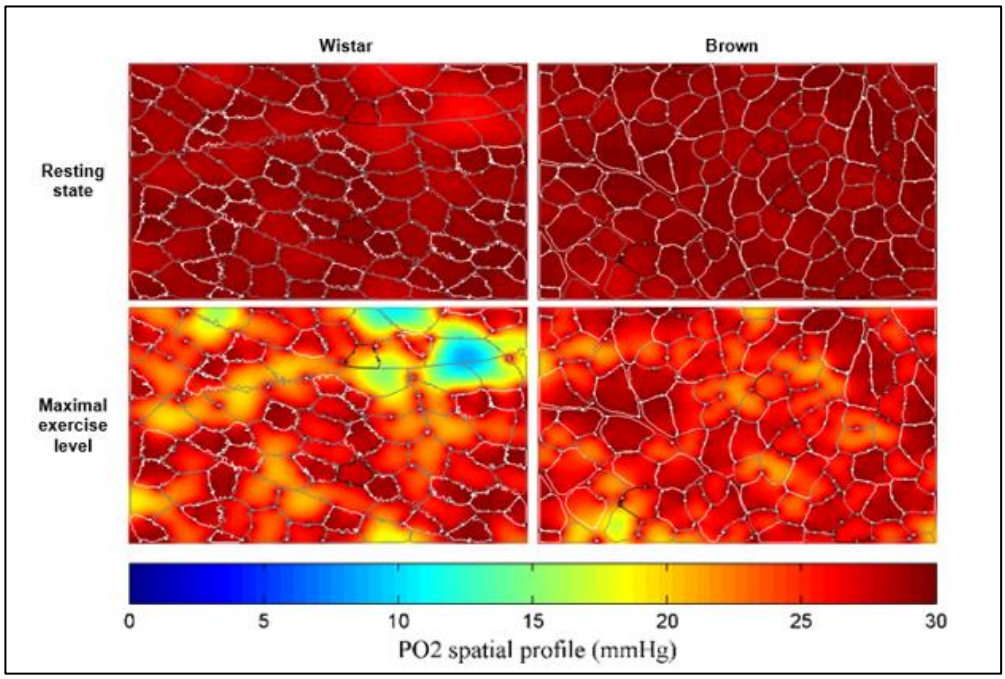

Figure 2.27: Legend as per figure 2.26 for TA core.

3. TA Cortex

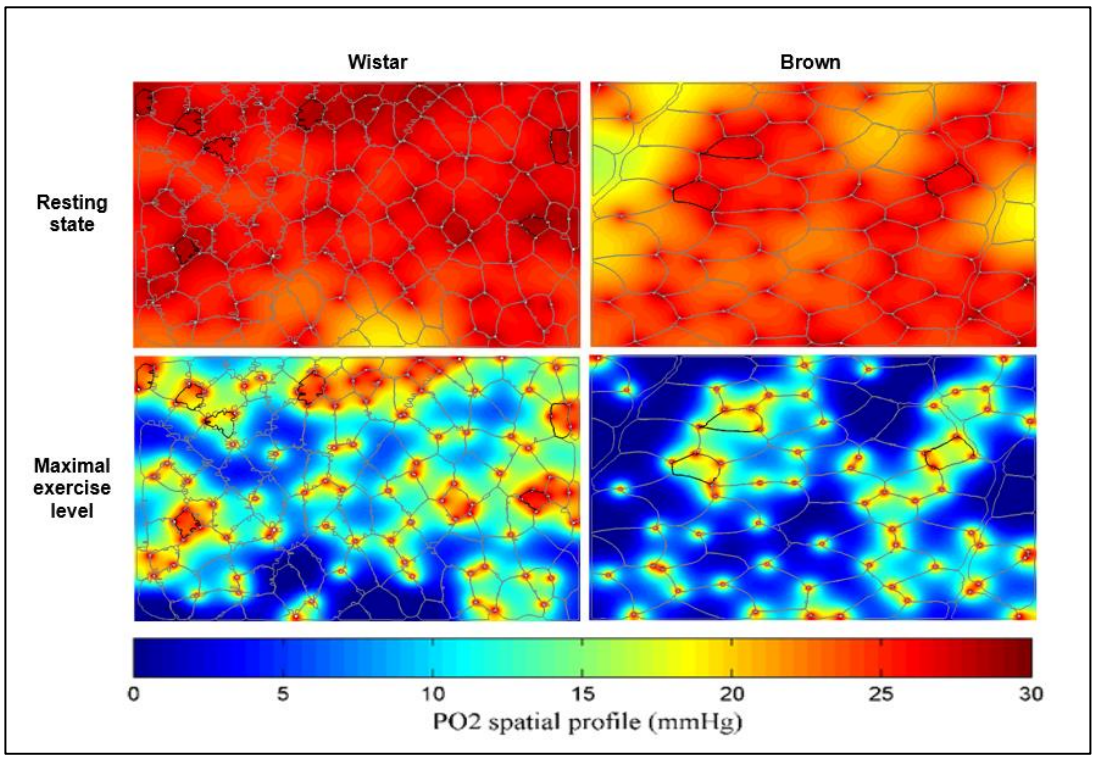

Figure 2.28: As per figure 2.26 for TA cortex.

4. Soleus

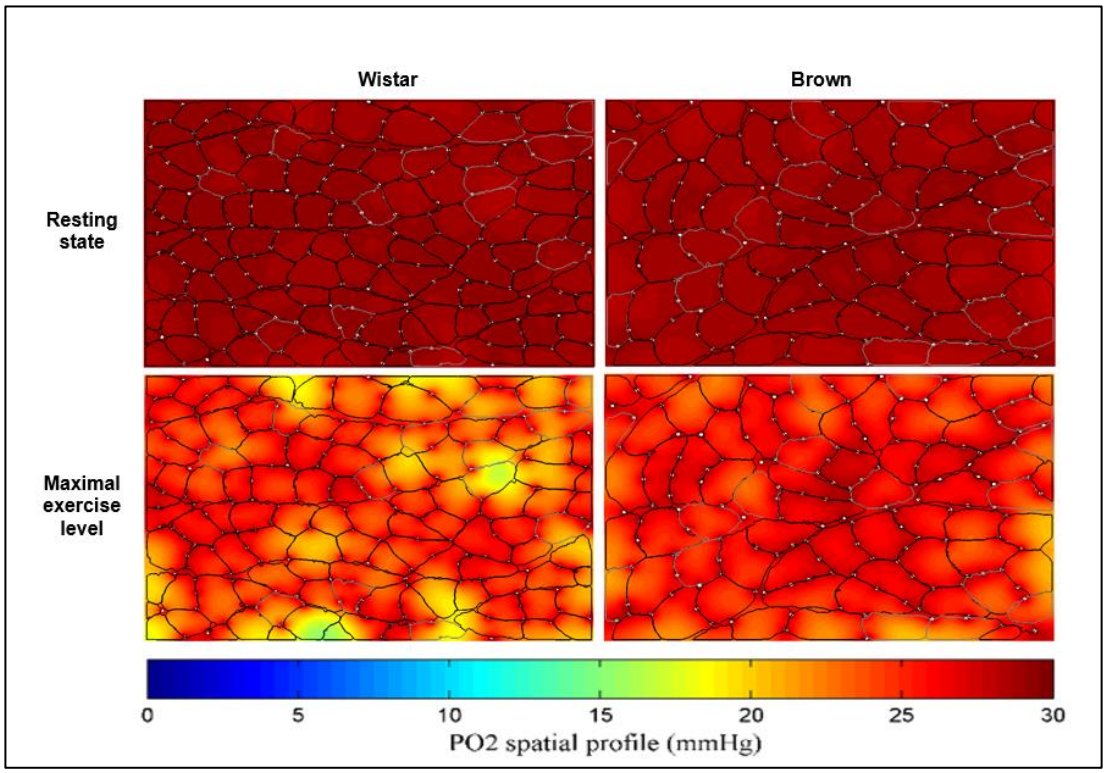

Figure 2.29: As per figure 2.26 for Soleus.

5. Diaphragm

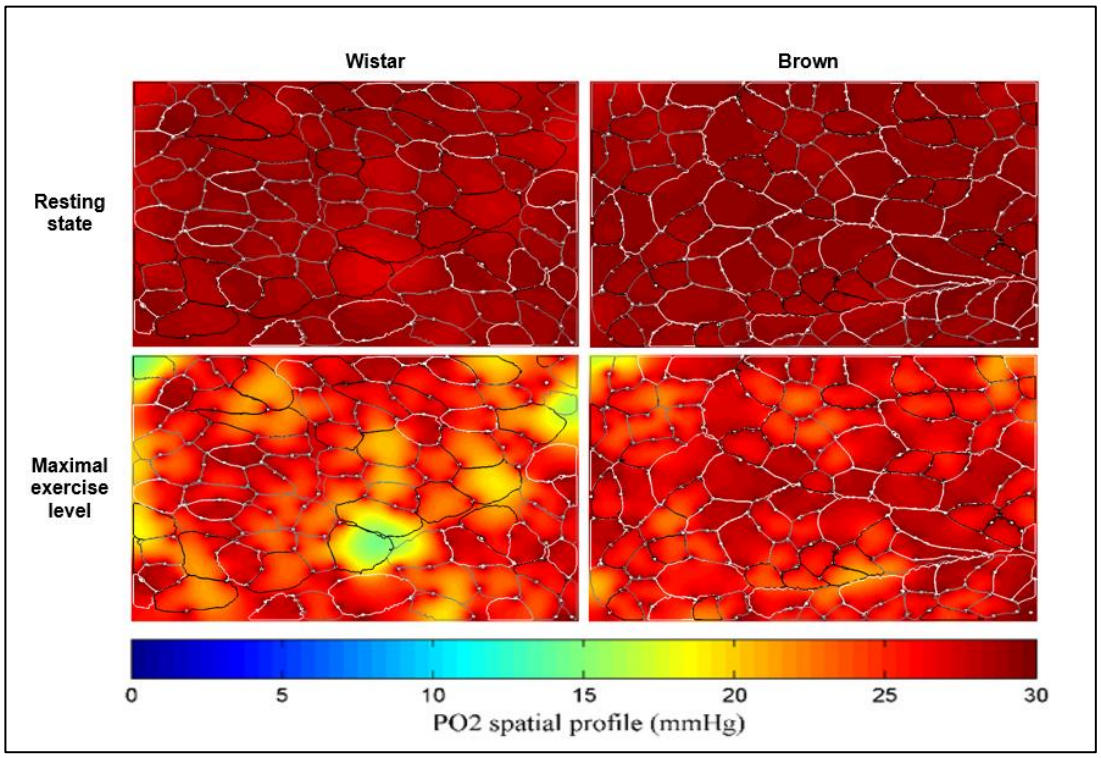

Figure 2.30: As per figure 2.26 for diaphragm.

2.12.4 **Discussion**

2.12.4.1 **Muscle survey**

mATPase staining at pH 10.4 (alkaline preincubation) for 20 minutes on TA sections was optimal to differentiate fibre types (Figure 2.19), and was performed on serial muscle sections to confirm fibre types in TA and Sol muscles thus reducing error in analysis. This was crucial step for determining fibre specific capillarity (global and regional) and fibre composition in the hind limb muscles.

The TA of Brown and Wistar strains showed a potentially similar capacity for performing sustained muscle activity as their global capillarity (C:F and CD) were not significantly different. Even though both have similar fibre area, Brown rat muscle mass was higher, so likely able to contribute more vigorous muscle contractions. Region and fibre specific LCFR indices showed that Brown rat Type I fibres in the TA core had a higher local capillary supply than in Wistar rats, potentially supporting sustained activity of these fibres.

Global angiogenic indices demonstrated that EDL of Brown rats had a greater aerobic capacity in this fast twitch muscle than Wistar rats, e.g. higher C:F, again likely able to support endurance activity even though this was associated with larger fibres. Gross LCFR of Brown EDL was greater than that in Wistars, indicating a better oxygen supply by capillaries was not just an averaged response, but evident at the individual fibre level. Soleus muscle of Brown strain was also more aerobic than the Wistar strain, as C:F and CD were higher with similar fibre size. Similarly, local capillarity of Brown rat Sol was also higher, showing better oxygenation than Wistars, further supporting the notion that the improved peripheral oxygen transport was finely tuned at a local level.

The greater relative muscle mass of Brown rat skeletal muscles and higher numerical and areal densities of Type IIa fibres (in Sol) were indicative of their higher capacity to produce more forceful and/or sustained muscle contractions, potentially contributing to increased running speed. Greater oxidative fibre composition in Brown rat EDL was similarly indicative of a higher oxygen demand to sustain an extended running activity, potentially improving endurance. However with a higher Type I fibre proportion, more exercise load may be needed to initiate a Type IIa fibre shift to Type I compared to that of Wistar strain EDL (Armstron et al., 1972; Demirel et al., 1999; Saltin et al., 1977).

Oxygen tension modelling was used to estimate muscle response to functional demand based on PO_2 distribution at simulated resting and maximal exercise levels. It was observed that at maximal exercising state the more aerobic fast muscles of Brown strain (TA cortex and EDL) demonstrated reduced hypoxic regions (Table 2.19), represented by less yellow and blue colour shading (Figure 2.26 and 2.28). Again, this is consistent with Brown rats displaying greater fatigue resistance than Wistar rats.

C:F of Brown rat cardiac muscle was higher than in Wistar, also having greater relative muscle mass to support sustained and forceful cardiac muscle contractions (and presumably cardiac output). This is consistent with a holistic improvement in aerobic capacity, with peripheral demand being supported by central supply. However, the Wistar rat diaphragm showed similar characteristic in terms of capillarisation and fibre types composition as observed in Brown rats, suggesting some decoupling of cardiorespiratory capacity.

In conclusion, the present data indicate that fast muscles of the Brown strain are phenotypically different, being relatively more aerobic than those of the Wistar strain. The aerobic capacity is muscle and region specific, potentially increasing exercise tolerance and facilitating a positive response to exercise intervention in both health and disease.

2.12.4.2 **Poor immunohistochemically stained images**

Images of stained sections from the muscle survey occasionally showed indistinguishable fibre types e.g. Type I and Type IIa fibres were sometimes difficult to differentiate in soleus, while Type IIa and IIb were poorly separable in cortex region of TA. Even though the incubation period for primary and secondary antibody for Type IIa was increased, difficulty in fibre type differentiation persisted thus optimal alkaline preincubation of mATPase staining was performed and used to confirm fibre types in orders to reduce error in analysis of fibre composition.

Observations from previous stained TA image quality found that Type IIa fibres were stained pale green in both staining protocols, leading to hypothesise that there was no cross reaction between anti-rabbit IgG (H+L), CF™ 405M and antibody SC-71, while not reducing binding sites for anti-mouse 488. Similar staining quality of the two TA sections indicated that use of Anti-rabbit IgG (H+L), CF™ 405M did not reduce reactivity between antibody SC-71 and Anti-mouse 488 in marking Type IIa fibres (Figure 2.15-2.16). Fluorescent labelling of Type IIa could be improved if a specific Type IIa antibody is applied.

The fixation period is matter of concern in immunohistochemistry, where too long preincubation would change amine group conformation of the epitope and reduce binding affinity between antibody and antigen binding sites (Puchtler & Meloan, 1985). It was feasible that the previous staining technique led to poor staining quality, due to a longer fixation phase experienced when dealing with many samples. It was observed in Sol that sections with longer (4 minutes) incubation tended to decrease binding affinity between SC-71 and Type IIa antigen, compromising differentiation. The modified epitope of Type IIa would potentially bind with antibody BA-D5 as red staining was seen on IIa fibres (Figure 2.18). Myosin ATPase staining was then used to confirm the red stained fibre were indeed Type IIa. This phenomenon was not seen in TA muscle where no Type IIa fibres ever stained with red after 4 minutes fixation (Figure 2.17). Therefore, incubation period was crucial and fibre type specific as they showed different rate of antigenicity loss (Barton et al., 1984; Hølund et al., 1981), and 2 minutes was a compromise duration to be used for all type of skeletal muscles of different fibre type composition in immunohistochemistry. Improved technique included incubation timing and washing performed rotationally would be applied when dealing with many samples.

Chapter 3 : Pilot study: 7-week voluntary wheel running exercise and its effect on muscle capillarity and fibre type composition in fast and slow muscles.

3.1 Introduction

Skeletal muscle has an impressive remodelling capacity in response to endurance exercise. This plasticity includes expansion of aerobic capacity by greater regional capillary supply, higher oxidative fibre type content, and reduced fibre size (affecting intramuscular diffusion distances) collectively improving oxygen delivery and utilisation. Voluntary wheel running exercise offers minimal stress on exercising animals, since they run freely without external stimulation, thus avoiding any stress response that may influence muscle phenotype (Leasure & Jones, 2008).

Exercise intensity and duration may influence the magnitude of muscle adaptation which varies across rats strain. 4 week olds Sprague Dawley rats showed increased running distance after five weeks wheel exercise, which drastically dropped by week 10 and plateaued in weeks 20 to 30 (Kariya et al., 2004). A study examined the influence of genetic on running capacity found that three inbred rats (ACI/Ztm, BH/Ztm and Lew/Ztm) of similar age showed different running distance as they produced significant different amount and duration of activity (Wollnik, 1991). In C57BL/6 mice a long term voluntary wheel running study indicated the running distance peaked at day 14 and doubled capillary density and capillary to fibre ratio of plantaris muscle after 4 weeks (Waters et al., 2004). We reasoned that inbred rat strain Wistar - currently used in this study – had relatively less aerobic capacity than Sprague Dawley rats would achieve peak exercise performance by 5^{th} week (Kariya et al., 2004), and that the performance would gradually drop when capillary rarefaction began. Increased exercise performance was thought to parallel improved aerobic capacity, as reflected by microcirculation expansion and/or improved oxidative capacity (e.g. increase in oxidative fibre content). Therefore, weekly running distance as a performance index would be monitored during the exercise period so that optimal muscle physiological adaptation could be examined before it started to be degraded.

Ergogenic supplementation of a composite carbohydrate, such as glucose and fructose, showed significant effect in exercise performance (Jeukendrup, 2014; O'Brien et al., 2013; Rowlands et al., 2012; Vandenbogaerde & Hopkins, 2011). Therefore, fructose may be used to increase exercise volume so that a maximal physiological response in skeletal muscle phenotype in order to meet body demand could be observed. Fructose is imported into tissue through the hexose transporter protein GLUT5. This transmembrane protein is found in the sarcolemma of muscle fibres and uptake of fructose is unaffected by the presence of insulin (Zierath et al., 1995), hence it is mediated in a concentration-dependent manner in contrast to glucose uptake that is dependent on GLUT4 content. Increased energy intake in fructose-treated rats led to higher muscle activity as it reduced muscle glycogen utilisation from reserves, termed the 'glycogen sparing effect' (Levine et al., 1983), which increased running distance and may stimulate further angiogenesis to support this higher metabolic demand.

There are several metabolically different locomotor muscles involved in running activity and they may adapt to a different extent when stimulated at different intensity and/or duration (Kariya et al., 2004; Ranjbar et al., 2017; Sexton, 1995; Waters et al., 2004). Therefore, this study looked at the exercise effects in a slow oxidative (soleus) and fast oxidative (EDL) muscle, describing defined degree of microcirculation changes in metabolically different muscles.

The aim of the study was to determine optimal voluntary wheel running exercise period in Wistar rats and its effect on muscle capillarity, fibre type composition and fibre area. This was achieved by monitoring daily running distance per week until an evident downturn was observed. Greater exercise volume generated by composite carbohydrate supplementation could provide an enhanced angiogenic stimulus that could be another indicator in determining optimal skeletal muscle response to the exercise modality. Muscle phenotype changes during adaptation would be explored, both gross averages or region-specific. We hypothesised that Wistar rats running performance would be at peak level by 5^{th} week, and fructose use would further increase angiogenic stimulus due to increase exercise volume that would result in enhanced running performance.

3.2 Methods

3.2.1 Animals

Nine male Wistar rats with initial body mass 137±2g (6 week old) were obtained from the animal unit of Central Biological Services of University of Leeds. They were randomly and equally divided into 3 groups; fructose-treated (20% concentrated in drinking water) trained rats, non-fructose-treated trained rats, and sedentary control groups. Exercised animals were housed individually in cages with free access to a running wheel connected to a logger and computer. Water and food were provided *ad libitum* food under a 12:12 light: dark cycle (lights on at 07:00). All procedures were conducted in accordance with the Animals (Scientific Procedures) Act 1986 and guiding principles approved by the Home Office. The animal room was entered twice daily during the day (morning and evening) to check the water bottles and food supply and once a week for cage cleaning, body mass, water and food consumption measurement.

3.2.2 Wheel dimension and setting

Refer to general methods section.

3.2.3 Running parameters

Refer to general methods section.

3.2.4 Schedule 1 killing and muscle dissection

Refer to general methods section. Rats body mass when sampled were 367±4g (SC), 396±27g (RW) and 360±53g (RWF).

3.2.5 Freezing and cryosections

Refer to general methods section.

3.2.6 Immunohistochemistry

Refer to general methods section.

3.2.7 Counting and calculation of capillary and fibre numbers, capillary density, capillary to fibre ratio and mean fibre area.

Capillaries and fibres of images were visualised through the Leica fluorescent microscope, Nikon E600 and captured based on region: R1, R2, R3, and R4 (EDL) and R1, R2 and R3 (Sol) under x20 and x16 magnification, respectively. They were then counted manually by using ImageJ software (NIH). Firstly, the image type was changed to RGB colour following image quality manipulation if needed, by adjusting colour balance and threshold values. Image scale was set (distance in pixels: 2560 and known distance: 439.86 and 549.39 µm) to give a total image area of 145,108.62 and 226,557 μm^2, respectively.

The region of interest (ROI) was set to 75% of total area, which included two dotted (inclusion) edges (Figure 2.4). Any capillary or fibre fully included in the ROI or that uniquely intersected the dotted lines were included in the analysis. Then a lattice grid was applied for fibre type counting that used 'Grid type: crosses' and 'Area per point: 1150$unit^2$'. The cell counter option was used as a systematic counter. The counted capillary and fibre were keyed into a spread-sheet for determination of capillary density (CD), capillary to fibre ratio (C:F), mean fibre area, and fibre type number.

3.2.8 Data analysis

Refer to general methods section.

3.3 Results

3.3.1 General characteristic and running activity of Wistar rats

A total of 9 male Wistar rats, 6 week old were used in this study that have been divided into three groups of three; running wheel (RW), running wheel with fructose (RWF) and sedentary controls (no access to wheel). Initial body mass were 138.00±4.04g and 137.00±2.00g, and gained weight as much as 221.67±49.06g and 259.67±27.14g for RW and RWF respectively. The body mass after 7 weeks were in the range of 356 to 392g; control animals were 367.33±3.21g.

Running distance was different for RW and RWF groups, that recorded 10.5±0.44 *vs.* 3.53±0.14 km and 516.27±171.81 *vs.* 173.21±48.89 km for daily and 7 weeks running, respectively (P<0.05). Exercise in the dark cycle produced markedly longer distances than the light period, RW group (149.80±47.34 *vs.* 7.55±3.44 km) and RWF (445.62±169.94 *vs.* 14.40±6.47 km), respectively. Running distance demonstrated an increasing pattern until the 4th week and then decreased gradually towards 7th week, with mean daily running distance per week 3.53±0.25 *vs.* 10.54±1.05 km for RW and RWF (average weekly value over the 7 week period). Food consumption was higher in RW group, 180.38±9.04 g/week compared to RWF, 121.00±13.65 g/week (P<0.05), while water consumption was only slightly higher in RW than RWF at 442.12±85.25 and 400.48±60.22 ml/week, respectively (P>0.05).

RW and RWF rats performed a similar number of running bouts per day, 26.84±0.89 and 28.05±0.01, respectively (P>0.05). However, the daily running duration, running distance and velocity were higher in RWF, 17.37±0.92 min, 0.42±0.02 km and 0.90±0.01 m/s, than RW, 8.91.00±0.26 min, 0.13±0.01 km and 0.76±0.01 m/s, respectively (all P<0.05; Table 3.1).

Table 3.1: General characteristics and running activity of rats. *P<0.05 *vs*. RW.

Parameter	Mean±SD		
	Running wheel (RW)	Running wheel fructose (RWF)	Sedentary control
Body mass			
Initial body mass (g)	137.00(2.00)	138.00(4.04)	-
After 7 weeks (g)	396.67(27.27)	359.67(52.67)	367.33(3.93)
Weight increment (g)	259.67(27.14)	221.67(49.06)	-
Weekly food consumption (g)	180.38(9.04)	121.00(13.65)*	-
Weekly water/fructose consumption (ml)	442.12(85.25)	400.48(60.22)	-
Running distance (km)			
Daily	3.53(0.14)	10.54(0.44)*	-
Dark	149.80(47.34)	445.62(169.94)*	-
Light	7.55 (3.44)	14.40(6.47)*	-
7 weeks	173.21(48.89)	516.27(171.81)*	-
Number of running trial (> 1 min period)	28.05(0.70)	26.84(0.89)	-
Daily running duration/ running trial(min)	4.88(2.06)	11.64(3.18)*	-
Running distance/running trial (km)	0.13(0.01)	0.42(0.02)*	-
Velocity (m/s)	0.76(0.01)	0.9(0.01)*	-

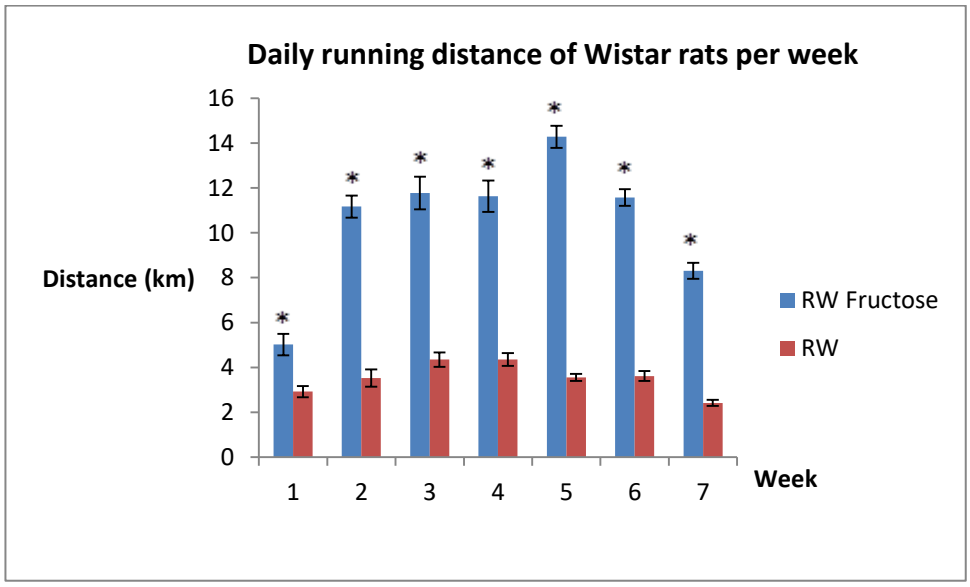

Figure 3.1: Daily running distance and weekly activity pattern with free access to wheel exercise. Abbreviation: RW= running wheel; RWF= running wheel with fructose. *P<0.05 *vs*. RW.

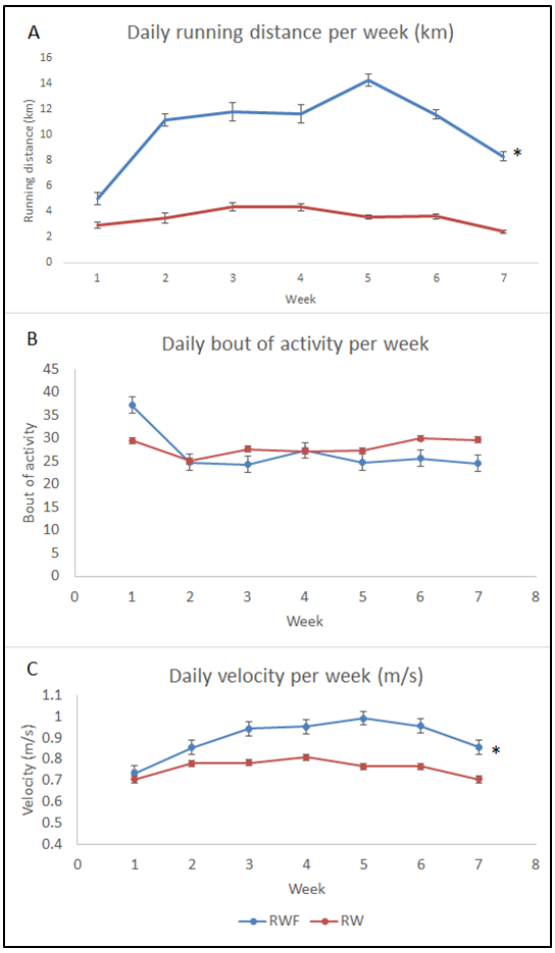

Figure 3.2: Running distance, number of activity bouts, and running velocity in Wistar rats with (RWF) and without (RW) fructose, added to drinking water. *P<0.05 *vs.* RW.

3.3.2 **Immunohistochemistry staining**

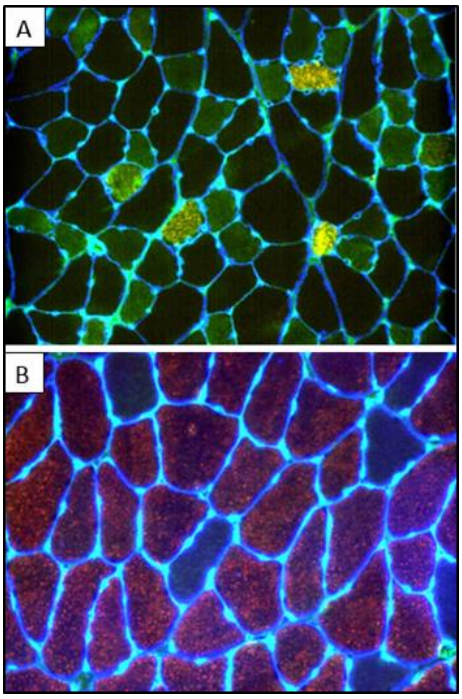

Figure 3.3: Cross section of EDL and Sol of Wistar rat shows the presence of different fibre types: Type I of slow motor unit (red), Type IIa (green) and Type IIb (black) of fast oxidative and fast glycolytic fibres respectively on the basis of monoclonal anti-MHC antibodies; BA-D5 for Type I fibre and SC-71 for Type IIa fibre. Blue fibre outlines represent laminin-stained sarcolemma. Images captured at x20.

3.3.3 **Global angiogenic indices**

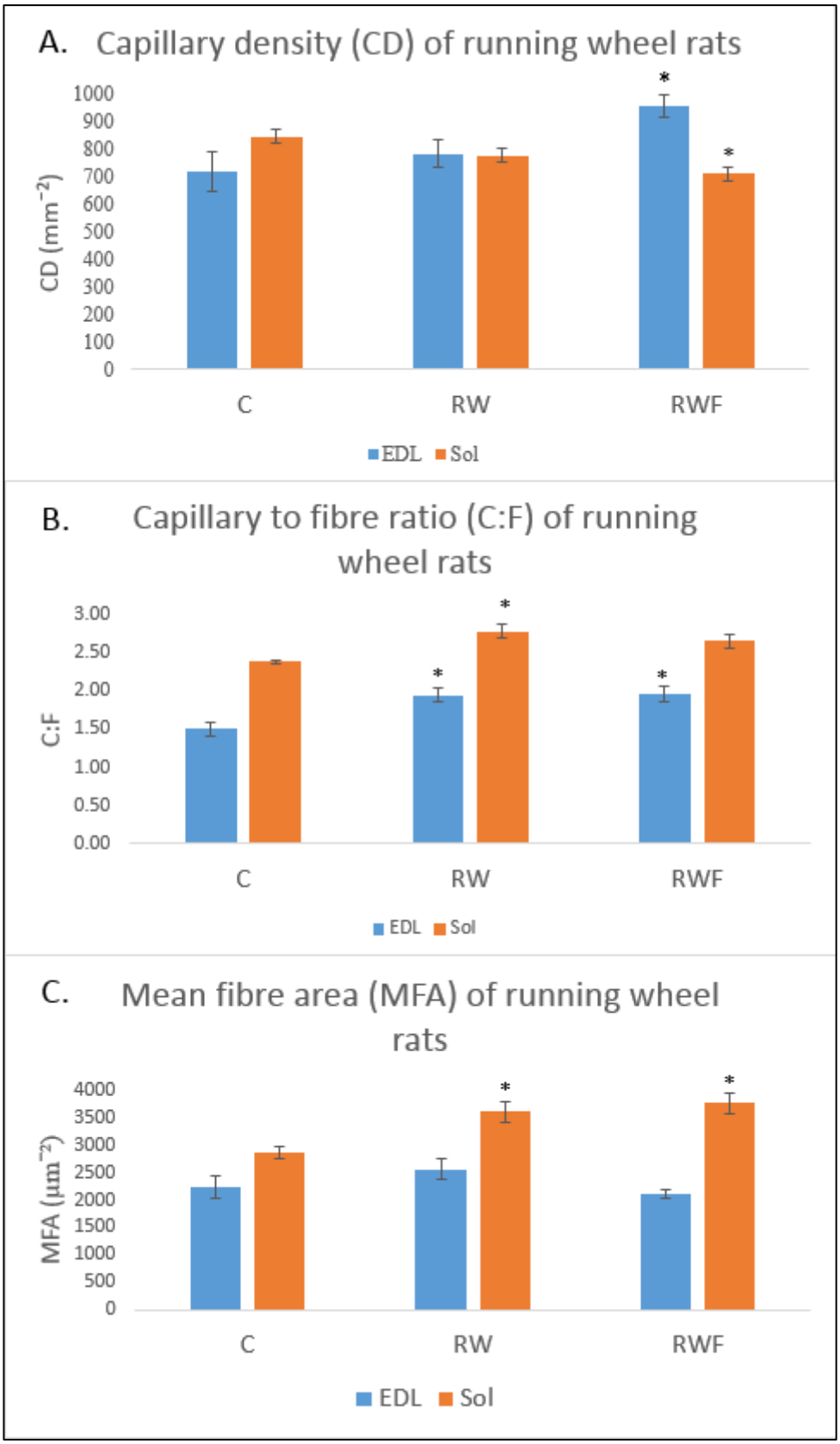

Figure 3.4: Capillary density (CD), capillary to fibre ratio (C:F) and mean fibre area (MFA) of EDL and Sol. *P<0.05 *vs.* control.

3.3.3.1 **Capillary density (CD)**

3.3.3.1.1 EDL

Capillary density (CD) steadily increased from sedentary, RW to RWF groups, 718.36±99.46, 784.16±72.65, and 956.29±114.52 mm^{-2}, respectively. The mean CD was significantly different between RWF and sedentary control (P<0.05) but not between RW and RWF or between RW and sedentary control (P>0.05) (Figure 3.4). In terms of region, only region 3 was significant among those groups, P=0.005 (Figure 3.5).

3.3.3.1.2 Sol

CD was decreased from 846±25 in sedentary controls to 778±25 and 711±26 mm^{-2} in RW and RWF, respectively. There was a decreasing trend of CD among exercising rats (RW) even though the mean CD was not significant different. Regional CD indicated that, both RW and RWF Sol reduced CD in region 2 (P<0.05, Figure 3.4).

Regional CD

1. EDL

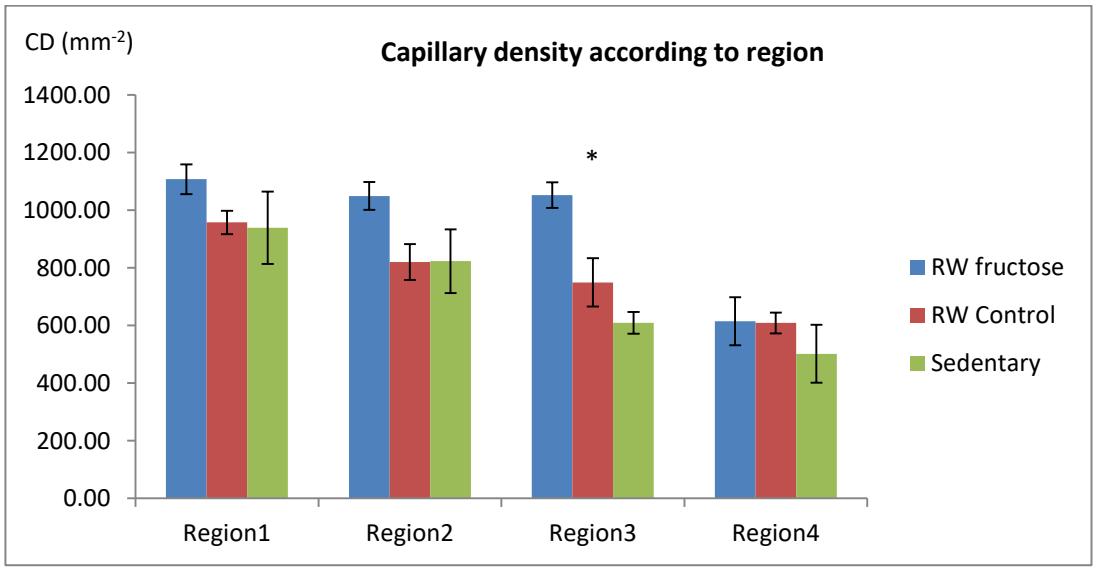

Figure 3.5: Capillary density of RWF, RW and sedentary control rats according to regions. *One-way ANOVA: the mean difference of capillary density is significant between RWF, RW and sedentary controls in region 3, *P=0.005.

2. Sol

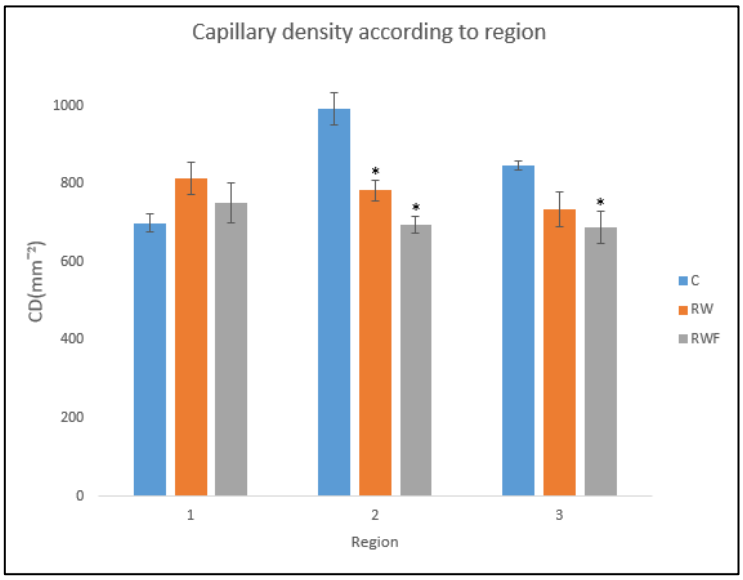

Figure 3.6: Capillary density of RWF, RW and sedentary rats according to regions. *One-way ANOVA: the mean difference of capillary density is significant between RWF, RW and sedentary controls in region 2 and 3, *P< 0.05.

3.3.3.2 **Capillary to fibre ratio (C:F)**

3.3.3.2.1 EDL

Wheel running exercise significantly increased C:F ratio of RW and RWF, 1.95±0.06 and 1.97±0.22 in, respectively compared to control group, 1.50±0.15 (both P<0.05).

3.3.3.2.2 Sol

RW recorded a significant increase in C:F from 2.39±0.02 to 2.79±0.10 (P<0.05), although the increased exercise volume of RWF group was not reflected in an increased C:F, 2.66±0.09 *vs.* 2.39±0.02 (*n.s.*).

3.3.3.3 **Mean fibre area (MFA)**

3.3.3.3.1 EDL

MFA was not significantly different among the groups, although there was a pattern of decreasing fibre area with increasing exercise volume (Figure 3.4C).

3.3.3.3.2 Sol

Wheel exercise increased MFA from 2870±110 to 3607±194 μm^2 (P<0.05) with no extended MFA in RWF, 3765±179 μm^2 (n.s.).

3.3.4 Relationship between angiogenic indices and MFA and running distance in EDL and Sol

Capillary density of both muscles was negatively associated with mean fibre area with a good strength of association, r=-0.6 (P=0.119) and -0.9 (P=0.001), respectively (Figure 3.7). EDL of RWF indicated the highest capillary density (956.30±49.71 mm^{-2}) with the lowest mean fibre area, (2108.50±177.56 μm^2) followed by sedentary and RW groups (Figure 3.7). Sol of RWF showed the lowest CD followed by RW. C:F of both muscles showed positive correlation with MFA (Figure 3.8). Meanwhile, CD and running distance of both EDL and sol demonstrated negative but non-significant relationships (EDL: r=-0.034, P=0.931; Sol: r=-0.409, P=0.274) (Figure 3.9). C:F of EDL and Sol and running distance were positively associated (r=0.380, P=0.314; r=-0.3, P=0.465, respectively) (Figure 3.10).

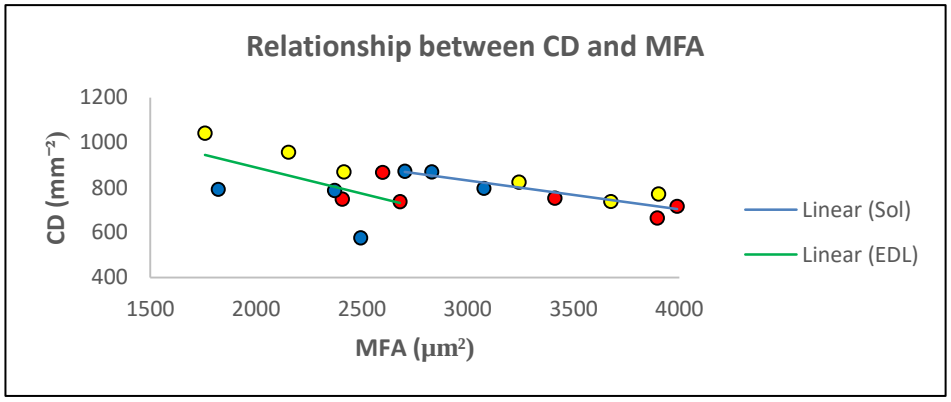

Figure 3.7: CD of EDL and Sol plotted against MFA. EDL: r=-0.557, P=0.119); Sol: r=-0.887, (P=0.001). (B) C:F of EDL and Sol, EDL: r=0.347, (n=9, P=0.36); Sol: r=0.840, (P=0.005). Red= RW, yellow= RWF, blue= control, green line= EDL and blue line= Sol.

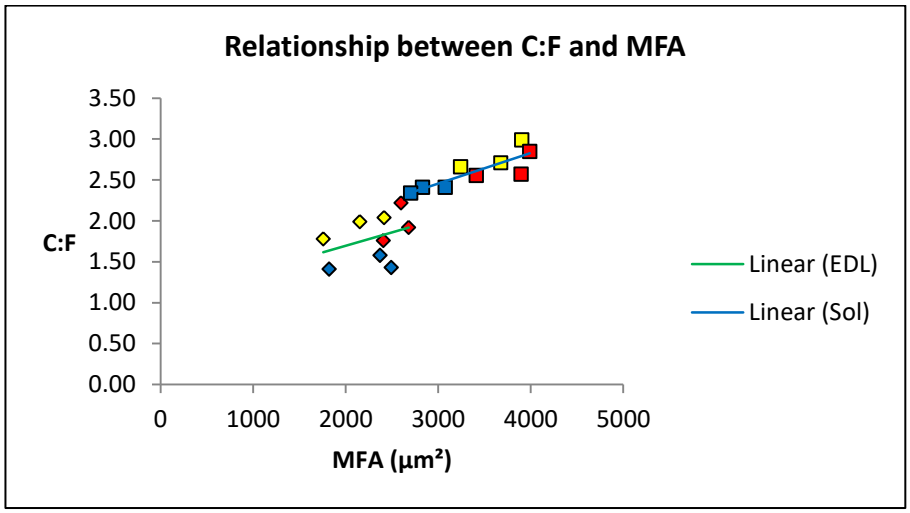

Figure 3.8: C:F of EDL and Sol, EDL: *r*=0.347, (n=9, *P*=0.36); Sol: *r*=0.840, (*P*=0.005). Red= RW, yellow= RWF, blue= control, green line= EDL and blue line= Sol.

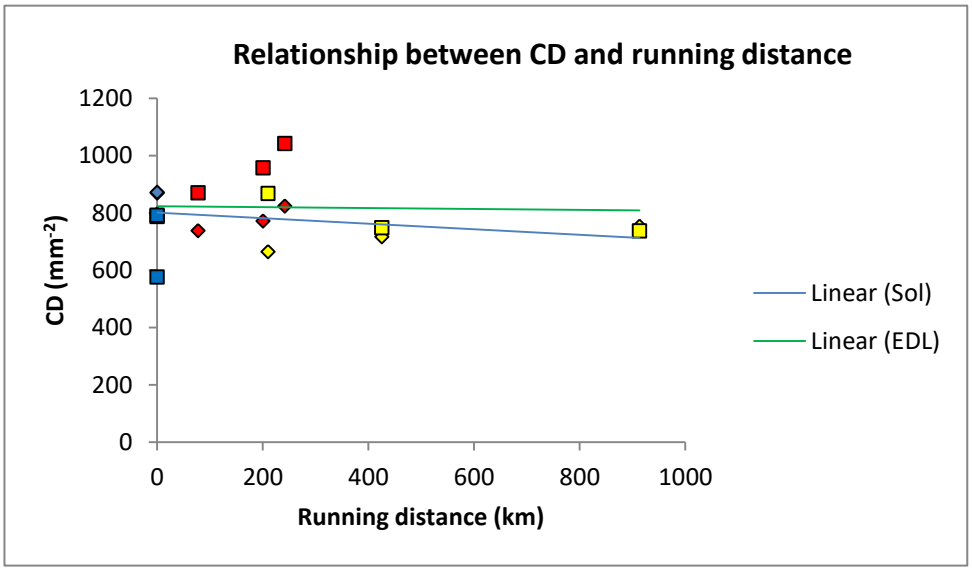

Figure 3.9: CD *vs.* running distance. EDL: *r*=-0.034, (*P*=0.931); Sol: *r*=-0.409, (*P*=0.274) respectively. Red= RW, yellow= RWF, blue= control, green line= EDL, blue line= Sol.

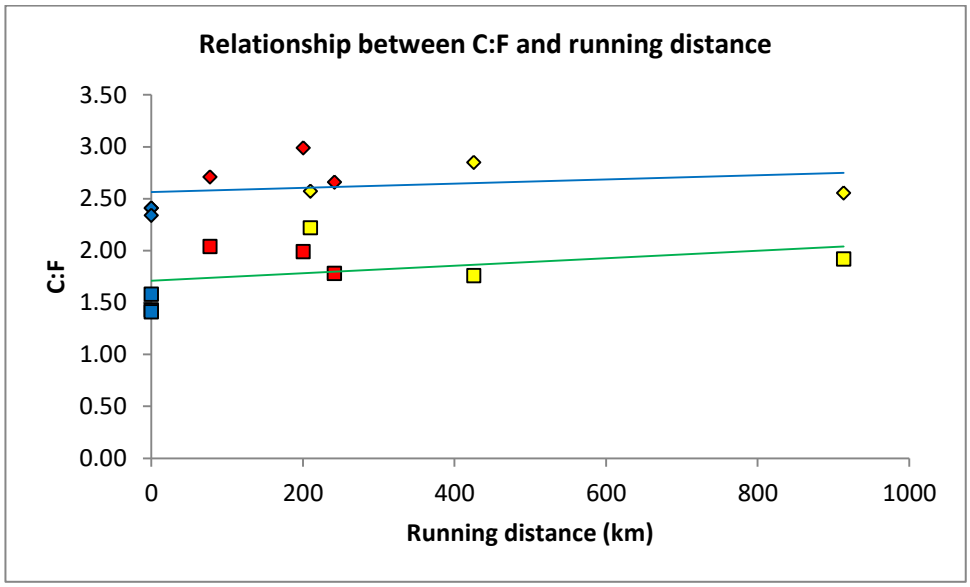

Figure 3.10: C:F *vs.* running distance. EDL: *r*=0.380, (*P*=0.314); Sol: *r*=-0.3, (*P*=0.465) respectively. Red= RW, yellow= RWF, blue= control, green line= EDL, blue line= Sol.

3.3.5 **Muscle fibre composition**

3.3.5.1 **EDL**

Numerical density (N_N) of Type I fibre was increased from RW, RWF to sedentary control, 0.04±0.01, 0.07±0.01 and 0.08±0.02. RWF contained the highest N_N of Type IIa, 0.42±0.03, followed by RW and sedentary control, 0.4±0.04 and 0.28±0.05, respectively. Sedentary control and RWF groups recorded the highest and lowest Type IIb N_N, 0.67±0.05 and 0.53±0.02, accordingly (Figure 3.11).

Numerical density of fibre Type IIa and IIb showed a significant difference among rat groups in region 4, (P<0.05), while none of regions were significantly different for Type I fibre content. The relative Type IIb reduction was 13% and 21% for RW and RWF, respectively (Figure 3.12 and Figure 3.13).

3.3.5.2 **Sol**

Numerical density of Type I fibre of RW and RWF were not significantly different compared to control, 0.971±0.029 *vs.* 0.990±0.010 and 0.993±0.007 *vs.* 0.990±0.010 (P>0.05), respectively. Type IIa fibre content of RW and RWF was similar 0.04±0.01 and 0.07±0.01, compared to control 0.08±0.02 (P>0.05; Figure 3.11).

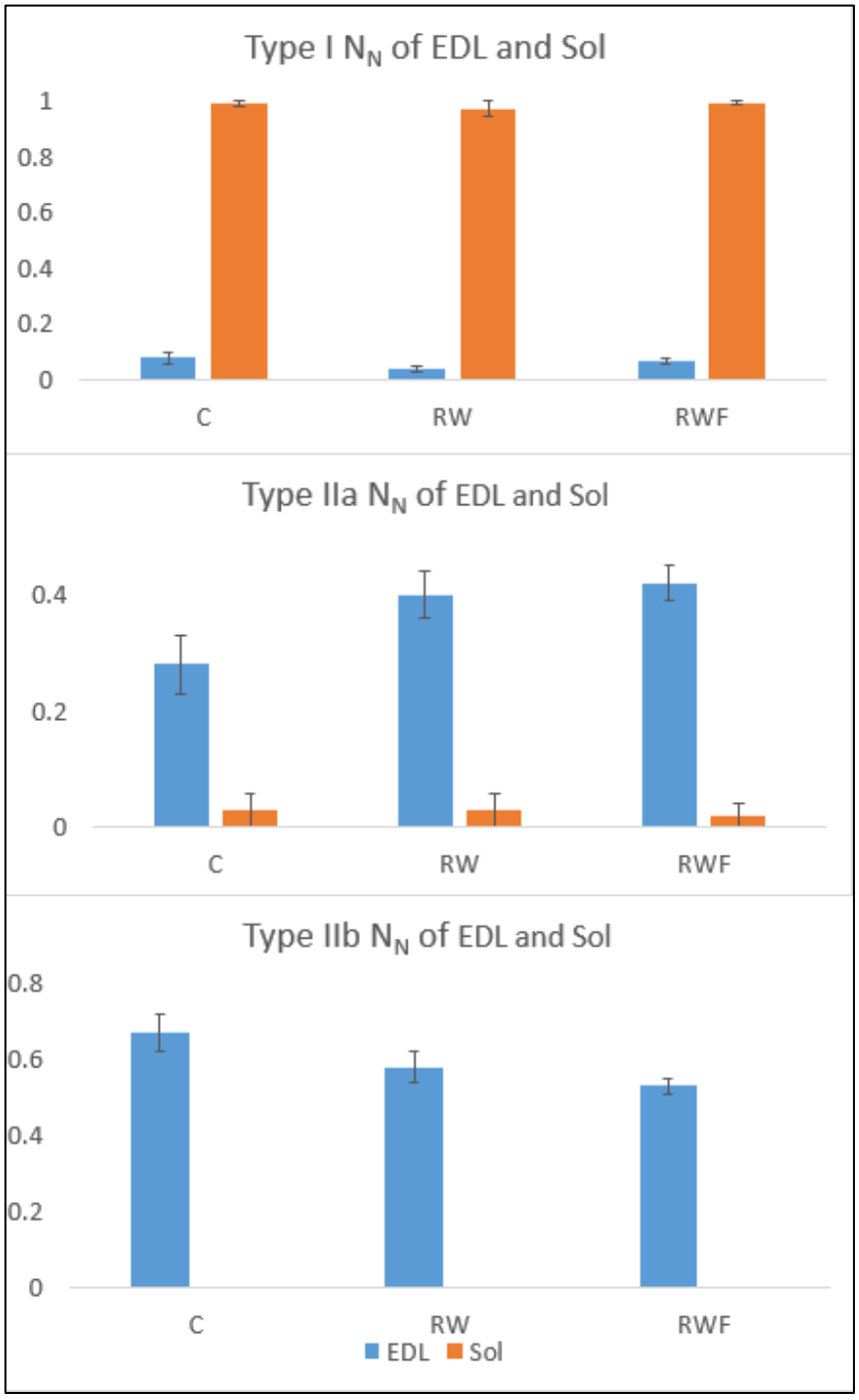

Figure 3.11: Fibre type composition in EDL and Sol of RW, RW fructose and sedentary rats (all *n.s.*)

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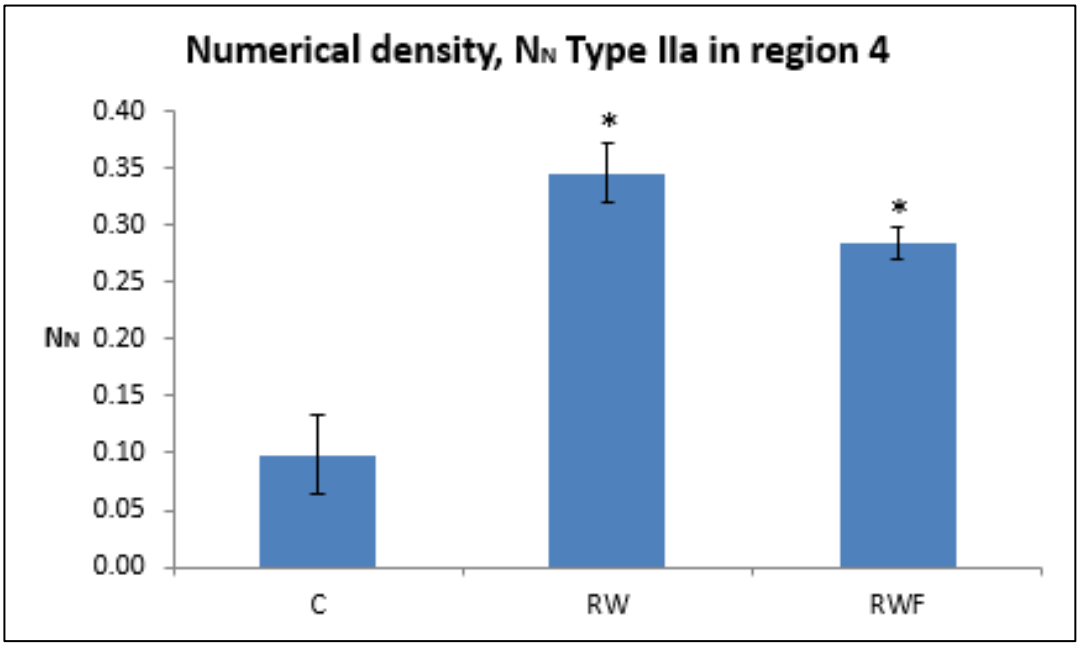

Figure 3.12: Numerical density, N_N of Type IIa fibre of EDL was significantly different among groups at region 4, (P<0.05).

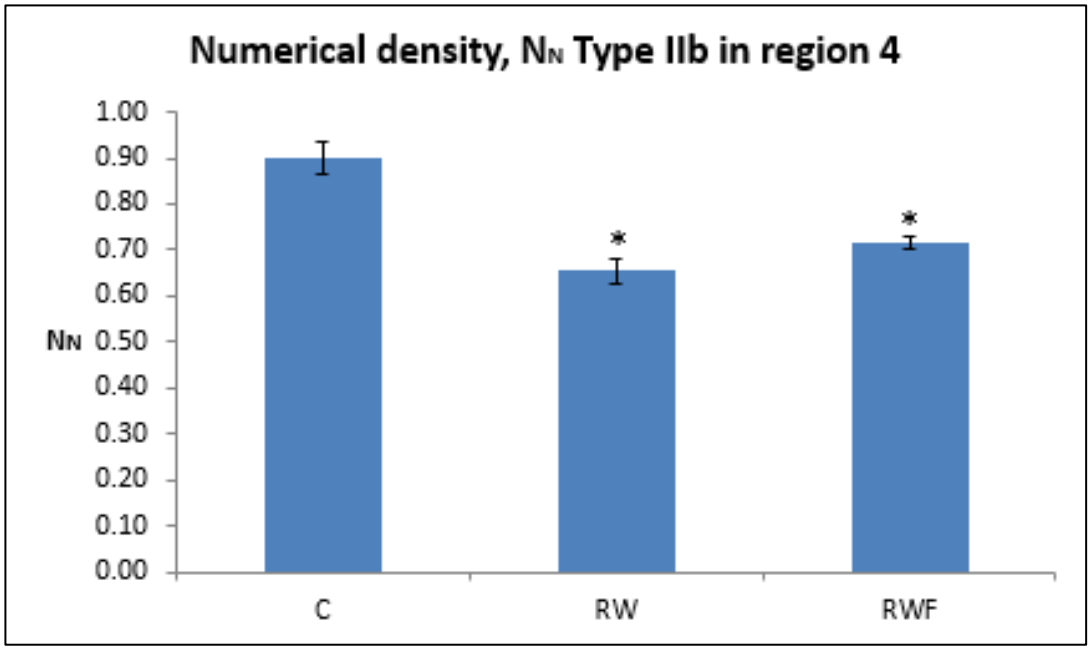

Figure 3.13: Numerical density, N_N of Type IIb fibre of EDL was significantly different among groups at region 4, (P<0.05).

3.4 Discussion

3.4.1 Voluntary wheel running activity

The total running distance over 7 weeks between RW and RWF rats were about 3-fold difference (516.3±171.8 *vs.* 173.2±48.9km): fructose consumption was able to enhance running capability and thus increase exercise volume. This was likely due to increased substrate intake that would aid aerobic ATP production needed for muscle contraction (Dillmann, 1984; Vrbova et al., 2008). In addition, the running pattern among groups was different as the fructose group ran longer than RW controls in terms of both duration and distance, although both performed almost the same amount of daily running activity (26.84±0.89 *vs*. 28.05±0.70 activity bouts). Even though Type IIa composition between RW and RWF was similar, the lighter RWF rats may have favoured a higher running speed, supporting a longer running activity.

In addition, the running distance gradually increased starting from the end of week 1 to week 4, and then slowly decreased until the seventh week. The first week of running activity was excluded from analysis to avoid bias when ambiguous data were recorded due to different rates of familiarisation. A similar pattern of running activity was observed in previous studies that showed increased running distance for the first four weeks of training, then decreased thereafter (Bigard et al., 1991; Kariya et al., 2004). There may be some scale effect involved in this reduced activity, as rat size began to approach the inner dimensions of the wheel, potentially meaning some discomfort restricted running activity. The integrated response, therefore, needs to take into account both behaviour and biology; the latter may be quite adaptive, which subsequent analyses probed.

3.4.2 Physiological adaptation to endurance exercise.

3.4.2.1 Capillary density and mean fibre area

Our data revealed that voluntary wheel running increased CD of EDL as much as 66 (RW) and 238 (RWF) capillaries/mm^2, although the increased volume of exercise did not provide an extra angiogenic stimulus, as capillary to fibre ratio (C:F) for RW and RWF were the same, 1.95. The RWF rats reduced mean fibre area (-6.3%) which results in an increased CD (+33%) instead of increasing capillary number, to enhance microvascular function to accommodate increased physiological demand during increased muscle activity. Thus, mean fibre area was reduced in exercising rats and accompanied by an increase in arteriole diameter (Lash & Bohlen, 1992), a reduction in peripheral resistance that would aid muscle perfusion. This finding was in agreement with a previous study that reported a twofold

increase in CD and C:F (466±16 to 909 ±55 capillaries/mm^2 and 0.95±0.04 to 1.70 ±0.08 capillaries/fibre) in plantaris muscle (metabolically similar to EDL) of mice after 4 weeks wheel running exercise (Waters et al., 2004). Swimming for 12 weeks, 5 days a week for 1 hour also gave the same result when CD of rat EDL muscle was increased from 727±45.9 to 1072±32.7 capillaries/ mm^2 (Bigard et al., 1991).

By contrast, wheel running exercise did not reduce CD of Sol but rather increased C:F to support increased metabolic demand during chronic exercise, accompanied by increased mean fibre area that balanced the extent of angiogenesis and hypertrophy. Similarly to EDL of RWF, increased exercise volume did not further increase capillarity of Sol of RWF as C:F was unchanged compared to RW with greater MFA leading to a reduced CD.

Capillary density of EDL was negatively correlated with mean fibre area. This suggests that wheel running exercise does not result in fibre hypertrophy in fast muscle (Dohm et al., 1977), where a positive relationship with C:F is seen, and resulting capillary bed adjustment will likely enhance its functional capacity and support increased muscle aerobic capacity (Boutellier, 1982; Hoppeler, 1990; Lash & Bohlen, 1992). A reduced mean fibre area will lead to reduced inter-capillary (e.g. oxygen) diffusion distance thus potentially increasing the rate of oxidative ATP production by mitochondria (Bigard et al., 1991). Sol muscle showed the same relationship but CD tended to be reduced with exercise, which was compensated by increasing C:F that showed a positive correlation. This is in accordance with a study that showed 25% increase in muscle enlargement of Sol after 6-8 weeks treadmill exercise post 4 weeks of synergist muscle (gastrocnemius) removal (Gollnick et al., 1981). However, the proportional change in CD and fibre size appears inadequate to fully compensate for increased oxidative demand, and an additional angiogenic response is therefore stimulated.

3.4.2.2 **Fibre type transformation**

In adapting to increased metabolic demand of long term endurance exercise, muscles were likely to shift to more oxidative type fibres and increase oxygen demand, which occurs in the order of Type IIb to Type IIa to Type I (Demirel et al., 1999; Saltin et al., 1977). Our results were consistent with previous data showing that numerical density of Type IIb fibres (expressing the relevant myosin heavy chain isoform) in EDL wheel running exercise, even with fructose, was somewhat lower than that in the sedentary group. Even though it was not significant in this pilot study, possibly due to inadequate statistical power, it was clear that the proportion of Type IIb fibres were less in RW and RWF relative to sedentary rats by 13% and 21%, respectively.

Regionally, there was a fibre type shift in region 4 of EDL as RW and RWF groups showed significant decrease and increase in Type IIb and IIa, respectively. This was in accordance with a rodent study of 35 weeks wheel running exercise that showed a significant decrease in Type IIb fibre content by as much as 19.5% less than control animals (Kariya et al., 2004). Interestingly, the difference in total running distance among RW and RWF groups (343.06 km) appears to be inadequate to drive gross change in fibre composition, since exercise-induced decrease in the Type IIb content was not significantly different among those groups, suggesting that increased exercise volume (facilitated by fructose consumption) was inadequate to decrease Type IIb content.

Type I fibre content of EDL was also not significantly different among those groups, in agreement with previous studies that showed unchanged Type I fibre content in voluntary running rats (Allen et al., 2001; Kariya et al., 2004). Other rodent voluntary wheel running studies also demonstrated a significant increase in Type IIa fibre and decrease in Type IIb content of plantaris muscle from 15% to 32% and 35% to 15% after 28 days and 35 weeks training period, respectively (Kariya et al., 2004; Waters et al., 2004). Low level physical activity was presumably the reason for no significant change in oxidative fibre number among groups (Dudley et al., 1982), a study that also demonstrated in Sol muscle a higher Type I and similar Type IIa composition across groups.

There were some animals in the wheel running groups that may not have adequately run to induce fibre transformation, indicated by the wide range of running distance due to biological variation. Therefore, it may be necessary to perform animal screening prior to exercise training, so poorly performing animals could be excluded to prevent data bias. Possibly, voluntary wheel running exercise of both groups may have induced fibre shift from Type IIb to Type IIa rather than to Type I fibres (Kariya et al., 2004), which may be effective at supporting fast endurance exercise as RWF run at higher speed (0.90±0.01 m/s) than RW (0.76±0.01 m/s).

Regionally, Type IIa and Type IIb fibres were significantly different in region 4 of RW, RWF and sedentary control animals, indicating the transformation may be dependent on initial composition of muscle fibre types. EDL is mostly composed of Type I and Type IIa in the medial region, and Type IIa and IIb towards the lateral aspect (depicted as region 1, 2, 3 and 4) with an increasing percentage of glycolytic fibre and mean fibre area (Deveci et al., 2001). The wheel running exercise was able to stimulate regional fibre type shift, where increased myosin heavy chain of Type IIb is induced by increased mechanical activity (Jaschinski et al., 1998). It is known that physical activity may cause increased skeletal

muscle blood blow (Flaim et al., 1979), which is a potential factor leading to increased capillary wall tension and shear stress, resulting in capillary growth in the presence of various growth factors (Egginton, 2009; Hudlicka, 1988). Similarly, mechanical strain across the muscle may induce complimentary adjustment in fibre type composition (Kissane et al., 2018).

3.5 Conclusion

Voluntary wheel running exercise for 7 weeks among Wistar rats was able to stimulate expansion of EDL and Sol muscle capillarity either by increased CD and/or C:F, and decreased or increased mean fibre area in the presence of fructose supplement (in EDL and Sol, respectively). It also changed muscle fibre type composition, which tends to shift to more oxidative types. Our finding demonstrated that exercise-induced angiogenesis and muscle fibre shift were muscle and region specific, determined by original muscle fibre type composition in that particular region. Remodelling in response to exercise may be enhanced by dietary supplementation to increase energy intake.

Chapter 4 : Four week voluntary wheel running: Running characteristics and its effect on angiogenic indices and fibre type composition

4.1 Introduction

Voluntary wheel running exercise is less used compared to other exercises modalities in studying the beneficial effects of activity on rodent physiological systems, particularly in hind limb skeletal muscles. Most studies used treadmill exercise as all parameters may be precisely controlled. With regards to wheel exercise, previous studies used more aerobic strains compared to Wistar to explore rat running characteristics, considering running distance in dark and light cycles, running speed and frequency of running episode, with different time periods ranging from 4 to 30 weeks periods (Allen et al., 2001; Kariya et al., 2004; Waters et al., 2004).

It was known that rat strain might affect running performance and behaviour, while two other factors (exercise intensity and period) may influence the degree of skeletal muscle adaptation (Kariya et al., 2004). Rodent studies have shown that different rat strains demonstrate distinct running characteristics (Bauer, 1990; Wollnik, 1991), to an extent that may affect the degree of hind limb muscles changes in term of capillarity and fibre type composition.

Using data from a pilot study of 7 weeks voluntary wheel running (Chapter 3), rat running activity peaked at week 4 before it gradually declined. Increased exercise volume demonstrated by running wheel fructose-fed rats was unable to further increase angiogenesis. Therefore, we established 4 weeks is probably an optimal period that adaptive changes could be observed in the muscle (capillary bed enhancement - increased C:F and CD - and decreased fibre area), thereby presumably maximising aerobic capacity of Wistar EDL muscle, before animals become unresponsive or too large for exercise or change in running pattern.

This study looked at other running variables rather than looking at total running activity, i.e. running activity would be further characterised for example splitting daily activity into active and inactive cycle, looking at bout of activity and link to running speed that would give information about volume, periodicity and intensity of exercise that are currently not available.

Changes in running behaviour or activity characteristics can be better explained when performing a parallel histological analysis such as association between physiological changes in muscle phenotype and running performance. So, degree of running performance and skeletal muscle remodelling (angiogenic response and MFA) of the least aerobic strain (Wistar) could be determined and compared with other more aerobic strains.

This study aimed to determine whether a less stressful (in comparison to the more usual treadmill) exercise protocol could elicit sufficient angiogenic response to enhance muscle microcirculation and improve running performance. This was achieved by applying voluntary wheel running exercise for 4 weeks and performing histological analysis of muscle phenotype changes. It was hypothesised that 4 weeks wheel exercise could increase running performance *via* capillary bed expansion. Having data on muscle phenotype changes and improvement in some running variables would provide information on factors that influence hind limb muscle remodelling in a low aerobic capacity strain of rat.

4.2 Materials and methods

4.2.1 Animals

21 male Wistar rats with similar body mass, 187±16 g, aged 6 week old were obtained from Animal Unit of Central Biological Services of University of Leeds. They were housed individually in cages with access of running wheel that connected to running wheel logger and computer. Water and food were provided ad lib food under a 12:12 light: dark cycle (lights on at 7:00). All procedures were conducted in accordance with the Guiding Principles in the Animals (Scientific Procedures) Act 1986 approved by the Home Office. The animal room was entered twice a day at random times during the day to check the water bottles and food supply. The cage bedding was replaced once a week during cage cleaning day.

4.2.2 Voluntary running wheel exercise

Animals were housed individually and randomly in standard cages attached to wheels (33cm diameter) that were interfaced to a computer and revolutions are recorded in 1min intervals continuously for 4 weeks.

4.2.3 Food and water consumption

Food and water consumed were weekly measured during cage cleaning day by subtracting initial value with amount left.

4.2.4 Running parameters

Refer to general method chapter

4.2.5 Schedule 1 killing and muscle dissection

The experimental and sedentary control animals were killed according to a Schedule 1 procedure. Rats were taken out from carrying boxes by holding their tail base, with the other hand used to support the rat's body. Immediately they were stunned by striking the cranium against a solid object. Animal death was then confirmed by neck dislocation. Extensor digitorum longus (EDL) and cardiac (left ventricle free wall) muscles were dissected. Adipose and connective tissue were removed from muscles which were then weighed.

4.2.6 Freezing and cryosections

Refer to general method chapter

4.2.7 Monoclonal-myosin heavy chain antibodies, lectin and laminin staining

Refer to general method chapter

4.2.8 Area of sampling region

Refer to general method chapter

4.2.9 Angiogenic indices and fibre type composition determination

Refer to general method chapter

4.2.10 Capillary domain area

Refer to general method chapter

4.2.11 Local capillarity

Refer to general method chapter

4.2.12 Estimated muscle function - oxygen transport modelling

Refer to general method chapter

4.2.13 Data analysis

Data were sorted and collated using a spread sheet (Excel 2010) and analysed using IBM SPSS statistical package v.20. Normality test was performed on the data prior to independent- samples T-Test to compare mean values among the rat groups. All results were presented in table and graph forms.

4.3 Results

Body mass, food and water consumption

Body mass of RW and sedentary rats increased over 4 week period (Figure 4.1); body mass of RW rats were lower than sedentary controls from week 2 onwards (11, 20, 26g, respectively), however they were not significantly different. Food consumption increased over time, although week 2 and 3 showed similar intake, however the rate of water intake consistently increased (Table 4.1).

4.3.1 Rat body and muscle mass, food and water consumption.

Table 4.1: Body mass, food and water intake of Wistar rats performing 4 weeks voluntary wheel running.

Parameter		Mean±SD		Significance (*P* Value)
	Period	SC	RW	
N		8	21	
Body mass (g)	Initial	191±13	197±10	0.651
	Week 1	221±19	230±25	0.850
	Week 2	266±23	263±25	0.391
	Week 3	306±23	288±33	0.180
	Week 4	338±28	312±42	0.692
Cardiac mass (g)	-	0.89±0.13	0.97±0.10	0.228
EDL mass (g)	-	0.16±0.03	0.20±0.03	0.022*
EDL:Cardiac ratio (mg/g)	-	0.51±0.10	0.63±0.06	0.017*
Food consumption (g)				
	Week 1	-	118±15	
	Week 2	-	173±24	
	Week 3	-	170±36	
	Week 4	-	189±15	
Water consumption (ml)				
	Week 1	-	174±43	
	Week 2	-	228±64	
	Week 3	-	246±58	
	Week 4	-	278±85	

4.3.2 **Running parameters**

Table 4.2: Daily mean value for all running parameters per week, mean±SD. Unshared letters denote statistical significance (*P*<0.05*)* between week 1 and week 2, week 2 and week3, and week 3 and week 4 as determined by Independent sample T-Test.

Running parameters	Week			
	1	2	3	4
	Mean±SD			
Total distance (km/day)	2.93±0.67^{a}	4.59±.23^{b}	5.41±.40^{c}	5.85±0.41^{c}
Dark cycle total distance (km)	2.62±.69^{a}	4.28±.24^{b}	5.19±0.49^{c}	5.63±0.41^{c}
Light cycle total distance (km)	0.31±.07^{a}	0.31±.07^{ab}	0.22±0.13^{bc}	0.22±0.09^{c}
Mean running velocity (m/s)	0.71±0.07^{a}	0.81±.01^{b}	0.85±0.01^{c}	0.86±0.01^{d}
Mode running velocity (m/s)	0.8±0.07^{a}	0.9±.05^{b}	0.89±0.06^{bc}	0.88±0.08^{c}
Median running velocity (m/s)	0.77±0.07^{a}	0.89±.01^{b}	0.93±0.01^{c}	0.95±0.01^{d}
Dark Mean running velocity (m/s)	0.72±0.07^{a}	0.82±.01^{b}	0.85±0.01^{c}	0.87±0.01^{d}
Light mean running velocity (m/s)	0.58±0.05^{a}	0.63±.03^{b}	0.62±0.03^{bc}	0.61±0.03^{c}
% time active (%)	16.4±0.7^{a}	18.3±.4^{b}	17.9±0.6^{bc}	18.2±1.5^{c}
Max activity duration (min)	22.8±5.3^{a}	29.2±1.5^{b}	30.3±3.7^{bc}	32.3±3.0^{c}
Bouts of activity	36±5^{a}	33±1^{a}	30±2^{b}	28±1^{c}
Mean of activity duration (min)	6.6±1.3^{a}	7.9±0.5^{b}	8.4±0.6^{b}	9.1±0.5^{c}
Mode of activity duration (min)	2.4±0.2^{a}	2.6±0.4^{ab}	2.9±0.7^{bc}	2.5±0.3^{c}
Dark % time active (%)	27.5±2.0^{a}	32±1.4^{b}	32.4±2.2^{bc}	32.5±1.4^{c}
Dark Bouts of activity	30±4^{a}	27±1^{ab}	26±1^{c}	25±1^{c}
Dark max activity duration (min)	22.1±5.9^{a}	28.9±1.5^{b}	29.9±3.8^{bc}	32.1±3.2^{c}
Dark mean activity duration (min)	6.9±1.6^{a}	8.4±0.5^{b}	9±0.6^{bc}	9.5±0.6^{c}
Dark mode activity duration (min)	2.5±0.1^{a}	2.8±0.4^{ab}	3.8±1.3^{bc}	2.6±0.4^{c}
Light % time active (%)	5.1±1.3^{a}	4.5±0.9^{ab}	3.4±1.8^{bc}	3.2±1.2^{c}
Light Bouts of activity	6±2^{a}	6±1^{ab}	4±2^{bc}	3±1^{c}
Light max activity duration (min)	10.9±1.4^{a}	11.1±1.4^{ab}	8.9±2.6^{bc}	9±2.2^{c}
Light mean activity duration (min)	5.2±0.7^{a}	5.5±0.6^{ab}	5.1±0.5^{bc}	5.6±1.0^{c}
Light mode activity duration (min)	2.9±.6^{a}	2.8±0.4^{ab}	3.5±0.9^{bc}	3.7±0.8^{c}

Running activity characteristics

Daily running distance averaged per week greatly varied between RW rats over the 4 week period. Differences in running distance magnitude was obvious at the end of the study period, showing capacity of the animals to perform more running activity following exercise training (Figure 4.2). Mean daily running distance per week increased over the time that similarly showed by dark cycle daily running distance, while light cycle demonstrated a downward trend (Figure 4.3). In terms of velocity, mean, median and mode of running velocity all increased until they plateaued between week 3 and 4. Dark and light running speed showed a similar trend, with light cycle was at lower speed (Figure 4.4).

Overall percentage time active increased over the period where rats performed more activity at night rather than during daytime, which was considerably lower reflecting their nocturnal habit (Figure 4.5). The mean number of running activity bouts gradually dropped over time, a similar trend seen in both dark and light cycles (Figure 4.6).

Maximal and mean running duration showed similar characteristic as they increased and peaked by the fourth week, while a decreasing trend was demonstrated during the light period (Figure 4.7). Mean and dark cycle running duration mode were high until they dropped at week 4 (Figure 4.8), however daytime mode of running duration was increased over the course (Figure 4.9).

4.3.2.1 **Individual daily running distance per week**

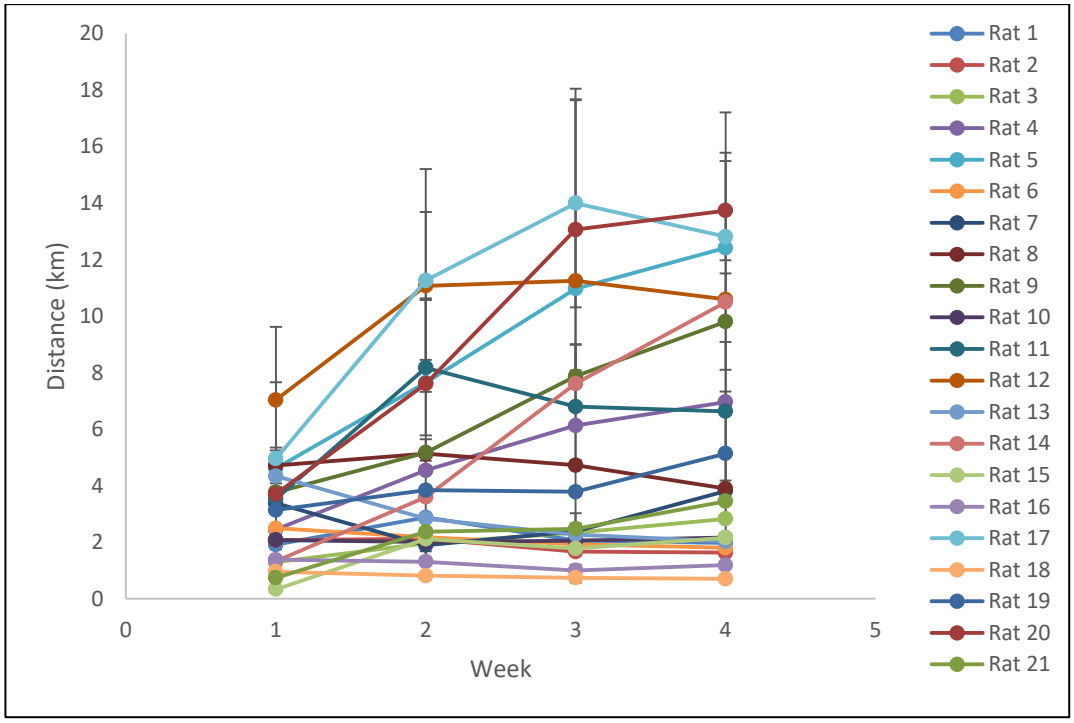

Figure 4.1: Individual daily running distance over 4 week period. Distance run increased up to week 4 with a great running distance variability among rats.

4.3.2.2 **Daily running distance per week**

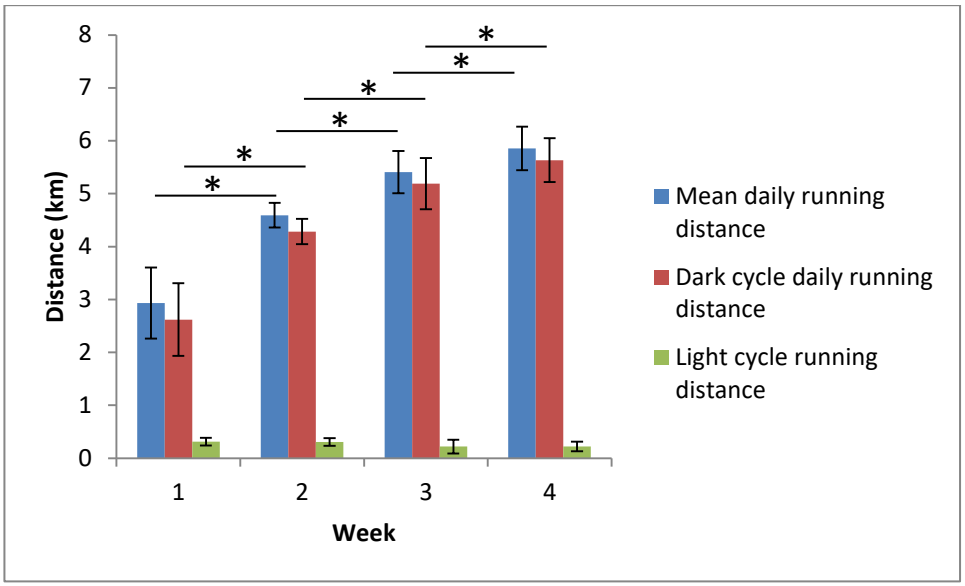

Figure 4.2: Mean, dark and light daily running distance per week of 4-week voluntary wheel running exercise. *P<0.05 between week 1 and week 2, week 2 and week3, and week 3 and week 4, determined by Independent sample T-Test.

4.3.2.3 **Running velocity per week**

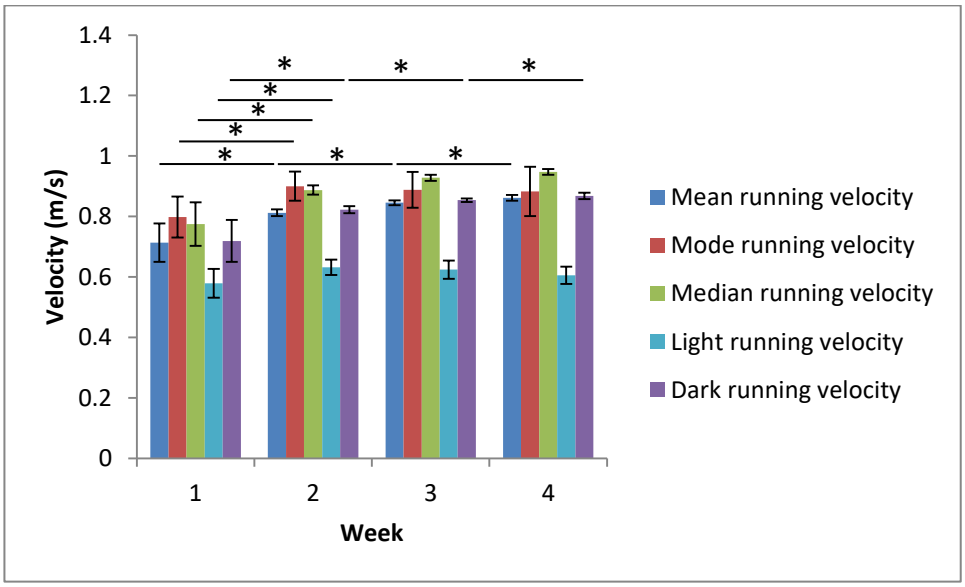

Figure 4.3: Mean, mode, median, light and dark running velocity per week of 4-week voluntary wheel running exercise. * P<0.05 between week 1 and week 2, week 2 and week3, and week 3 and week 4, determined by Independent sample T-Test.

4.3.2.4 **Percentage time active**

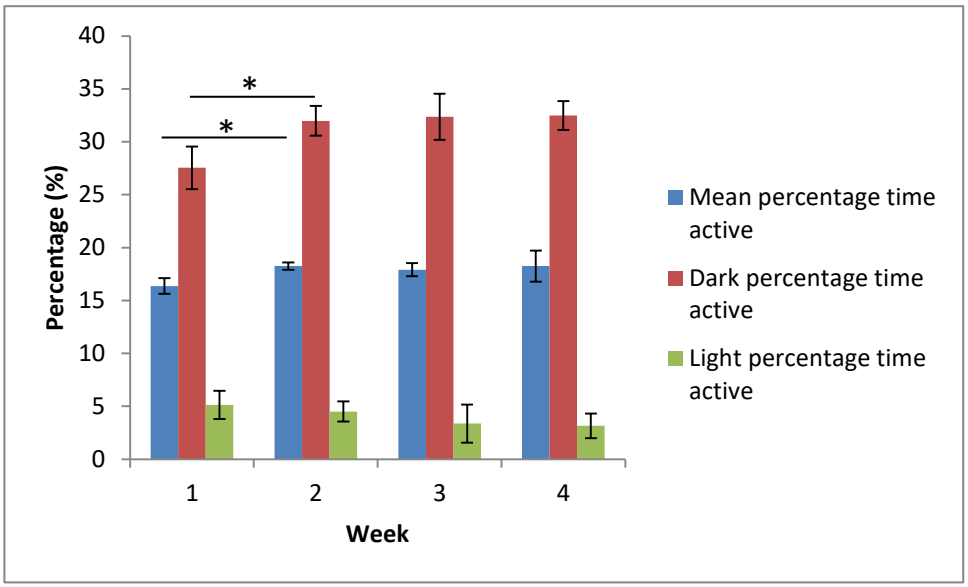

Figure 4.4: Mean, dark and light percentage of time active per week of 4-week voluntary wheel running exercise. *P<0.05 between week 1 and week 2, week 2 and week3, and week 3 and week4, determined by Independent sample T-Test.

4.3.2.5 **Bouts of running activity**

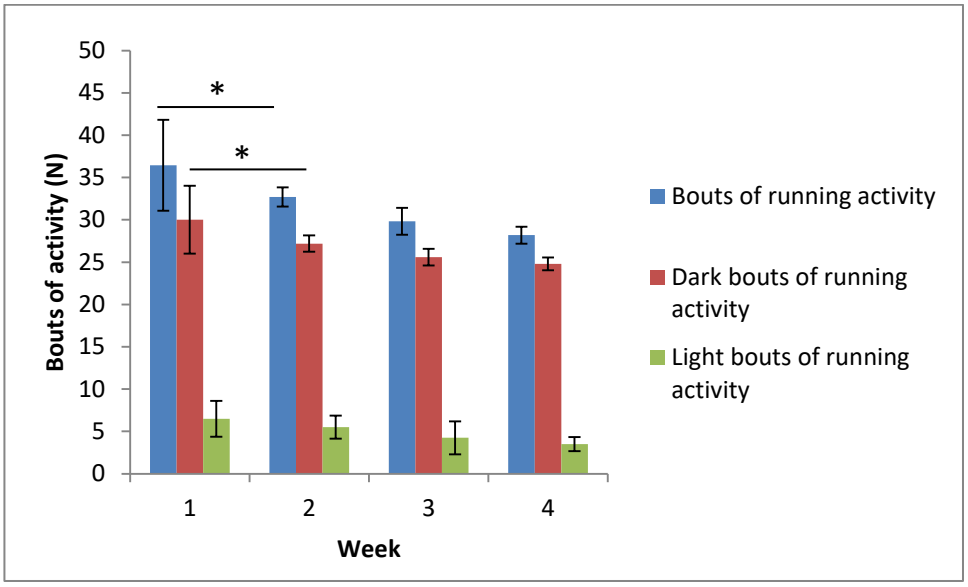

Figure 4.5: Mean, dark and light bouts of running activity per week of 4-week voluntary wheel running exercise. *P<0.05 between week 1 and week 2, week 2 and week3, and week 3 and week4, determined by Independent sample T-Test.

4.3.2.6 **Maximal running duration**

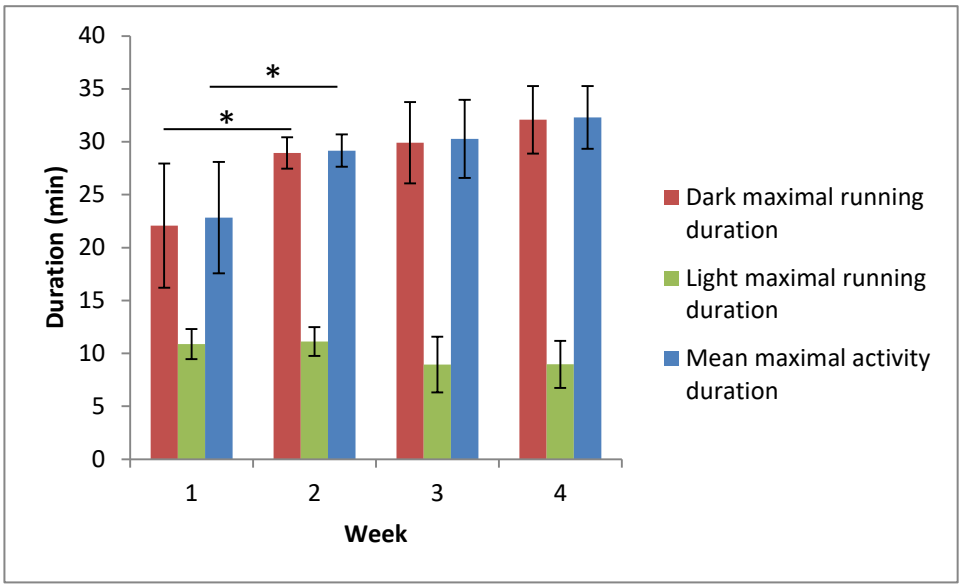

Figure 4.6: Maximal running duration per week of 4-week voluntary wheel running exercise. *P<0.05 between week 1 and week 2, week 2 and week3, and week 3 and week4, determined by Independent sample T-Test.

4.3.2.7 **Mean running duration**

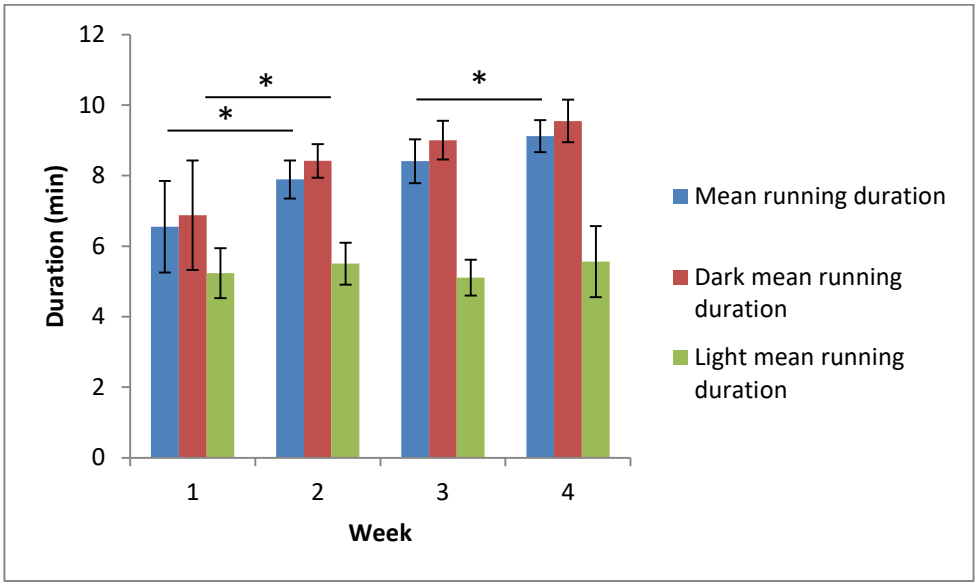

Figure 4.7: Mean, dark mean and light mean of running duration per week of 4-week voluntary wheel running exercise. *P<0.05 between week 1 and week 2, week 2 and week3, and week 3 and week4, determined by Independent sample T-Test.

4.3.2.8 **Mode of running duration**

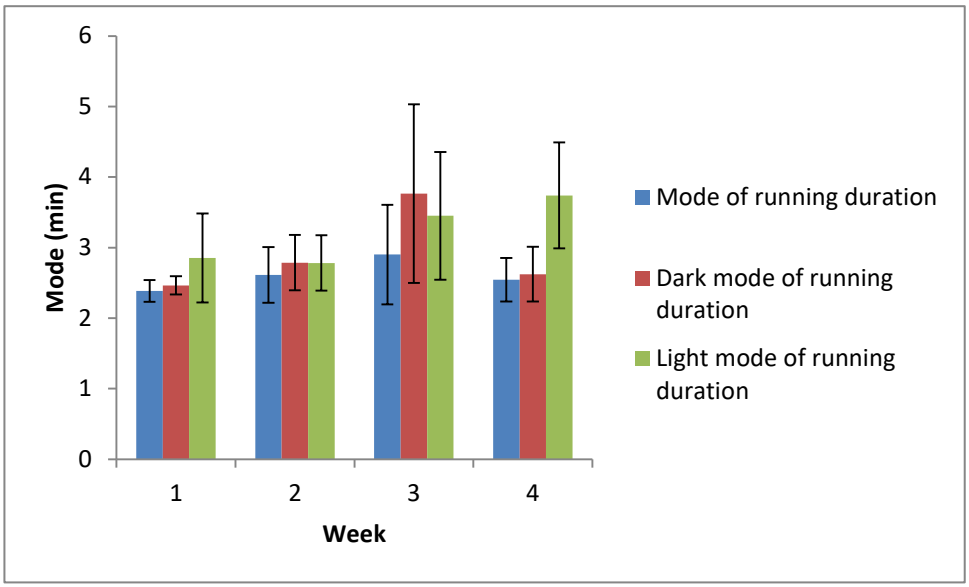

Figure 4.8: Mode, dark mode and light mode of running duration per week of 4-week voluntary wheel running exercise. *P<0.05 between week 1 and week 2, week 2 and week3, and week 3 and week4, determined by Independent sample T-Test.

4.3.3 **Global angiogenic indices**

The capillary to fibre ratio (C:F) of EDL of RW rat was significantly increased compared to SC, 1.79±0.07 *vs*. 1.62±0.01 (P<0.05), while CD of RW rats was similar SC group, 631±34 *vs*. 773±39 mm^{-2} (P>0.05), as MFA was greater in RW rats indicating fibre hypertrophy took place during the adaptation, 2966±283 *vs*. 2138±128 μm^2 (P<0.05).

Table 4.3: Global angiogenic indices of EDL of sedentary control and running wheel rats. *P<0.05 *vs*. SC.

Angiogenic indices	N	Group	Mean±SD	Significance
C:F	6	SC	1.44±0.04	
	7	RW	1.67±0.06	*0.009
CD, mm^{-2}	6	SC	666±151	
	7	RW	587±84	0.301
MFA, μm^2	6	SC	2322±546	
	7	RW	2971±273	*0.05

4.3.4 **Fibre type composition**

Fibre type composition

N_N of all fibre types of EDL of RW were similar to SC. TI A_A of RW was higher, but similar for TIIa and TIIBbcompared to SC (Table 4.4).

Table 4.4: Muscle fibre type composition of EDL of sedentary control and RW rats. *P<0.05 *vs*. SC.

Fibre composition	N	Group	Mean±SD	Significance
N_N				
TIIa	6	SC	0.291±0.10	
	7	RW	0.293±0.03	0.973
TIIb	6	SC	0.671±0.10	
	7	RW	0.657±0.02	0.725
TI	6	SC	0.050±0.01	
	7	RW	0.053±0.01	0.637
A_A				
TIIa	6	SC	0.176±0.09	
	7	RW	0.177±0.04	0.98
TIIb	6	SC	0.808±0.09	
	7	RW	0.801±0.04	0.874
TI	6	SC	0.021±0.003	
	7	RW	0.029±0.009	*0.009

4.3.5 **Capillary domain area (CDA)**

CDA distribution was not significantly different between groups (P>0.05). RW tended to show a higher frequency of larger CDA although the difference was not statistically significant (Figure 4.10). CDA of RW was more heterogeneous than SC, *P*<0.05 (Figure 4.11).

4.3.5.1 **CDA frequency distribution**

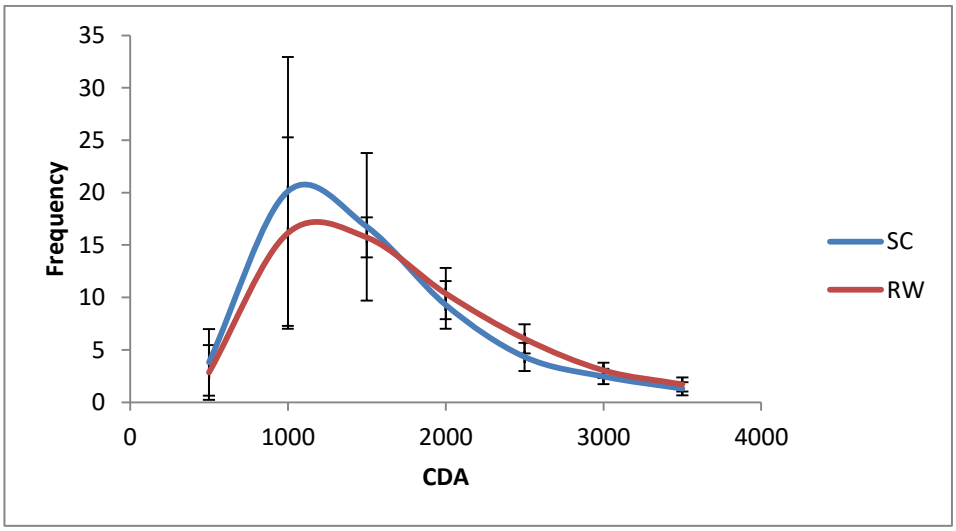

Figure 4.9: Frequency distribution of capillary domain area (CDA, µm^2) of SC and RW rats. Independent sample t-test: the mean difference is not significant for each class of CDA (all *n.s.*).

4.3.5.2 **Heterogeneity of CDA in EDL**

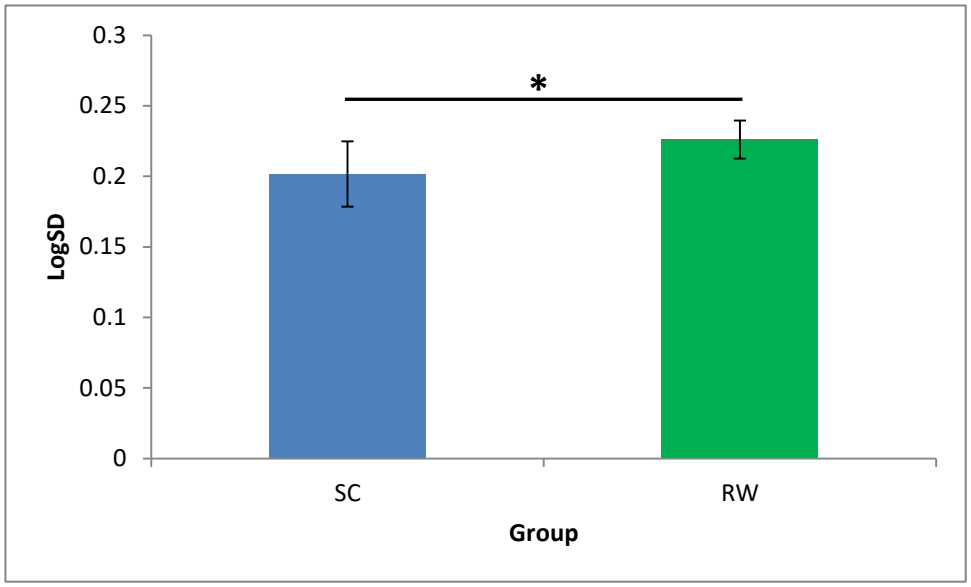

Figure 4.10: Heterogeneity of capillary domain area (LogSD CDA, µm^2) between SC and RW. Independent sample t-test: the mean difference of CDA heterogeneity were significant between *RW *vs*. SC.

4.3.6 **Local capillarity**

The local capillary to fibre ratio (LCFR) of RW was significantly higher than SC (1.45 *vs*. 1.16, *P*<0.05), a 25% rise. However, LCD was similar between groups (*P*>0.05) although RW showed a slightly lower density compared to SC group (Table 4.5).

Table 4.5: LCFR was higher in RW *P*<0.05 and LCD was similar *P*>0.05.

Parameter	Group	N	Mean±SD	Significance (*P* value)
LCFR	SC	6	1.16±0.17	
	RW	7	1.45±0.13	*0.007
LCD (μm^{-2})	SC	6	851±221	
	RW	7	768±126	0.436

4.3.7 **Estimated muscle functional capacity - oxygen transport modelling**

Representative histological images were selected from each study groups, with C:F close to the respective group mean value, to estimate EDL muscle oxygenation in a resting state and at simulated maximal exercise levels. Overall, RW rats showed relatively higher oxygen partial pressure than SC at rest. There was no evidence of hypoxia for either individual fibre types or the whole tissue. At maximal exercise, oxygen partial pressure of Type I, IIA and IIB of RW rats were relatively higher than SC (by 13, 18 and 51%, respectively). EDL muscles of RW rats had a greater physical oxygen supply (capillarity) compared to SC rats, and as a result hypoxia levels were lower (1.26 *vs*. 7.92%). Type IIB fibres were the only one that demonstrated the presence of hypoxia in both groups, but still this was lower in RW than SC rats (1.53 *vs*. 9.29%). Figure 4.12 depicts RW rats with reduced hypoxia indicated by faded blue colour compared to SC rats.

4.3.7.1 **Resting state**

Table 4.6: Oxygen partial pressure and percentage of hypoxia for each fibre types of EDL of RW and SC rats at resting state.

Compartment	PO_2 (mmHg)		% Hypoxia	
	SC	RW	SC	RW
Interstitium	26.5±1.9	28.01±1.3	0.00	0.00
Type I	28.1±0.8	29.0±0.4	0.00	0.00
Type IIa	27.6±1.0	28.6±0.6	0.00	0.00
Type IIb	25.8±1.7	27.5±1.1	0.00	0.00
All fibres	26.0±1.7	27.7±1.1	0.00	0.00
Tissue	26.1±1.8	27.8±1.1	0.00	0.00

4.3.7.2 **Maximal exercise level**

Table 4.7: Oxygen partial pressure and percentage of hypoxia for each fibre types of EDL of RW and SC rats at maximal exercise level.

Compartment	PO_2 (mmHg)		% Hypoxia	
	SC	RW	SC	RW
Interstitium	16.1±8.3	21.1±7.2	7.24	1.51
Type I	22.3±4.3	25.2±1.7	0.00	0.00
Type IIA	20.9±4.3	24.6±2.8	0.00	0.00
Type IIB	12.1±6.9	18.3±5.9	9.29	1.53
All fibres	13.3±7.3	19.5±5.9	7.99	1.24
Tissue	13.6±7.5	19.6±6.0	7.92	1.26

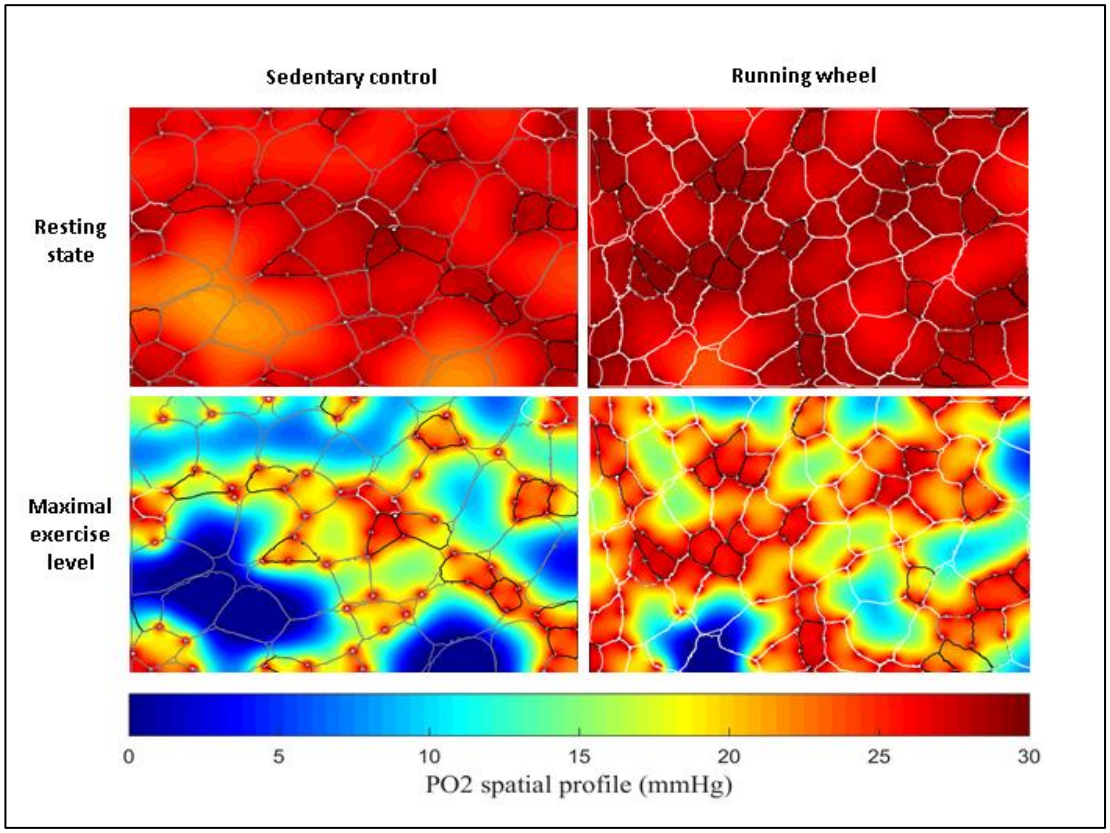

Figure 4.11: Estimated function of EDL of 4 week voluntary wheel exercise through mathematical modelling of muscle O_2 partial pressure, with O_2 consumption modelled at rest and during maximal exercise level.

4.4 Discussion

4.4.1 Rat general characteristics

The mean value for weekly body mass was not significantly different among groups due to appreciable biological variation among animals, as some of the rats ran less based on individual distance data that likely resulted in a higher body mass. Final rat body mass after 4 week wheel running exercise was relatively lower than SC, while the relative body mass decrease was similar compared to final body mass of 7 week voluntary wheel running, 7.7 *vs*. 6.4% (P>0.05). Therefore, 4 week running wheel data was in agreement with previous studies where this mode of exercise reduced body mass for both adult and young rats (Goodrick, 1980; Retzlaff et al., 1966)

4.4.2 Running wheel characteristic

Running distance of Wistar rats increased steadily over the study period, consistent with a previous study that found running wheel activity of male Wistar rats peaked 33-35 days after access to wheels, using a similar initial age i.e. 7 week old (Goodrick, 1980). A similar response was seen in a more aerobic strain, male Sprague-Dawley rats, where running distance increased until week 4 before declining towards week 12 (Sexton, 1995). Male Wistar rats in this study were classified as average performers based on mean weekly running distance, i.e. 28.9 km/week; compared to the norms of high performers (51.6±7.4 km/week), average performers (22.4±1.6 km/week), and low performers (6.4±0.8 km/week) as outlined by (Lambert & Noakes, 1990) and (Rodnick et al., 1989). High total running distance variability was also seen in a more aerobic strain - Sprague-Dawley - that were also grouped as high, average and low performers (Sexton, 1995). Mean daily running distance increased over the study period, again peaking at week 4 before it dropped in the following weeks (data from our preliminary study recorded distance run of RW rats gradually decreased from week 5 onwards). The same trend of running activity were also observed in studies that showed increased distance run of first four week exercise training before it declined (Harber et al., 2002; Laughlin, 1999). Pearson correlation test showed that running distance was moderately and negatively correlated with body mass (R= -0.672, P=0.001).

Rodents are nocturnal species, where this strain like other strains spent most on dark cycle engaged in running activity compared with the light period, 32.5±1.4% or 3.9 hours compared with 3.2±1.2% or 0.38 hours during daytime, indicating natural activity

pattern of rats was during the dark cycle. Mean time spent on running activity increased up to week 2 and it plateaued over the following weeks, while daytime running time kept decreasing across the period. Frequency of running bouts was negatively related with mean running duration. The number of running bouts slowly decreased with increasing mean running duration, while improved running endurance was supported by greater efficiency of oxygen delivery supported by an increased C:F in RW rats.

4.4.3 **Global angiogenic indices**

4 week voluntary wheel exercise increased C:F from 1.62 to 1.79, able to improve tissue aerobic capacity and support increased demand during running activity. Capillary density was not significantly different following training, and was 18% lower than SC, due to increased MFA which was significantly higher than SC, 2966±283 *vs*. 2138±128 (P=0.007). This finding was different to the 7 week pilot study, where the RW group showed relatively slight increase of CD of 9% compared to SC (P>0.05). This could be due to shorter period of exercise regime, thus limiting further muscle adaptation including reducing MFA and increasing CD, as shown by RWF rats (pilot study) and chronic swimming exercise rats (Bigard et al., 1991). However, a similar study of wheel exercise training using plantaris muscle of C57BL/6J mice showed increases in both C:F and CD with lower MFA after 4 weeks (Waters et al., 2004), although differences in body mass may introduce an allometric scaling issue.

4.4.4 **Fibre type composition**

Even though not significant, N_N of Type IIb of RW rats was 2% lower than SC. Percentage reduction of Type IIb of RW rats was less than in the pilot study, which recorded a 13% reduction. The smaller change might be due to insufficient exercise intensity to initiate significant fibre type shift to oxidative fibres, even at the first level of shifting order (Demirel et al., 1999; Saltin et al., 1977) where muscles tend to transform to oxidative type fibre to increase oxygen demand, which occurs in the order of Type IIb to Type IIa to Type I. While areal density (A_A) showed significant increase of Type I, indicating Type I fibre performed hypertrophy to support sustained muscle contraction during running activity, which was also seen in a study that demonstrated fibre type hypertrophy after 5 weeks running exercise (Andersen & Henriksson, 1977). N_N of Type IIa or I may increase if running wheel activity is extended, as shown by EDL muscle of RWF in the pilot study. Similarly, it was observed in a study where a significant increase in Type IIa fibre proportion was evident after 10 weeks wheel running exercise that muscle fibre shifted from glycolytic to fast oxidative fibres (Kariya et al., 2004).

Overall increase in mean fibre area, particularly in oxidative muscle fibres, may support increased running speed in RW rats. Even though only a small proportion, Type IIa fibre composition seemed to be relatively higher (6%) and TIIb lower (2%) in RW compared to SC rats, indicating increased capacity to produce forceful and sustained running characteristic (Bilodeau et al., 2009; Guderley et al., 2006). The effect of 4 weeks running exercise in Wistar rats appears insufficient to trigger significant fibre shift to oxidative fibre types, even though distance run was at peak (week 4).

4.4.5 **CDA frequency distribution**

Our result showed that mean capillary domain area was similar between RW and SC across class of capillary supply area, $P>0.05$. 4 week wheel running exercise produced no change in distribution of the area of muscle supplied by individual capillaries (capillary domain area). That mean CDA was not different from SC might be due to running distance variability, as low exercise volume offers only moderate stimulation for neovascularisation by capillary growth, and so could not significantly reduce the supply area for individual capillaries to enhance muscle oxygenation of exercising muscle (Al-Shammari et al., 2014; Kissane & Egginton, 2019). It was observed that muscle fibre hypertrophy accompanying capillary growth was a likely cause of the heterogeneous capillary spacing compared to SC, indicating long intercapillary distance underpinning local oxygen supply variability (Al-Shammari et al., 2019).

4.4.6 **Local capillarity and oxygen transport modelling**

Locally, chronic wheel exercise increased EDL muscle aerobic capacity by increasing LCFR to enhance muscle oxygenation supply along with fibre hypertrophy. Overall, the exercise regime was unable to reduce oxygen diffusion distance of the tissue as no reduction in MFA failed to increase LCD (Al-Shammari et al., 2019; Appell, 1990; Hortobágyi, 2000; Tomanek & Lund, 1974). Oxygen transport modelling demonstrated oxygen saturation of all fibre types in RW rat were relatively higher than SC, both at rest and maximal exercise levels. However, lower hypoxia levels in RW group at maximal exercise compared to SC (Figure 4.12) showed that improved local capillary supply area through increased LCFR was able to bring about a better oxygen tension distribution across the EDL muscle.

4.4.7 **Comparison between good and bad runners in the RW group: running performance and muscle phenotype changes.**

Massive individual variation among running rats led to great difference in exercise volume that they could run less than 10 km/week and more than 90 km/week (Lambert & Noakes, 1990; Mondon, Dolkas, Sims, & Reaven, 1985; Rodnick et al., 1989). Duration of endurance exercise did not enhance rat running desire as there was still great running distance variation after 8 and 12 weeks voluntary wheel running exercise, 3-77 and 4-74 km/week, respectively (Lambert & Noakes, 1990; Sexton, 1995). Running performance, angiogenic indices and fibre composition of good and bad runners were directly compared. A good runner produced 7-fold longer running distance compared to a bad runner which resulted in relatively greater C:F and CD accompanied by fibre atrophy. This effect could not be observed when average value of RW group produced similar CD and fibre hypertrophy (*vs*. control) that might be underestimated by lower running performance rats. The good runner empowered its aerobic capacity by increasing CD for extended running activity showing that continuous angiogenic stimuli (increased exercise volume) further enhanced muscle capillarity by reducing MFA of skeletal muscle. This was in line with a mice study that showed an average daily running distance of 10.2±0.7 km increased both C:F and CD of plantaris muscle (Waters et al., 2004). Therefore, two-fold lower in mean exercise volume in this study (4.7±1.3 km/day) could only increase C:F to support the muscle activity. The good runner exhibited higher proportion slow twitch fibres, +19.8% than the bad runner that indicated that fibre type transformation was dependent on exercise volume. Therefore, it was suggested that the variation in running performance among Wistar rat could be minimised by increasing sample size, thus improving data accuracy.

Table 4.8: Running performance, angiogenic indices and fibre type composition between good and bad runners.

	Good runner	Bad runner
Daily running distance (km)	5.74	0.81
C:F	1.86	1.54
MFA (µm²)	3162	3237
CD (mm^{-2})	622	509
NN		
TIIa	0.279	0.301
TIIb	0.665	0.663
TI	0.057	0.048
AA		
TIIa	0.167	0.184
TIIb	0.801	0.799
TI	0.032	0.024
LCFR	1.57	1.32
LCD (mm^{-2})	842	655

4.5 Conclusion

Voluntary wheel running exercise for 4 weeks among Wistar rats was able to stimulate global and local capillary expansion in EDL; C:F and LCFR. Increased mean fibre area in the tissue is crucial at early stage to support heavy body mass, which might be reduced if exercise activity was extended thus increasing CD to further enhance aerobic capacity for more sustained running activity. Rat biological variation in running capacity was an important factor in determining the magnitude of angiogenesis. This exercise regime tends to shift to more oxidative types as Type IIb fibres relatively decreased compared to SC composition.

Chapter 5 : Effect of 4-week voluntary wheel running exercise on rat hind limb muscles following unilateral femoral artery ligation.

5.1 Introduction

Exercise intolerance is prominent in arterial occlusive disease such as PAD and it is the main cause of critical limb ischemia. Reduction in blood supply occurs due to stenosis of conduit artery, thus limiting capillary perfusion and lowering oxygenation capacity to respiring tissues (Deveci & Egginton, 2002). Anatomic adaptations for this condition include an increase in size and/or density of muscle capillary network to enhance oxygen diffusion (Couffinhal, 1998; Roberts et al., 1997), but an inadequate stimulus would limit angiogenesis rate due to impaired nitric oxide production (Brown et al., 2003), or enlargement of collateral vessels to bypass e.g. femoral artery occlusion, thus lowering large artery resistance (Brown et al., 2003; Conrad, 1977). The main target in PAD treatment is to enhance circulatory reserve in skeletal muscle to enhance oxygen delivery, thus improving exercise tolerance for prolong muscle activity (Conrad, 1977; Deveci & Egginton, 2002). Vasodilators may increase blood flow to ischaemic muscle by dilatation or increasing collateral vessel development, which maintains a persistent increase of blood flow velocity. A similar effect to chronic vasodilatation on collateral expansion was also observed in exercise training (Lloyd et al., 2001; Schirmer, 2015), however use of vasodilators *per se* did not increase exercise tolerance (Conrad, 1977) showing oxygen diffusion capacity is unaltered at a downstream level to support increased muscle activity. However, other studies showed capillarity and fatigue resistance of ischaemic muscle were improved following treatment with vasodilators (Fulgenzi et al., 1998).

Chronic ligation of a single lower limb artery reduced capillarity in soleus and gastrocnemius muscles after 8 weeks (Yang et al., 1991). Although 3 weeks ligation did not change C:F of rabbit TA and soleus, tissue oxygenation was impaired (Cherwek et al., 2000). A similar capillary density was reported in mice soleus muscle after 28 days of ligation, due to muscle necrosis (Couffinhal et al., 1998; Yamaguchi et al., 1994) which resulted in smaller fibre area (Couffinhal et al., 1998; Hudlicka & Torres, 1990), especially in Type IIb fibres (Regensteiner et al., 1993a). The impairment was also evident in oxidative fibres, thus attenuating muscle performance in both slow and fast skeletal muscles (Dawson & Hudlicka, 1990).

Exercise is non-invasive approach to treat PAD with potentially fewer side effects. Impaired capillary perfusion due to insufficient blood supply would affect optimal tissue

function, and alteration in metabolic demand by increasing muscle function may result in adaptive remodelling in working muscles (Deveci & Egginton, 2002). Exercise activity in the ischaemic model would increase blood flow in collateral-dependent vessels supplying tissues, and increased shear stress would enhance capillarity *via* mechanical stimuli in active skeletal muscle with microcirculation dysfunction (Lloyd et al., 2001), showing the potential of therapeutic angiogenesis in tacking the impairment (Ferrara & Alitalo, 1999; Lloyd, Prior, Yang, & Terjung, 2003; Lloyd et al., 2001).

It is reported that exaggerated stimulation of ischaemic muscle activity leads to detrimental effects, for instance a higher percentage of damaged muscle fibres accompanied longer walking distances (Sjöström, 1982). Ischaemic rat muscle exhibited loss of muscle mass, fibre atrophy and showed abnormal centrally located nuclei in fibres following chronic electrical stimulation at high frequency (Hudlicka, Price, Hoppeler, Uhlmann, & Egginton, 1988), and even impaired capillary growth normally seen with stimulation (Hudlická & Price, 1990). Extensive damage to capillary endothelial cells occurred following induced ischaemic, both in human (Gidlöf, Lewis, & Hammersen, 1988) and rat muscle (Egginton, Hudlicka, & Glover, 1993; Sjöström, 1982). A moderate exercise intensity or less stressful exercise modality may be appropriate for treating such tissue. Therefore, it is proposed that a natural and physically tolerable approach, i.e. through voluntary wheel running exercise, would be a better option for exercising activity in a rat model of PAD.

Exercise intolerance was also evident in PAD patients which contributes to morbidity and mortality among the elderly (Golomb, Dang, & Criqui, 2006). Decreased muscle function and severity of claudication in PAD patients are highly associated with a sedentary lifestyle (McDermott, 2006; McDermott et al., 2011). The attenuated muscle function of individuals with vascular insufficiency was observed in both clinical (Zetterquist, 1970) and animal studies (unilateral or bilateral femoral ligation; (Conrad, 1977). Exercise training among patients with intermittent claudication indicated an improved muscle function as there was significant increase in maximal walking-distance (Cucato, 2013; Larsen & Lassen, 1966; Leicht, Crowther, & Golledge, 2015; Schlager, 2012; Tew et al., 2009, 2015). Enhanced maximum walking time (Hiatt et al., 1994; Larsen & Lassen, 1966; Sanderson, 2006) and pain-free walking distance (Collins, 2005; McDermott et al., 2008; Wood, 2006) were also reported in PAD patients who underwent supervised treadmill walking exercise. At present it is unclear to what extent this may be attributed to macro- or microvascular improvements.

Most previous studies in rats showed that voluntary wheel exercise produced significant physiological improvement to cardiovascular and musculoskeletal systems (Lambert, Van Zyl, Jaunky, Lambert, & Noakes, 1996; Lerman et al., 2002; Rodnick et al., 1989). This showed potential use of this less stressful exercise modality as a treatment for cardiovascular disease so as to enhance or restore affected physiological function. Our preliminary work on 7-week voluntary wheel exercise demonstrated that rats significantly improved running distance (exercise volume), which peaked at week 4.

This parallels significant capillary expansion in EDL muscle accompanying fibre hypertrophy, and showed potential of voluntary wheel exercise in treating PAD disease by stimulating capillary bed expansion *via* increasing C:F and CD. Therefore, this study aimed to determine the effect of 4-week voluntary wheel running exercise on skeletal muscle during femoral artery stenosis (*via* unilateral ligation), a rat model of PAD. Running behaviour after arterial ligation would be assessed to determine whether the ligation could impact running performance of Wistar rats.

The aims of this study was to determine femoral ligated rats' performance in 4-week voluntary wheel running exercise that attenuates deleterious effects on skeletal muscle fatigue resistance resulting from local vascular insufficiency (unilateral femoral artery ligation), improving extent of the microcirculation and preventing atrophy of skeletal muscle. We hypothesised that: 1) running wheel exercise has a preventative effect on the adverse effects of local blood flow reduction on skeletal muscle performance. 2) Rats with unilateral femoral artery ligation perform wheel exercise to a lesser extent due to limited running capacity, improving with exercise training due to improved blood and oxygen supply. 3) Mean fibre area of EDL muscle in unilateral femoral artery-ligated hind limbs rats decrease, and fibre type composition change to more oxidative fibres following exercise intervention. By measuring the effects of exercise on skeletal muscle phenotype following arterial ligation, we provide new data on the underlying factors that may determine exercise intolerance in disease and how these impairments may be better targetted by therapeutic interventions.

5.2 Materials and methods

5.2.1 Animals

25 male Wistar rats with similar body mass, 195±10 g (6 week old) were obtained from Animal Unit of Central Biological Services of University of Leeds. They were divided into 4 groups; sedentary control (SC), ligation control (LC), running wheel (RW) and ligation running wheel (LRW); n= 5, 7, 7 and 7 rats, respectively. RW and LRW were housed individually in cages with access to a running wheel connected to a logger and computer. Water and food were provided *ad libitum* under a 12:12 light: dark cycle (lights on at 7:00). All procedures were conducted in accordance with the Guiding Principles in the Animals (Scientific Procedures) Act 1986 approved by the Home Office. The animal room was entered twice a day at random times during the day to check the water bottles and food supply. The cage bedding was replaced once a week during cage cleaning day.

5.2.2 Voluntary running wheel exercise

Animals were housed individually and randomly in standard metal cages attached to wheels (33cm diameter) that were interfaced to a computer and revolutions are recorded in 1min intervals continuously for 4 weeks.

5.2.3 Food and water consumption

Food and water consumed were weekly measured during cage cleaning day by subtracting initial value with amount left.

5.2.4 Running parameters

Refer to general method chapter

5.2.5 Femoral artery ligation

Male Wistar rats of LC and LRW groups were anaesthetised using 4% isoflurane with 4l/min of oxygen flow through the chamber for induction. Left thigh area was shaved and body mass was recorded. On a surgery mat (temperature set at 37°C), an anaesthetic mask was placed and isoflurane concentration was reduced to 1.5-2.5% with 1l/min O2 (maintenance) followed by 0.1ml analgesia (buprenorphine (Vetergesic®, Ceva, Amersham, UK) 0.05 mg kg-1) by subcutaneous injection at the back of the neck. Ethanol was sprayed on the shaved skin and wiped with paper tissues. Under isoflurane anaesthesia, an incision was made (about 1.5 cm in length) at the midline of the thigh. Then, a ligation on left femoral arteries was performed aseptically distal to the inguinal ligament with a 3.0 surgical

suture i.e. about 5mm to superficial epigastric artery (H. Yang, Ogilvie, & Terjung, 1995b). Two drops of antibiotic (Enrofloxacin (Baytril®, Bayer, Reading, UK) 2.5 mg kg-1) were applied on the incision, and opened skin was sutured. Blood on the skin was cleaned using sterile saline and the animals were placed in recovery chambers (set at 37°C).

5.2.6 Schedule 1 killing and muscle dissection

Refer to general method chapter

5.2.7 Freezing and cryosections

Refer to general method chapter

5.2.8 Monoclonal-myosin heavy chain antibodies, lectin and laminin staining

Refer to general method chapter

5.2.9 Area of sampling region

Refer to general method chapter

5.2.10 Capillary and fibre composition; gross and regional CD, C: F and MFA.

Refer to general method chapter

5.2.11 Local capillarity and capillary domain area determination

Refer to general method chapter

5.2.12 Data analysis

Differences in running performance between RW and LRW were determined using independent samples T-Test. Differences in cardiovascular parameters, fatigue index (FI) and blood flow between groups were determined using ANOVA with Tukey post hoc tests. 2-way ANOVA with Tukey *post hoc* tests were used to determine interaction between femoral artery ligation and exercise volume on muscle capillarity. Statistical testing was conducted in SPSS (v.25). Data are presented throughout as mean ± SD and statistical tests were considered significant where $P \leq 0.05$.

5.3 Results

5.3.1 Cardiovascular characteristics of SC, LC, RW and LRW rats

A total of 30 rats were divided into four groups; sedentary control (SC) - 6 rats, ligation control (LC) - 7 rats, running wheel (RW) - 9 rats and ligation running wheel (LRW) - 7rats. All body mass were similar; 191±13, 188±7, 197±10 and 202±5 g, respectively (P>0.05).The final body mass at the end of 4 weeks were similar between LC, RW and LRW groups; 317±13, 312±42 and 328±31 g, respectively (P>0.05). Non-exercise heart mass was lower than exercise groups (P<0.05); cardiac enlargement was significant in RW, but not in LRW (P>0.05). The lack of a significant difference of LRW compared to LC was due to large individual heart mass variability. Carotid BP and heart rate were similar across the groups (P>0.05). Tail BP and carotid:tail BP were higher and lower, respectively in RW, LC and LRW groups than control group (Table 5.1). EDL muscle of RW group underwent hypertrophy as its muscle mass was greater than SC group (P<0.05), and it was relatively higher than LC and LRW EDL mass.

Table 5.1: Cardiovascular characteristics of SC, LC, RW and LRW rats. Unshared letters denote statistical significance (P<0.05) as determined by ANOVA with Tukey post-hoc tests.

Parameter		Mean±SD			
		SC	LC	RW	LRW
N		4	7	9	7
Body mass (g)	Initial	191±13^{a}	188±7^{a}	197±10^{a}	202±5^{a}
	Final	338±28^{a}	317±13^{b}	312±42^{b}	328±31^{b}
Heart mass (% Mb)		0.27 ± 0.04^{a}	0.27±0.02^{ac}	0.32 ± 0.04^{b}	0.29±0.03^{abc}
EDL mass (g)		0.162±0.03	0.174±0.01	0.199±0.03^{a}	0.18±0.02
BP (carotid; mmHg)		122 ± 3^{a}	129±9^{a}	124 ± 13^{a}	120 ± 14^{a}
BP (tail; mmHg)		92 ± 7^{a}	121±12^{b}	115 ± 11^{b}	107 ± 15^{ab}
carotid:tail BP		1.33 ± 0.13^{a}	1.05±0.04^{b}	1.08 ± 0.11^{ab}	1.09 ± 0.13^{b}
HR (bpm)		394 ± 43^{a}	381±24^{a}	383 ± 53^{a}	396 ± 32^{a}

5.3.2 Food and water consumption of RW and LRW

First week food consumption was greater in LRW compared to RW (P<0.05), however RW rats consumed more food after 4 weeks of wheel running exercise (P<0.05). Water intake was similar between RW and LRW rats, but gradually increased in RW group over the period.

Table 5.1: Food and water consumption of RW and LRW over 4 week period. Food and water intakes by RW and LRW rats were different during the study period. * P<0.05 vs. RW.

Parameter		Mean±SD		*P*-Value
		RW	LRW	
N		9	7	
Food consumption (g)				
	Week 1	111±14	161±11	*<0.05
	Week 2	182±33	167±30	>0.05
	Week 3	181±16	176±30	>0.05
	Week 4	190±17	152±16	*<0.05
Water consumption (ml)				
	Week 1	179±53	239±102	>0.05
	Week 2	225±78	221±68	>0.05
	Week 3	234±54	237±94	>0.05
	Week 4	274±117	179±72	>0.05

5.3.3 **Running characteristic of unilateral femoral artery ligated rats**

Total running distance increased over the period and was significantly higher in RW group (for every week) compared to LRW group, which ran half the distance (P<0.05). Night-time running distance showed a similar trend as demonstrated by total running distance, with RW group significantly higher than LRW for every week (P<0.05). During the first week dark cycle mean running velocity was similar between groups, but was significantly different from week 2 to week 4 in which both showed increasing trend, while daytime mean running velocity was similar between groups over the period (Table 5.2). Light cycle running distance was similar between RW and LRW at the end of study period in which there was a marked increase in LRW group from week 1 to week 2 (+63%, 0.07 to 0.20 km), the distance then was similar for week 3 and week 4 compared to RW group. Mean running velocity was similar between groups for week 1 and significantly different from week 2 onwards (P<0.05), with LRW running slower than RW rats. However, the mode of running velocity was similar between groups at first two week and the end of 4 week wheel exercise (P>0.05), while median running velocity for both groups showed an increasing trend for first three weeks and a similar speed at the end of study period (Table 5.2). Dark cycle mean running velocity was similar between groups in the first week, and then it was significantly different from week 2 to week 4 in which both showed an increasing trend, while daytime mean running velocity was similar between groups over the period.

LRW rats were less active than RW rats in performing exercise, depending on the measure used (Table 5.2). The frequency of running activity was similar between groups, with a decreasing trend demonstrated by both groups, while maximal running duration was showed an upwards trend in both groups. However, LRW maximal running capacity was significantly lower than RW over the study period, where RW plateaued from week 2. Mean bout duration was significantly lower in LRW than RW rats over the study period; although the LRW group showed a slow and steady increase they only reached half of RW duration by the end of study. Mode of running duration was similar for both groups at week 4; while LRW ran consistently with a similar mode of running duration for the whole study, RW mode was slightly higher at week 3.

LRW rats were less active than RW rats in performing exercise at night after the second week (Table 5.2). The number of bouts was similar during the study period, with a decreasing trend showed by both groups, but an upwards trend for maximal running duration was seen in both groups. However, LRW maximal running capacity was significantly lower than RW across the study (P<0.05), where RW plateaued from week 2, as

was mean running duration. Although LRW group showed a slow and steadily increase in mean running duration, they reached only half of RW group by the end of the study (P<0.05). However, mode of running duration was similar for both groups at week 4; while LRW ran consistently with a similar mode of for the, RW mode was slightly higher at week 3 at greater.

LRW rats were as active as RW rats during daytime across the study period (P>0.05). Light running bout of LRW was similar to RW rats. Even though it was not statistically significant, LRW seemed to run more frequently compared to RW from the second to the fourth week, which may be biologically relevant. Light maximal, mean and mode running duration were similar between LRW and RW until wheel running exercise was completed (P>0.05; Table 5.2).

5.3.3.1 **Running parameters**

Table 5.2: Running parameters of RW and LRW rats of 4 week voluntary wheel running exercise. Independent sample T-Test: *P<0.05 *vs*. RW.

Running parameters	Group	N	Week			
			1	2	3	4
			Mean±SD			
Total distance (km)	RW	7	2.09±0.73	4.52±060	5.96±0.40	6.70±0.67
	LRW	7	1.05±0.49*	1.61±0.22*	1.74±0.18*	2.66±0.26*
Dark cycle total distance (km)	RW	7	2.13±0.86	4.37±0.55	5.84±0.34	6.33±0.56
	LRW	7	0.99±0.52*	1.42±0.20*	1.57±0.25*	2.51±0.31*
Light cycle total distance (km)	RW	7	0.13±0.09	0.16±0.08	0.12±0.10	0.18±0.13
	LRW	7	0.07±0.04	0.20±0.05	0.17±0.16	0.14±0.16
Mean pulse velocity (ms^{-1})	RW	7	0.66±0.08	0.77±0.02	0.82±0.02	0.84±0.01
	LRW	7	0.59±0.08	0.72±0.02*	0.74±0.03*	0.78±0.01*
Mode pulse velocity (ms^{-1})	RW	7	0.70±0.10	0.82±0.08	0.81±0.06	0.83±0.10
	LRW	7	0.58±0.25	0.71±0.10	0.61±0.20*	0.87±0.19
Median Pulse velocity (ms^{-1})	RW	7	0.70±0.09	0.82±0.03	0.88±0.02	0.89±0.01
	LRW	7	0.62±0.10	0.78±0.02*	0.82±0.04*	0.87±0.02
Dark Mean Pulse velocity (ms^{-1})	RW	7	0.67±0.09	0.78±0.02	0.83±0.01	0.84±0.01
	LRW	7	0.60±0.08	0.73±0.02*	0.75±0.03*	0.79±0.01*
Light mean pulse velocity (ms^{-1})	RW	7	0.48±0.10	0.54±0.04	0.52±0.09	0.56±0.10
	LRW	7	0.40±0.06	0.53±0.01	0.52±0.07	0.53±0.10
Time active (%)	RW	7	13.52±1.40	17.88±1.61	18.88±1.31	18.95±1.53
	LRW	7	10.94±2.01*	11.38±1.44*	11.74±0.82*	13.02±1.27*
Max activity duration (min)	RW	7	18.30±5.29	32.75±7.36	33.4±6.59	33.19±3.24
	LRW	7	10.52±3.84*	14.6±1.85*	16.4±1.10*	22.01±2.19*
Bouts of activity (N)	RW	7	34±4	30±5	29±2	28±2
	LRW	7	38±4	32±4	28±2	27±4
Mean of activity duration (min)	RW	7	5.62±1.31	8.63±1.27	9.14±0.76	9.47±0.37
	LRW	7	3.77±0.74*	4.79±0.27*	5.29±0.43*	6.28±0.41*
Mode of activity duration (min)	RW	7	2.30±0.35	2.23±0.20	3.29±1.94	2.44±0.28
	LRW	7	2.20±0.23	2.41±0.25	2.22±0.16	2.64±0.40
Dark time active (%)	RW	7	23.86±3.29	32.66±2.01	35.25±1.88	35.06±3.73
	LRW	7	19.54±4.62*	18.94±1.95*	19.97±1.77*	23.04±1.09*
Dark Bouts of activity (N)	RW	7	29±4	26±3	25±1	25±3
	LRW	7	33±5	26±2	24±2	23±1
Dark max activity duration (min)	RW	7	17.81±5.61	32.56±7.49	33.34±6.65	32.74±3.33
	LRW	7	10.24±4.01*	13.33±1.77*	15.99±1.05*	21.19±3.26*

Dark mean activity duration (min)	RW	7	5.82±1.43	9.27±1.32	9.78±0.91	9.75±0.29
	LRW	7	3.83±0.82*	4.78±0.34*	5.44±0.50*	6.55±0.48*
Dark mode activity duration (min)	RW	7	2.41±0.28	2.22±0.27	4.69±3.36	2.36±0.18
	LRW	7	2.28±0.32	2.41±0.22	2.32±0.25	2.87±0.35
Light time active (%)	RW	7	3.11±1.67	3.08±1.41	2.47±1.27	2.82±1.95
	LRW	7	2.31±1.04	3.8±1.23	3.49±2.51	2.97±2.88
Light Bouts of activity	RW	7	5±2	4±2	3±2	3±2
	LRW	7	5±2	6±2	5±3	4±3
Light max activity duration (min)	RW	7	6.41±3.21	7.83±	6.80±3.39	7.99±4.19
	LRW	7	4.52±1.34	7.08±1.78	6.90±2.96	7.88±5.13
Light mean activity duration (min)	RW	7	3.82±0.96	4.62±0.80	4.43±0.81	5.53±1.73
	LRW	7	3.28±0.52	4.11±0.42	4.01±0.85	4.08±0.90
Light mode activity duration (min)	RW	7	2.47±0.42	2.49±0.66	3.41±0.93	4.38±2.28
	LRW	7	2.72±0.77	2.63±0.71	2.88±0.34	2.66±0.38

5.3.4 **Global angiogenic indices**

C:F of RW was significantly higher (1.79±0.07) than SC, LC and LRW (1.44±0.04, 1.33±0.07 and 1.48±0.07, respectively, *P*<0.05). LRW C:F was greater than LC (*P*<0.05) but lower than SC (*P*<0.05). CD was 26% lower in LC (*P*<0.05) and similar in RW and LRW compared to SC (*P*>0.05). MFA of RW was higher compared to SC, LC and LRW (2322±546, 2264±277 and 2319±297 *vs*. 2966±283 μm², respectively, *P*<0.05); LRW was 9% higher than LC. Specific MFA showed that Type I MFA was similar between SC, LC and LRW (803±149, 896±100 and 932±142 μm², respectively), and significantly higher in RW (1180±209 μm²) (*P*<0.05). Type IIa fibres of RW (1478±242 μm²) and LC (1246±148 μm²) were larger than SC and LRW (1129±174 and 1195±191 μm²) (*P*<0.05). Fast-glycolytic muscle fibres were similar between groups (*P*>0.05).

Table 5.3: Global angiogenic indices of SC, LC, RW and LRW rats post 4 week voluntary wheel running exercise. Unshared letters denote statistical significance (*P*<0.05) as determined by ANOVA with Tukey post-hoc-tests.

Group	N	C:F	CD (mm^{-2})	MFA (μm²)
SC	6	1.44±0.04[a]	666±151[e]	2322±546[g]
LC	7	1.33±0.07[b]	614±86[f]	2264±277[g]
LRW	7	1.48±0.07[c]	667±46[e]	2319±297[g]
RW	9	1.79±0.07[d]	631±34[e]	2966±283[h]

Table 5.4: MFA of fibre types of EDL of SC, LC, LRW and RW rats post 4 week voluntary wheel running exercise. Unshared letters denote statistical significance (*P*<0.05) as determined by ANOVA with Tukey post-hoc-tests.

Group	N	MFA (μm²)		
		Type I	Type IIa	Type IIb
SC	6	803±149[a]	1129±174[c]	2511±510
LC	7	896±100[a]	1246±148[cd]	2593±257
LRW	7	932±142[a]	1195±191[c]	2536±347
RW	9	1180±209[b]	1478±242[d]	3024±261

5.3.5 **Fibre type composition**

Numerical density (N_N) of type I, IIA and IIB fibres were similar between groups (P>0.05). N_N TI of LC was relatively lower than SC while N_N TI was relatively higher in RW and LRW than SC and LC. N_N of all fibre types of LRW was similar to RW (Figure 5.5). Areal density A_A was not significantly different between groups (Figure 5.5). However, LRW and RW were relatively greater in A_A TI compared to SC. TI fibres of LRW and RW seemed to undergo hypertrophy as their areal density were greater than SC and LC. LC appeared to adapt with the condition by increasing type IIA areal and numerical density (Table 5.5).

Table 5.5: Fibre type composition; numerical density and areal density of fibre type I, IIA and IIB of SC, LC, RW and LRW following 4 week voluntary wheel running exercise. Unshared letters denote statistical significance (P<0.05) as determined by ANOVA with Tukey post-hoc-tests.

Fibre composition	Group	N	Mean±SD
N_NTI	SC	6	0.022±0.01[a]
	LC	7	0.020±0.01[a]
	RW	7	0.028±0.02[a]
	LRW	7	0.028±0.01[a]
N_NTIIA	SC	6	0.176±0.09[b]
	LC	7	0.213±0.04[b]
	RW	7	0.177±0.04[b]
	LRW	7	0.163±0.02[b]
N_NTIIB	SC	6	0.808±0.09[c]
	LC	7	0.772±0.04[c]
	RW	7	0.802±0.04[c]
	LRW	7	0.818±0.02[c]
A_ATI	SC	6	0.050±0.01[a]
	LC	7	0.043±0.01[a]
	RW	7	0.053±0.01[a]
	LRW	7	0.056±0.01[a]
A_ATIIA	SC	6	0.291±0.10[b]
	LC	7	0.346±0.04[b]
	RW	7	0.293±0.03[b]
	LRW	7	0.280±0.03[b]
A_ATIIB	SC	6	0.671±0.10[c]
	LC	7	0.623±0.05[c]
	RW	7	0.666±0.03[c]
	LRW	7	0.682±0.03[c]

5.3.6 **Capillary domain area**

5.3.6.1 **CDA distribution**

CDA distribution was not significantly different between groups (P>0.05). LRW showed higher frequency of small CDA (500, 1000 and 1500 µm² CDA), and RW tend to have greater CDA distribution at 2000, 2500, 3000 and 3500 µm² (Figure 5.6).

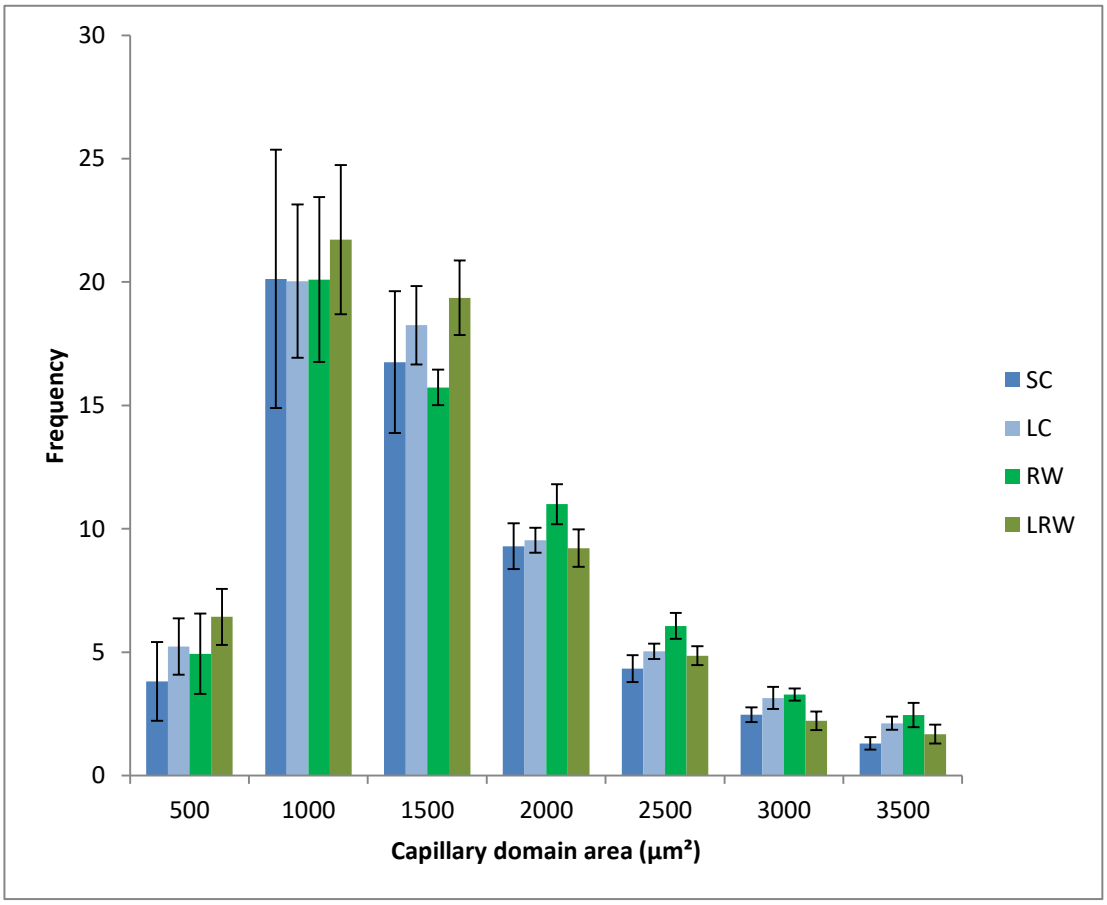

Figure 5.1: Frequency of capillary domain area (CDA) of SC, LC, RW and LRW. One-way ANOVA: the mean difference is not significant for each class of CDA (all *n.s.*).

5.3.6.2 **Heterogeneity of CDA between groups.**

CDA of LRW and RW was more heterogeneous than SC and LC (P<0.05). Homogeneity of CDA was observed in both control (SC and LC) and exercised groups (RW and LRW), P>0.05.

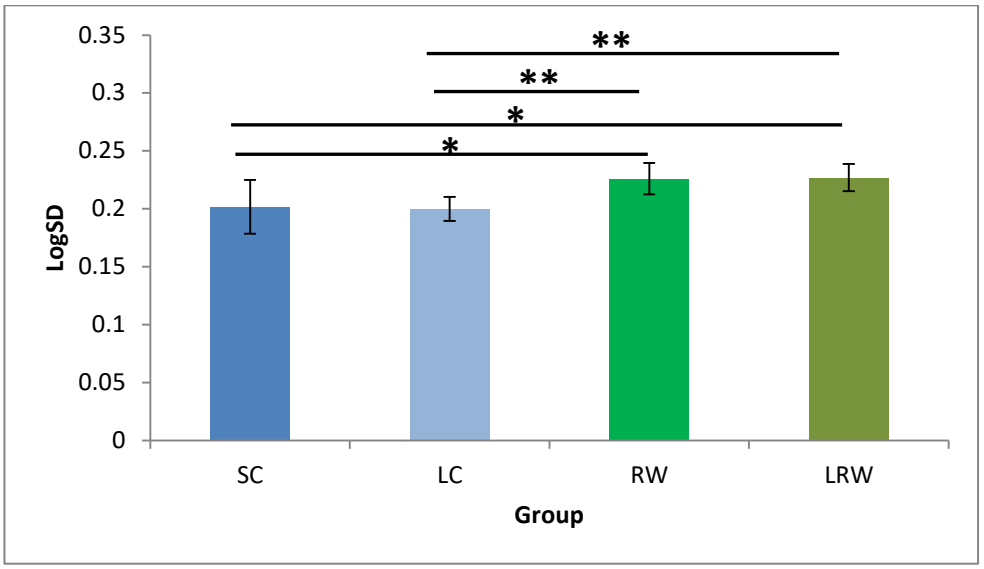

Figure 5.2: Heterogeneity of capillary domain area between SC, LC, RW and LRW. One-way ANOVA: the mean difference of CDA heterogeneity were significant *P<0.05 between RW and LRW *vs*. SC, and **P<0.01 between RW and LRW *vs.* LC.

5.3.7 **Local capillarity**

5.3.7.1 **Local capillary to fibre ratio (LCFR) and local capillary density (LCD)**

LCFR and LCD of LRW were not significantly different compared with SC (1.38 *vs*. 1.16 and 886±132 *vs.* 852±221 μm^{-2}, P>0.05; Table 5.6). Relative increase in LCFR of LRW was 19% and 7% greater than SC and LC, respectively (Figure 5.8). LCD was similar across groups (P>0.05), although RW showed a decreased trend compared to SC group (Figure 5.9).

Table 5.6: LCFR and LCD of SC, LC, RW and LRW. The mean difference of LCFR is significant between RW and SC, $_P$<0.05 and LCD is not significant among groups (all *n.s.*) as determined by One-way ANOVA with Tukey *post-hoc* tests.

Parameters	Group	N	Mean±SD
LFCR	SC	6	1.16 ± 0.17^{a}
	LC	7	1.29 ± 0.14^{ab}
	RW	7	1.45 ± 0.13^{b}
	LRW	7	1.38 ± 0.26^{ab}
LCD (mm^{-2})	SC	6	852 ± 221^{a}
	LC	7	806 ± 123^{a}
	RW	7	768 ± 126^{a}
	LRW	7	886 ± 132^{a}

5.3.7.2 **Relationship between MFA and LCFR**

MFA was positively associated with mean LCFR with a week strength of association, *r*=0.257 (*P*=0.206) (Figure 5.3). Exercise intervention increased MFA of RW EDL along with a higher LCFR. Similar pattern was observed in LRW EDL that demonstrated expansion of local capillarity with fibre hypertrophy (Figure 5.4).

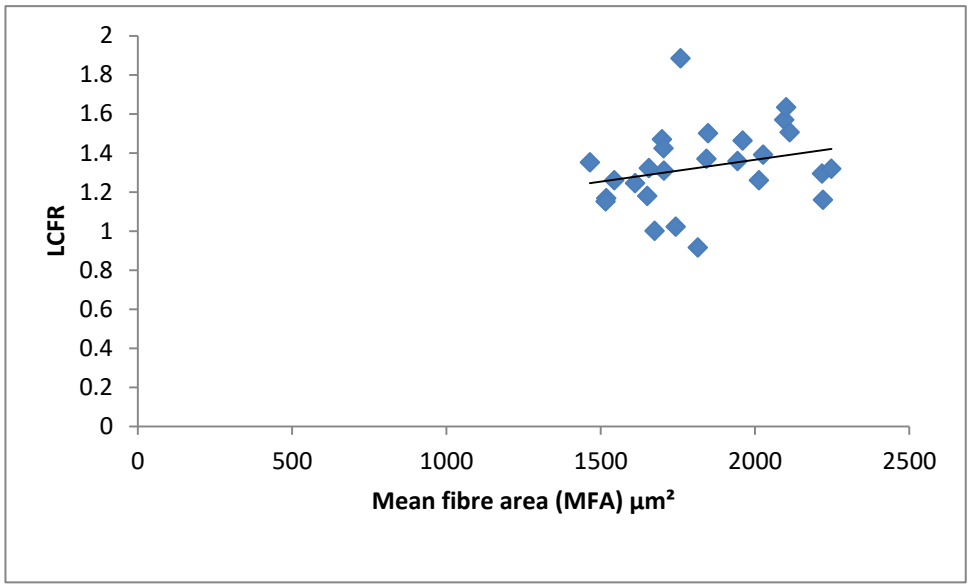

Figure 5.3: MFA of EDL plotted against LCFR that MFA was directly proportional to LCFR, r=0.257, *P*=0.206.

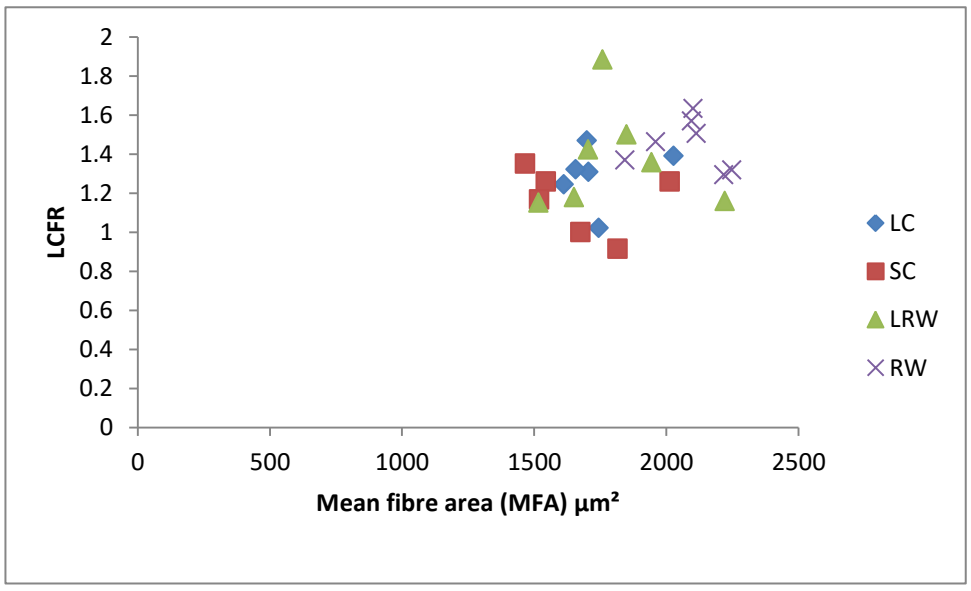

Figure 5.4: MFA of EDL of exercise rats was positively correlated with LCFR, r=0.877, *P*=0.123.

5.3.8 **Effects of femoral artery ligation and exercise volume on muscle capillarity**

A two-way between groups analysis of variance (2-way ANOVA) was conducted to explore the impact of femoral artery ligation and exercise volume on muscle capillarity (C:F). Rats were divided into three groups according to their running distance (low: 0-2 km/day; moderate: 3-7 km/day; high: 8 and above km/day). The interaction effect between arterial ligation and exercise volume was not statistically significant, F (1, 6) = 1.885, *P* = 0.219. There was a statistically significant main effect for exercise volume, F (2, 6) = 17.067, *P*=0.003, the effect size was large (partial eta squared=0.850). Post-hoc comparisons using the Tukey HSD test indicated that the mean C:F for high (10.8km) and moderate running distance (M = 5.24, SD =1.13) was significantly different from the low running distance (M = 1.43, SD = 0.17). The high running distance group did not differ significantly from moderate runners. The main effect for femoral artery ligation, F (1, 6) = 10.52, *P*= 0.018, reached statistical significance, the effect size was large (partial eta squared=0.637).

5.3.9 **Estimated muscle function - oxygen transport modelling**

Overall, the wheel running rats (RW and LRW) showed a relatively higher estimated oxygen tension than sedentary rats (SC and LC) at simulated rest (Table 5.7). There was no evidence of hypoxia for either individual fibres or the whole tissue. At simulated maximal exercise levels, oxygen tension of wheel running rats was relatively higher than SC and LC, enriching oxygenation of working muscles. The extent of hypoxia was relatively greater in SC and LC rats at maximal exercise level compared to exercised rats, particularly in type IIB fibres as indicated by presence of blue colour with higher intensity depicted by oxygen transport modelling images (Figure 5.10). Only LC demonstrated the presence of hypoxia in type IIA fibres during maximal exercise level (Table 5.8).

5.3.9.1 **Resting state**

Table 5.7: Oxygen partial pressure and percentage of hypoxia for each fibre types of EDL of SC, LC, RW and LRW at resting state.

Compartment	PO_2 (mmHg)				% Hypoxia			
	SC	LC	RW	LRW	SC	LC	RW	LRW
Interstitia	26.5±1.9	27.3±1.6	28.0±1.3	27.2±2.4	0	0	0	0
Type I	28.1±0.8	28.2±0.4	29.0±0.4	29.5±0.1	0	0	0	0
Type IIa	27.6±1.0	27.7±1.0	28.6±0.6	28.8±0.4	0	0	0	0
Type IIb	25.8±1.7	26.5±1.6	27.5±1.1	27.2±1.8	0	0	0	0
All fibres	26.0±1.7	26.9±1.5	27.7±1.1	27.5±1.7	0	0	0	0
Tissue	26.1±1.8	26.9±1.6	27.8±1.1	27.5±1.9	0	0	0	0

5.3.9.2 **Maximal exercise level**

Table 5.8: Oxygen partial pressure and percentage of hypoxia for each fibre types of EDL of SC, LC, RW and LRW at maximal exercise level.

Compartment	PO_2 (mmHg)				% Hypoxia			
	SC	LC	RW	LRW	SC	LC	RW	LRW
Interstitia	16.1±8.3	18.3±7.4	21.1±7.2	18.6±9.7	7.24	3.25	1.51	13.70
Type I	22.3±4.3	22.4±1.9	25.2±1.7	28.4±0.35	0	0	0	0
Type IIa	20.9±4.3	20.2±4.8	24.6±2.8	25.5±2.1	0	0.22	0	0
Type IIb	12.1±6.9	14.4±6.7	18.3±5.9	17.5±7.4	9.29	4.61	1.53	4.58
All fibres	13.3±7.3	16.4±6.7	19.5±5.9	19.1±7.4	7.99	3.16	1.24	3.65
Tissue	13.6±7.5	16.6±6.9	19.6±6.0	19.0±7.8	7.92	3.17	1.26	5.27

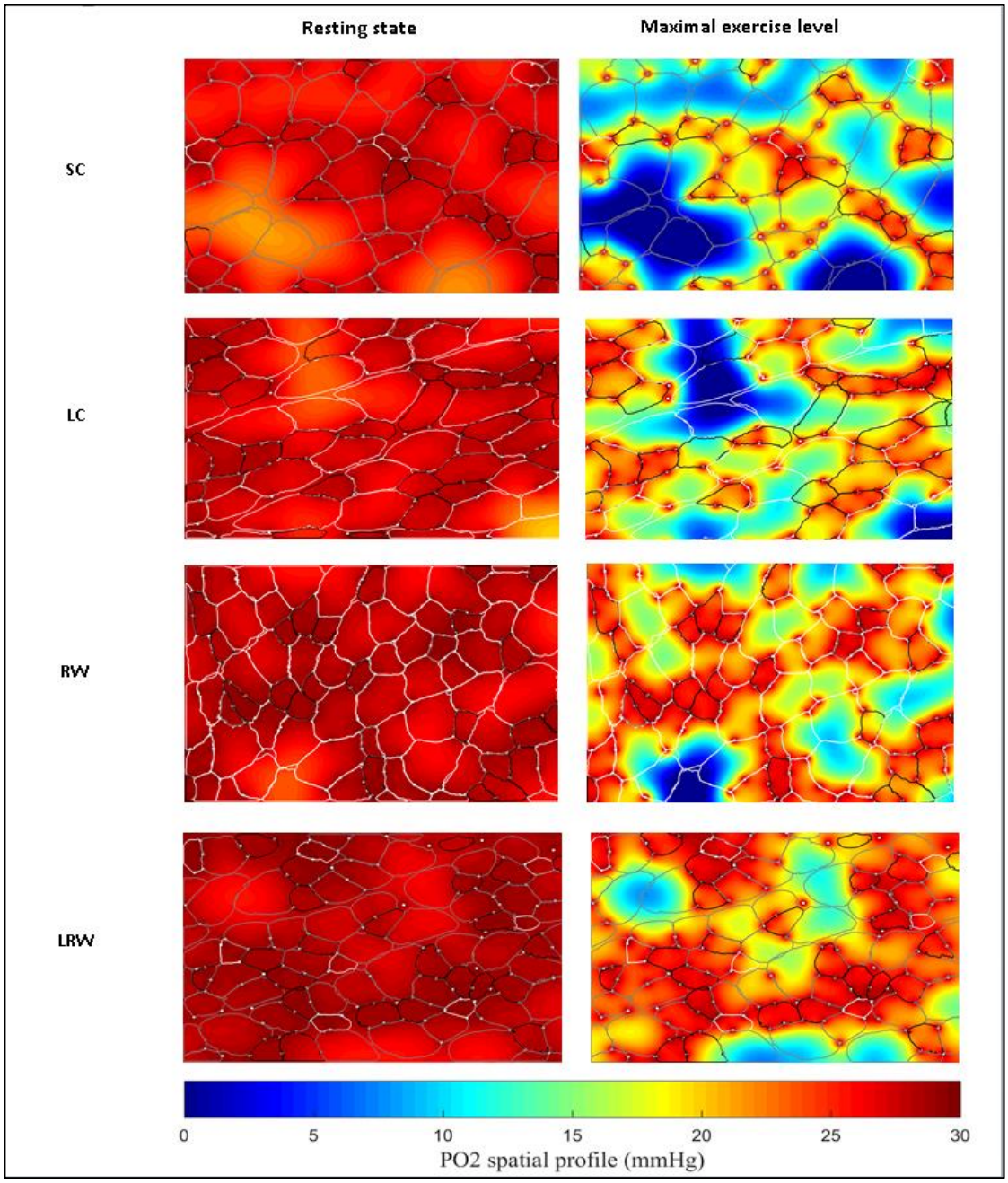

Figure 5.5: Estimated function of EDL of SC, LC, RW and LRW rats through mathematical modelling of muscle O_2 partial pressure, with O_2 consumption modelled at rest and during maximal exercise level.

5.3.10 **Muscle performance (FI)**

Fatigue index of LC was less than control (0.38±0.05 *vs*. 0.49 ± 0.06), while exercise induced a modest increase in muscle performance of LRW rats with a 21% increase in fatigue resistance compared to control rats (Table 5.10). Exercise itself did not significantly enhance muscle performance but maintained aerobic capacity to similar level of control rats (P>0.05). Femoral blood flow of exercised rats was comparable to controls, while wheel exercise activity in LRW did not improve blood flow which was similar to LC rats. Rest and stimulated flow of non-ligated right femoral artery were similar between LC and LRW groups (P>0.05).

Table 5.9: EDL fatigue resistance and hindlimb blood flow. Unshared letters denote statistical significance (P<0.05) as determined by ANOVA with Tukey post-hoc tests.

		Control	RW	LC	LRW	LC	LRW
				ligated		Non-ligated	
Fatigue index		0.49 ± 0.06[a]	0.45 ± 0.05[a]	0.38±0.05[b]	0.46±0.05[ab]	-	-
Femoral flow (ml/min)	rest	1.09 ± 0.22[a]	1.32 ± 0.39[a]	0.08±0.05[b]	0.09±0.02[b]	1.30±0.57[a]	1.27±0.46[a]
	end-stimulation	1.74 ± 0.23[a]	2.07 ± 0.81[a]	0.08±0.04[b]	0.09±0.03[b]	1.75±0.76[a]	1.83±0.91[a]

5.4 Discussion

5.4.1 **Animal characteristics**

The mean final body mass of all groups were not significantly different from each other due to intrinsic biological variability, e.g. running distance capacity, that likely led to different morphology. The low relative final body mass of LRW compared with SC is consistent with a reduced ability of ligated rats to perform exercise due to limited aerobic capacity (especially O_2 delivery). This finding was similar to non-ligated rats, where sedentary control Sprague Dawley rats had similar body mass as those with free access to running wheels for 10 or 30 weeks (Kariya et al., 2004). However, other studies of at least 6 weeks running wheel exercise recorded a significant reduction in final body mass, in which exercise volume still varied among individuals, observed in both male and female rats (Goodrick, 1980; Rodnick et al., 1989; Sexton, 1995). This indicates that longer duration of wheel exercise may motivate animals to perform sustainable exercise activity, in which case the influence of biological variation on the capacity for locomotor activity could be reduced by increasing sample size and/or intragroup normalisation of physiological responses. It is worth noting that low mean running distance was associated with higher body mass in exercised rats, and negatively associated with final body mass as rats were classified into low, medium and high running capacity after 6 week training period, performed 3.1±0.4, 7.6±0.5 and 12.6±0.4 km/day with final body mass 385±12, 368±13 and 332±8 g, respectively (Rodnick et al., 1989).

Muscle mass of LRW was relatively greater than both SC and LC animals, by 13 and 5%, respectively, paralleling the low degree of fibre hypertrophy took place during chronic running 2319±297 *vs*. 2138±128 (SC) and 2264±277 (LC) μm^2, respectively. This is consistent with other exercise studies showing increased muscles mass in slow-twitch soleus muscle, ranged from 13% (medium runners) to 20% (low runners) of male Sprague-Dawley rats but not in fast-twitch plantaris after 6 weeks running (Rodnick et al., 1989), and that muscle to body mass ratio of rat plantaris increased by 13% after 30 weeks wheel exercise (Kariya et al., 2004).

A central determinant of aerobic capacity is cardiac mass (CM), as a surrogate for potential cardiac output and hence blood flow delivery to locomotor muscles. CM of LRW was not significantly different from SC, LC and RW groups and unchanged CM was also found in running wheel rats following 6 weeks (Rodnick et al., 1989) and 12 weeks (Sexton, 1995) voluntary wheel running. This shows that wheel exercise for 4 weeks was not of

sufficient intensity to challenge the cardiovascular system to work beyond its normal capacity, avoiding e.g. volume overload hypertrophy associated with endurance performance. This ensures that estimates of local tissue remodelling are appropriate measures to assess physiological limits to activity pattern.

5.4.2 **Exercise characteristics**

To our knowledge this is the first study using the less aerobic strain of rat, Wistar, to study the effects of chronic voluntary wheel running exercise on muscle capillarity and fibre type shift following unilateral femoral artery ligation, and behaviour of ligated rats run in this manner. Weekly running distance of LRW rats showed an increasing trend, indicating their capacity was improving and muscle adaptation was still continuing during at week 4. However, although they ran less than RW rats and at slower pace – i.e. displayed lower exercise volume (-60%) and lower exercise intensity (-7%) - they demonstrated a similar trend of daily running distance increment as RW animals, with peak running distance reached after 4 weeks training. The lower distance ran by LRW rats (which ranged from 0.91 to 3.04km) was likely due to shorter running duration (-32.8%), but with a similar number of running bouts during the dark cycle, than RW rats, suggesting muscle fatigue due to limited hindlimb blood flow and/or attenuated capillary perfusion. Unilateral femoral artery ligation did not change the diurnal running behaviour, as they predominantly run at night and were much less active during day time, similar to RW rats. The mean running speed of LRW was -7% lower than RW, which could be explained by a relatively low Type IIa composition (0.163±0.02 *vs*. 0.177±0.04 N_N and 0.280±0.03 *vs*. 0.293±0.03 A_A). However, as running speed tended to gradually increase over the period this suggests that LRW EDL muscle might be adapting to greater force development, and improving running capacity by transforming fibre composition to a higher proportion of fast oxidative fibres.

5.4.3 **Global angiogenic indices**

Four week voluntary wheel exercise increased C:F in RW rats (1.79±0.07 *vs*. 1.62±0.01), reflecting an angiogenic response, but fibre hypertrophy in EDL (involving a 6% rise in Type IIa fibres compared to SC) resulting in a similar CD, thus maintaining potential oxygen delivery to support endurance exercise. This is similar to a 4 week wheel running study that showed increases of C:F and CD in RW mice (Waters et al., 2004). The current study showed no increase in CD, likely due to a lower training volume performed by RW rat compared to mice (5.85±0.41 *vs*. 10.2±0.7 km/day), suggesting that this animal model mimics mild-moderate running exercise. A human study showed similar findings, where cycling exercise

training for 5 weeks led to fibre hypertrophy accompanied by an increase in C:F but unchanged CD (Andersen & Henriksson, 1977).

Capillarity of LC EDL was greatly decreased, as both C:F and CD were lower than SC (-22 and -26%, respectively), indicating a failure to recruit angiogenesis and hence not compensate for reduced blood flow by increasing diffusive exchange. MFA remained intact confirming that true capillary rarefaction occurred without muscle atrophy. Rat slow-twitch muscle (soleus) showed muscle atrophy following 28 day unilateral ligation that resulted in increased CD rather than real capillary growth (Yamaguchi et al., 1994), suggesting muscle adaptation under attenuated blood supply is muscle specific.

Four week voluntary wheel running was insufficient to stimulate angiogenesis in LRW (C:F was 8.6% lower compared to SC) in contrast to RW (C:F was 10.5% higher). However, compared to LC rats, wheel running exercise was able to stimulate mild capillary bed expansion in LRW rats by increasing C:F, potentially supporting an increased exercise demand across the study period. Neither exercise volume nor running intensity was sufficient to increase C:F to the same extent as RW vs. SC rats (Δ10.5%). Therefore, a progressive increase in running distance in LRW rats was apparently not limited by diffusive capacity of the microvasculature, as CD was reduced compared to SC due to fibre hypertrophy.

Hindlimb-ligated male Sprague-Dawley rats exercised on a treadmill at 20m/min (5-10 minutes at 15% grade) for 5 days recorded a 25% increase in capillary contacts per fibre of white gastrocnemius muscle (Lloyd et al., 2001). The effect was more evident when rats ran at 20 to 25m/min, four times a day (one hour per session), which then increased capillarity by 57% (Lloyd et al., 2003), demonstrating that exercise intensity affected the extent of muscle adaptation.

Capillary growth seemed to take place in fast muscle as C:F of LRW increased in plantaris which were run on a treadmill, but not in slow soleus muscle (Roberts et al., 1997). However, in this 4-week wheel study there was little capillary growth observed in EDL muscle of LRW (lower than SC, higher than LC) that might be caused by insufficient angiogenic stimulus due to low exercise volume. A low to moderate intensity of endurance exercise mainly recruits slow twitch fibres (Abernethy, Thayer, & Taylor, 1990) that the current exercise modality may favour. However, MFA of Type I fibre in LRW rats was similar to LC and SC, but RW rats showed a greater value than other groups (P<0.05) (Table 5.4) showing an extended duration of exercise is needed for a significant MFA change in slow twitch fibres in the ligation group.

2-way ANOVA analysis showed that femoral artery ligation reduced overall running performance of LRW rats compared to RW rats. There was only one LRW rat that was classified as a moderate runner, 3.1 km/day running distance, compared to slow runners (0.6- 2.1 km/day, N=6). In this study, the angiogenic response in LRW after wheel exercise was sufficient to prevent capillary rarefaction, thus attenuating the development of muscle ischaemia. There was a statistically significant main effect for exercise volume on C:F, with a greater C:F in moderate and good runners, mostly from RW rats. Therefore, more samples are needed for future work to increase the likelihood of having good LRW runners, and with an improved dynamic range of responders the resolution of the analysis will be improved.

5.4.4 **Fibre type composition**

EDL muscle has a heterogeneous composition, for example in Sprague Dawley rats composed of about 42% fast oxidative glycolytic (FOG), 56% fast glycolytic (FG) and 2% slow oxidative (SO) fibres (Ariano, Edgerton, & Armstrong, 1973; Armstrong & Phelps, 1984). SC of Wistar rat EDL composition was 20% (FOG) 78% (FG) and 2% (SO) fibres, suggesting they are a lower aerobic capacity strain (Armstrong & Phelps, 1984). The present study showed that 4-week running did not change fibre type proportion in EDL of LRW rats, compared to SC. A shorter exercise period could be the reason for no significant fibre composition alteration, as a 35-week wheel running led to a 20% reduction in number of Type IIb fibres (Kariya et al., 2004).

The present study showed that fibre type composition of LRW (N_N and A_A) after 4 weeks was not different to SC, LC or RW rats, indicating that this exercise programme was insufficient to initiate fibre type shift. Fibre composition of LC was similar to SC, which is consistent with reported data on fibre type distribution in gastrocnemius muscle of PAD patients compared to age-matched controls (Clyne, 1982; Henriksson et al., 1980; Jansson et al., 1988). Type IIa A_A of LC EDL was relatively higher than SC (by 19%), which parallels a study that reported a 35% higher percentage of Type IIa fibres in muscles of patients with intermittent claudication (Hammarsten et al., 1980). Low exercise intensity and volume performed by LRW caused Type I hypertrophy (relatively higher N_N and A_A density, 40 and 30% higher than LC, respectively), that was compensated by decreased Type IIa N_N and A_A density (24% and 19% lower than LC, respectively). Hypertrophy in Type I fibres might be attributed to slow-twitch fibre recruitment during submaximal exercise exerted by LRW rats during running wheel exercise (Abernethy et al., 1990).

5.4.5 **Capillary domain area heterogeneity**

The 4 week exercise programme did not change CDA of LRW, and its distribution was similar to other groups for each CDA class; this parameter is important in demonstrating that local heterogeneities in O_2 supply were not exacerbated by treatment. This was similar to the pilot study (7-week voluntary wheel exercise) where RW rats showed no shift in CDA distribution. Mean exercise intensity and/ or volume with different running capacity among RW rats led to insignificant CDA distribution change. The running capacity of LRW rats was half that of RW rats, which might reduce the magnitude of angiogenic stimulus even further, thus being unable to decrease the muscle supply area due to an absence of new capillaries (Al-Shammari et al., 2014).

5.4.6 **Local capillarity and oxygen transport modelling**

The exercise regime resulted in a significant LCFR enhancement in RW rats (25% higher than SC). Muscle local adaptation to the chronic exercise involved capillary growth which led to a greater fraction of capillary domain (supply) areas overlapping a fibre, thus improving individual fibres ability to function aerobically i.e. oxygen delivery and metabolite removal is likely improved (Al-Shammari et al., 2019). Application of ligation did not change LCFR of ischaemic muscle (LC) compared to SC but it was relatively lower (7%) than LRW, suggesting that local capillary supply of LC rats was not severely altered following 4-week attenuated femoral blood supply, possibly a result of hypokinesia. In LRW, the exercise resulted in a modest LCFR increase compared to SC group value. This showed that wheel running activity had the potential to stimulate local capillary expansion *via* LCFR augmentation, i.e. 19% increase *vs*. SC, to support increased oxygen demand during exercise. The lack of a statistically significant effect of exercise may be due to biological variability in exercise performance, but it may still be physiologically relevant. A similar lack of difference in the scale-independent index of local capillary supply (LCD) suggests that any alteration in fibre size had little effect on sharing O_2 among different fibre types in this mixed muscle.

Calculated oxygen tension in the resting state of RW was relatively similar between all groups, mean PO_2= 27.8±1.1 *vs*. 26.1±1.8 (SC), 26.9±1.6 (LC) and 27.5±1.9 (LRW) mmHg. Overt microcirculation improvement in RW EDL muscle was observed only at maximal exercise levels, illustrated by a 44% (*vs*. SC) and 18% (*vs*. LC) increase in oxygen partial pressure. It was noted that oxygen tension in the resting state and exercise level of LRW EDL were similar to RW rats, indicating an obvious improvement in microcirculation

efficiency due to chronic wheel exercise training where oxygenation increased by 43% (*vs*. SC) and 17% (*vs*. LC).

RW rats improved muscle functional capacity, indicated by lower tissue regions of hypoxia compared with SC and LC (1.26, 7.92 and 3.17%, respectively), attributed to an enhanced capillary bed expansion. Overall, the exercise programme was not able to ameliorate the extent of muscle hypoxia in LRW during maximal exercise levels, as this was relatively higher than LC (5.27 *vs*. 3.17%). A greater proportion of muscle experiencing hypoxia in LRW relative to LC could be due to presence of interstitial values that might influence the mean tissue value, as the degree of hypoxia between the two groups was similar when expressed as average fibre hypoxia percentage, although this influence is likely to be small. Fibre specific hypoxia of LRW showed Type IIa fibres tend to be less hypoxic (-22%) and Type IIb were similar (3.65 *vs*. 3.16%) compared with LC, demonstrating some capacity to ameliorate the effects of attenuated hindlimb blood flow that may become significant with an increased intensity and/or duration of exercise.

5.4.7 **Muscle performance**

Significant running activity levels in RW rats increased the relative heart mass (expressed as body mass percentage), likely in response to increased cardiac output needed during prolong muscle activity to support greater physiological demand. However, the significantly lower running distance in LRW, i.e. one-third of RW intensity, resulted in a similar heart body mass ratio compared to control and LC rats. It is thought that regular exercise protects against myocyte loss, with no loss of mass, and favours reactive hypertrophy in the left ventricle (Kwak, Song, & Lawler, 2006).

EDL muscle of LC rats with limited limb blood supply showed attenuated muscle performance, as fatigue index (FI) was 22% lower compared to control group (i.e. reduced endurance capacity). This was paralleled by a significant reduction in femoral blood flow, up to 95% during muscle stimulation. A similar effect was observed in a previous study that showed 76-93% blood flow reduction in rat following unilateral iliac artery ligation (Elander, Idstrom, Schersten, & Bylund-Fellenius, 1985), and a similar magnitude of blood flow restriction was recorded during leg exercise in peripheral arterial disease (PAD) patients with claudication pain (Bylund-Fellenius, 1981). Isolated ischaemic canine gastrocnemius muscle demonstrated a 30% reduction in muscle force that correlated with a 67% reduction in arterial blood flow (Hogan, Kurdak, & Arthur, 1996). With all these findings, it was suggested that muscle performance was blood flow dependent (SjØgaard, 1987), and an adequate blood supply is essential to support sustained muscle activity.

Exercise alone (RW) had little effect on FI, as presumably the animals were exercising within their physiological capacity and did not exert themselves into a heavy exercise domain. In other words, the exercise may have maintained muscle, cardiorespiratory and/or metabolic health, but did not challenge the system – i.e. exceed capacity - sufficiently to induce any obvious adaptive response.

Wheel exercise did indeed improve FI of LRW rats, but this was independent of any improvement in limb blood flow (the functional hyperaemia was minimal), which suggests an improved efficiency in peripheral oxygen transport to support aerobic-based activity: presumably a combination of microvascular adaptations (Roberts, Nixon, Unthank, & Lash, 1997), muscle fibre size and/or mitochondrial content (fibre type, levels of oxidative enzymes) that improved oxygen diffusion (and possibly metabolite removal) (Andersen & Henriksson, 1977) (Yang, Ogilvie, & Terjung, 1994). Femoral ligated rats increased muscle performance following treadmill walking exercise, with a significant increase in blood flow to the hindlimbs due to an increase in collateral artery blood flow after the primary vessel (femoral artery) was surgically ligated (Yang, Ogilvie, & Terjung, 1995a). This suggests that 4 week wheel running sufficiently restored muscle function by stimulating only a modest angiogenic response, but was inadequate to stimulate noticeable collateral artery formation.

5.5 Conclusion

In conclusion, femoral artery ligated Wistar rats were able to voluntarily and naturally exercise in the running wheels, though at lower speed and with reduced distance, both of which showed improvement over the study period. Voluntary wheel running exercise for 4 weeks had a minimal effect on LRW capillary bed expansion, indicated by C:F, and the training period was insufficient to elicit the same effect as in intact rats. Increased exercise volume would be an important factor to further enhance angiogenesis in LRW rats so that aerobic capacity could be improved, and thus increase running capacity of the ischaemic muscles. EDL muscle performance of LRW was able to be restored after 4-week voluntary wheel running exercise which improved fatigue resistance; further improvement might be supported by collateral blood vessel formation to match increased metabolic demand during more intense or prolonged running activity.

Chapter 6 : Effect of 4-week voluntary wheel running exercise on rat hind limb muscles following aortic banding.

6.1 Introduction

Persistent exercise intolerance is one of the manifestations of cardiac dysfunction that is linked with chronic heart failure (CHF), compromising quality of life and contributing to widespread hospitalisation and death worldwide. The main factors that lead to CHF are ischaemia, acute inflammation, arterial hypertension and valve incompetence (Francis, 2001). One animal model used to study these effects is the application of arterial stenosis by banding the abdominal aorta, which causes haemodynamic pressure overload to the heart (Olivetti, 2000). The heart may adapt to this condition including molecular, cellular and interstitial myocardial changes - cardiac remodelling - causing changes in cardiac size, form and function (Cohn, Ferrari, & Sharpe, 2000).

Microvascular rarefaction is evident in cardiac and vascular disease which may contribute to declining skeletal muscle performance. Muscle fatiguability is, however, poorly associated with left ventricular ejection fraction in CHF (Franciosa, Park, & Levine, 1981; Rogers, 2001), suggesting that it is largely determined by peripheral factors (Pandey, 2015). Impaired muscle function resulting from CHF is characterised by appearance of sarcopenia and fibre type transformation from Type I to Type IIa (Drexler, 1992) or IIb (Mancini, 1989; Sullivan, Green, & Cobb, 1990) such changes are associated with reduced oxidative capacity, and limited muscle function is likely due to structural and functional changes in the microcirculation during exercise (Duscha, 1999; Richardson, Kindig, Musch, & Poole, 2003; Tickle et al., 2020). Low skeletal muscle capillary density was seen in experimental studies (Bowen, 2017; Kindig, Musch, Basaraba, & Poole, 1999; Nusz, 2003; Richardson et al., 2003) and clinical cases (Duscha et al., 1999; Schaufelberger, Eriksson, Grimby, Held, & Swedberg, 1995; Wadowski, 2018) of CHF, and this diminished capillary bed resulted in limited oxygenation in working muscle, thus reducing endurance capacity in performing sustained (aerobic) activity (Hauton, 2015; Tickle et al., 2020).

Cardiac dysfunction that induces peripheral muscle capillary rarefaction indicates systemic effects may occur rapidly (< 21 days) after development of perturbed heart function (Nusz et al., 2003). Furthermore, a disturbed anatomical distribution of capillaries may further compromise muscle function because increased heterogeneity of capillary spacing can impair tissue oxygenation (Degens et al., 2006; Piiper & Scheid, 1991). Reduced muscle activity may result in reduction of oxidative enzyme content of skeletal muscle,

muscle mass and capillary density (Fleg & Lakatta, 1988; Frontera, Meredith, O'Reilly, Knuttgen, & Evans, 1988; Shephard, Bouhlel, Vandewalle, & Monod, 1988). Hence, muscle fibre atrophy is expected in this compensated cardiac hypertrophy model.

Altered fibre type percentage was observed in slow twitch soleus muscle of rats where CHF was induced by aortic banding for 7 months with a decrease in Type I and an increase in Type IIA proportion, consistent with MHC type I content being reduced with a higher MHC type IIA content. However, the proportion of fast MHC in gastrocnemius remained unchanged (De Sousa, Veksler, Bigard, Mateo, & Ventura-Clapier, 2000). Similar morphological changes in soleus have also been noticed, even during the transition period to CHF development (Carvalho, 2003), while Type IIA fibre atrophy was found in the muscle during overt CHF even though similar muscle mass was recorded (Carvalho et al., 2003; Sullivan et al., 1990), which might result from fibrosis (Filippatos, 2003).

Muscle capillarity apparently decreased both in patients with CHF and rats where CHF was induced by aortic banding (De Sousa et al., 2000; Duscha et al., 1999; Sullivan et al., 1990). The impairment has been shown in slow and fast fibres of aortic banded rats, with a decrease in C:F (De Sousa et al., 2000) indicating a true capillary rarefaction due to muscle atrophy as a result of decreased functional demand (Nusz et al., 2003).

It has been proposed by previous studies that physical training has a beneficial effect on cardiac remodelling for patients with heart disease and severe cardiac dysfunction (Diwan & Dorn, 2007; Erbs, 2010; Giannuzzi, Temporelli, Corrà, & Tavazzi, 2003; Kavanagh, 2002; Smart, 2011). It is also recommended for patients with stenotic lesions based on clinical examination and haemodynamic data (Bonow, Cheitlin, Crawford, & Douglas, 2005). In animal studies, 8 week voluntary wheel running exercise in aortic constricted mice found no significant effect on LV function (van Deel, 2011). However, aortic-banded swine preserved left ventricle function upon performing a low-intensity treadmill running, 3 days/week, 55 min/day for 15 weeks, which was associated with reduced fibrosis and collagen levels, and attenuated mitochondrial dysfunction, proving a beneficial effect of exercise in restoring cardiac hypertrophy (Emter & Baines, 2010). A similar treadmill training programme for 10 weeks was also able to alleviate cardiac remodelling and severity of heart failure in Wistar rats (Pacagnelli, 2014).

It has been showed by previous work on the effect of 4-week voluntary wheel exercise on unilateral femoral arterial ligation rats that EDL muscle of LRW rats increased oxygen supply by enhancing C:F and maintaining MFA, even though running distance was about half that of RW rats (Chapter 5). However, the exercise effect on EDL muscle with

systemic stenosis is not well known, particularly during the transition period towards heart failure induced by aortic banding, which is associated with compensatory cardiac hypertrophy (CCH). It is also unclear to what extent the EDL of aortic banded rats has the capacity to adapt to this exercise modality and compensate for a reduced systemic blood supply. Specifically, there is limited information on whether this exercise programme could be tolerated by aortic banded rats, what the characteristics of running behaviour are, and if it would increase peripheral blood supply to lower limb muscles i.e. by triggering pro-angiogenic stimuli and thus ameliorating the effect of cardiac hypertrophy.

A reduction in exercise capacity (exercise intolerance) is one of the manifestations of cardiovascular disease and it is a major predictor of death in the elderly (Leeper, 2013). This muscle impairment following cardiac dysfunction is associated with attenuated blood or oxygen supply to working muscle (Hauton et al., 2015; Tickle et al., 2020) leading to low capillary density in skeletal muscle of an animal model of CHF (Bowen et al., 2017; Wadowski et al., 2018), resulting in reduced fatigue resistance (Tickle et al., 2020). Therefore, angiotherapy may be an alternative or supplementary approach to pharmaceutical intervention. Expansion of the microcirculation (Waters et al., 2004; Olesen et al., 2010) has been shown following prolonged aerobic exercise training (Andersen and Henriksson, 1977), suggesting that physiological regulation of blood vessel proliferation is locally controlled. There are a variety of angiogenic stimuli that stimulate exercise-derived angiogenesis including mechanical (e.g. muscle stretch, increased shear stress) and metabolic (e.g. hypoxia, elevated glucose metabolism) (Olfert et al., 2016) factors, providing potential therapeutic approach for restoring disease-impaired muscle function (Egginton, 2009).

In clinical (Gustafsson et al., 2001; Esposito et al., 2010; Esposito et al., 2018) and experimental (Ranjbar et al., 2017) CHF, enhanced capillarity and increased angiogenic signalling (e.g. *via* vascular endothelial growth factor, VEGF) are found after exercise therapy, indicating that the capacity for remodelling is still present even in established disease. Improvements in peak oxygen consumption after exercise therapy in CHF patients are also considered to be driven by improvements in peripheral microvascular function (Haykowsky et al., 2012). In CHF patients without exercise therapy, contradictory data on angiogenic marker expression have been reported, whereby elevated VEGF (Valgimigli et al., 2004) is thought to repair endothelial damage rather than expanding microvascular density *per se* (Chong et al., 2004). However, reduced VEGF expression has also been reported (Arakawa et al., 2003), indicating regulatory dysfunction and the potential of multiple factors (e.g. severity and specific type of CHF) to affect capillary proliferation in

muscle. Nevertheless, the beneficial effects of exercise-induced angiogenesis for restoring muscle function suggests that intrinsic remodelling capacity may be harnessed, but this remains a poorly exploited therapeutic target.

The aims of this study was to determine aortic banded rats' performance in 4-week voluntary wheel exercise which whether or not can attenuate compensatory cardiac hypertrophy, improve microcirculation and prevent atrophy of EDL muscle. This was achieved by measuring the functional effect of such angiogenic stimulus on skeletal muscle under systemic impaired muscle blood flow (during development of compensatory cardiac hypertrophy (CCH), a model of incipient cardiac dysfunction). We hypothesised that: 1) Aortic banded (AB) rats were able to perform voluntary wheel running exercise to a lesser extent than intact running wheel (RW) rats due to limited blood supply which their running performance improve over time. 2) CCH has deleterious effects on skeletal muscle fatigue resistance. 3) Wheel exercise has a preventative effect on the adverse effects of systemic blood flow reduction on skeletal muscle performance. By quantifying the effects of cardiac dysfunction and vascular insufficiency on skeletal muscle response following wheel exercise, we provide new data on the underlying factors that determine exercise intolerance in disease and how these impairments may be better targetted by therapeutic interventions.

6.2 Materials and methods

6.2.1 Animals

36 male Wistar rats with similar body mass, 196±14g, and aged 6 week old were obtained from the animal unit of Central Biological Services of University of Leeds. They were divided into 4 groups; sedentary control (SC; 7 rats), aortic banded control (AB Control; 5 rats), running wheel (RW; 8 rats) and aortic banded running wheel (ABRW; 16 rats). An acute AB group was added, (N=6) with body mass 245±40g, in which the immediate effects of banding on muscle performance were determined that used same protocol as the former groups. RW and ABRW rats were housed individually in cages with free access to a running wheel that was connected to an activity logger and computer. SC and ABC were placed in similar cages but without access to running wheels. Water and food were provided *ad lib* under a 12:12 light:dark cycle (lights on at 07:00). All procedures were conducted in accordance with the guiding principles in the Animals (Scientific Procedures) Act 1986 approved by the Home Office. The animal room was entered twice a day at random times to check the water bottles and food supply; bedding was replaced once a week during cage cleaning day.

6.2.2 Voluntary running wheel exercise

Animals were housed individually and randomly in standard cages attached to wheels (33cm diameter) that were interfaced to a computer and revolutions are recorded in 1min intervals continuously for 4 weeks.

6.2.3 Food and water consumption

Food and water consumed were weekly measured during cage cleaning day by subtracting initial value with amount left.

6.2.4 Running parameters

Refer to general method chapter for details.

6.2.5 **Clip placement on abdominal aorta (aortic banding)**

Male Wistar rats of AB control and ABRW groups were anaesthetised using 4% isoflurane with 4l/min of oxygen flow through the chamber for induction. The targetted area was shaved and body mass was recorded. On a surgery mat (temperature set at 37°C), an anaesthetic mask was placed and isoflurane concentration was reduced to 1.5-2.5% with 1l/min O_2 (maintenance) followed by 0.1ml analgesia (buprenorphine (Vetergesic®, Ceva, Amersham, UK) 0.05 mg kg^{-1}) by subcutaneous injection at the back of the neck. Ethanol was sprayed on the shaved skin and wiped with paper tissues. Under isoflurane anaesthesia, access was *via* a left-sided dorso-lateral incision where titanium clips with 0.51±0.03mm diameter (Ligaclip®; Ethicon Endo-Surgery Inc., Cincinnati, OH, USA) were placed around the abdominal aorta immediately cranial to the renal artery bifurcations (Cornelussen, 1994; Degens, 2006; Levy, Avkiran, Ferrari, & Hearse, 1996). Two drops of antibiotic (Enrofloxacin (Baytril®, Bayer, Reading, UK) 2.5 mg kg^{-1}) were applied on the incision, and opened skin was sutured. Blood on the skin was cleaned using sterile saline and the animals were placed in recovery chambers (set at 37°C). Clip placement for acute AB rats followed the same protocols but they were not placed in recovery chambers as they were sampled on the same day.

6.2.6 **Fatigue resistance and hindlimb perfusion**

Protocols for measuring muscle fatigue resistance and arterial blood flow followed those described in Tickle et al (2020). In brief, anaesthesia was induced with isoflurane (4% in 100% O^2) and thereafter maintained by constant Alfaxalone (Jurox, Crawley, UK) infusion (30-35mg.kg.hr^{-1}) delivered *via* an external jugular vein catheter. Implanted carotid and tail artery catheters allowed continuous measurement (BP transducer: AD Instruments, UK) of central and peripheral blood pressure and heart rate.

Bilateral EDL isometric twitch force was quantified by linking each muscle to a lever arm force transducer (305B-LR: Aurora Scientific, Aurora, ON, Canada) and providing indirect electrical stimulation *via* the lateral popliteal nerve (Hudlická et al., 1977). Unimpeded access to the EDL was facilitated by extirpation of the overlying synergist tibialis anterior (TA). EDL fatigue resistance was quantified according to a standard procedure whereby a 30s period of 1 Hz twitches to activate the metabolic machinery was followed by 10Hz impulses (0.3ms pulse width, supramaximal voltage) for 180s to elicit fatigue (Egginton and Hudlická, 1999; Tickle et al., 2020). Optimal muscle length and electrical current delivery were determined before fatigue measurements to allow for maximal isometric twitch force development. A fatigue index (FI) was calculated as muscle force at

the end of stimulation/peak muscle tension as recorded at the beginning of the test. A mean of five consecutive twitches was selected to represent end stimulation and peak force data.

Bilateral femoral flow was also quantified throughout each experiment by placement of perivascular flow probes (0.7PSB; Transonic, Ithaca, NY, USA) at the proximal aspect of the *profunda femoris* arterial bifurcation (Tickle et al., 2020). Resting and end-stimulation flows were quantified, allowing calculation of the functional hyperaemic scope in each experimental group. Muscle mass-specific calculation of the increase in flow, i.e. the hyperaemic increment above resting, was made to account for potential differences in extent of EDL hypertrophy, and normalised to femoral vascular conductance using arterial pressure measured in the tail.

6.2.7 **Schedule 1 killing and muscle dissection**

Refer to general method chapter. For acute AB rats, they were sacrificed immediately post FI measurement (clips placement, FI measurement and muscles sampling performed on the same day). They were stunned by striking the cranium against a solid object and their death was then confirmed by neck dislocation. Extensor digitorum longus (EDL) and cardiac (left ventricle free wall) muscles were dissected. Adipose and connective tissue were removed from muscles which were then weighed.

6.2.8 **Freezing and cryosections**

Refer to general method chapter

6.2.9 **Monoclonal-myosin heavy chain antibodies, lectin and laminin staining**

Refer to general method chapter

6.2.10 **Area of sampling region**

Refer to general method chapter

6.2.11 **Capillary and fibre composition; gross and regional CD, C: F and MFA.**

Refer to general method chapter

6.2.12 **Fibre type composition**

Refer to general method chapter

6.2.13 **Local capillarity**

Refer to general method chapter

6.2.14 **Capillary domain area frequency and heterogeneity**

Refer to general method chapter

6.2.15 **Estimation muscle function**

Refer to general method chapter

6.2.16 **Data analysis**

Differences in running performance between RW and ABRW were determined using independent samples T-Test. Differences in cardiovascular parameters, fatigue index (FI) and blood flow between groups were determined using ANOVA with Tukey post hoc tests. ANCOVA with Sidak *post hoc* tests were used to test if aortic banding influenced heart mass after controlling for the effect of body mass (Solomon & Bengele, 1973). 2-way ANOVA with Tukey *post hoc* tests were used to determine interaction between aortic banding application and exercise volume on muscle capillarity. Statistical testing was conducted in SPSS (v.25). Data are presented throughout as mean ± SD and statistical tests were considered significant where $P \leq 0.05$.

6.3 Results

6.3.1 **Clips placement (aortic banding surgery)**

Clips were successfully placed at the abdominal aorta on 21 Wistar rats. However, 8 rats died during the 24 hour post-surgery period; 2 due to a twisted clip, 2 due to internal bleeding, and 4 within 48 hour post-surgery of unknown causes.

6.3.2 **Rat body and muscles mass, food and water consumptions.**

All rat body masses were similar: sedentary control (SC) - 191±13g (n=7), aortic-banded control (AB Control) - 202±4g (n=5), running wheel (RW) - 197±10g (n=8) and aortic-banded running wheel (ABRW) – 198±16g (n=8) ($P>0.05$). The final body mass at the end of 4 weeks were also similar between groups; 338±28g, 301±19g, 312±42g and 301±33g, respectively ($P>0.05$). Even though the final body mass difference was not significant, AB control, RW and ABRW final body mass were relatively lower than SC group (-11, -8 and -11%, respectively). Cardiac mass was relatively higher in exercise groups than SC ($P<0.05$), and AB control showed the largest hearts (Table 6.1). Normalisation of rat body mass showed the heart to body mass ratio was significantly higher in AB control and ABRW rats compared to SC rats ($P<0.05$), with RW rat 12% higher than SC ($P>0.05$). EDL mass of ABRW

was unchanged compared to SC and AB control rats (P>0.05), and 19 % lower than RW rats (P<0.05). A similar food intake was recorded in the first week by all groups (P>0.05), however consumption was lower in ABRW rats after 4 weeks (P<0.05) although water intake was similar to RW rats (Table 6.1).

Table 6.1: General characteristic, rats' body, cardiac and muscle mass of SC, AB control, RW and ABRW over 4 weeks period. Food and water intakes by RW and ABRW rats were different along the study period. $^{*}P$<0.05 *vs.* SC, ^{a}P<0.05 *vs*. AB control and ^{b}P<0.05 *vs.* RW.

Group		SC	AB control	RW	ABRW	*P*-Value
N		7	5	8	8	
Body mass (gram)	Initial	191±13	202±4	197±10	198±16	P>0.05
	Week 1	221±19	-	230±25	210±13	
	Week 2	266±23	-	263±25	257±23	
	Week 3	306±23	-	288±33	285±30	
	Week 4	338±28	301±19	312±42	301±33	P>0.05
Cardiac mass (g)		0.89±0.13	1.18±0.17*	1.00±0.11^{a}	1.06±0.09	
Heart body mass ratio (mg/g)		2.76±0.33	3.94±0.82^{*b}	3.08±0.29	3.57±0.33*	
EDL mass (g)		0.162±0.029	0.169±0.012	0.197±0.025*	0.166±0.015^{b}	
EDL body mass ratio (mg/g)		0.51±0.10	0.56±0.04	0.63±0.06*	0.56±0.06	
Food consumption (g)						
	Week 1	-	-	118±15	123±44	P>0.05
	Week 2	-	-	173±24	149±33	P<0.05
	Week 3	-	-	170±36	161±35	P<0.05
	Week 4	-	-	189±15	131±30	P<0.05
Water consumption (ml)						
	Week 1	-	-	174±43	98±35	P<0.05
	Week 2	-	-	228±64	240±71	P>0.05
	Week 3	-	-	246±58	246±55	P>0.05
	Week 4	-	-	278±85	218±44	P>0.05

6.3.3 **Running parameters**

Total daily running distance per week was significantly lower in ABRW group compared to RW group, around 3 -fold less at the end of the training period (P<0.05; Table 6.2), although running distance of ABRW rats increased ~42% weekly (Figure 6.1). Night-time running distance rate showed a similar trend with ABRW rats significantly less active than RW each week (P<0.05; Figure 6.2). Light cycle running distance was similar between ABRW and RW for every week at a constant rate. Mean running velocity of ABRW rats was significantly lower than RW rats, although both increased steadily during the study (Figure 6.4). ABRW and RW run at a similar modal pace during the study period (P>0.05; Figure 6.5), while weekly median running velocity for both groups showed an increasing trend but with ABRW rats at a lower rate (P<0.05; Figure 6.6). During the first week dark cycle mean running velocity was similar between groups, then significantly different from week 2 to week 4 in increasing but in ABRW group to a lesser extent (Figure 6.7). While daytime mean running velocity was similar between groups over the period (Figure 6.8), average active time among ABRW rats were ~50% less than RW rats (Figure 6.9), and this was similarly present the during dark cycle (Figure 6.14). Meanwhile, light cycle percentage active time was similar between the groups, with a decreasing trend towards the end of the study period (Figure 6.19). Overall, bouts of running activity were similar between groups but decreasing by the fourth week. Dark cycle indicated ABRW produced fewer bouts of activity than RW at week 1, but were similar at week 2 onwards with ABRW plateaued within last 3 weeks, while running episode during daytime of both groups seemed to be lower over time (Figures 6.10, 6.15 and 6.20).

Maximal running duration of ABRW and RW rats increased 81% and 92% at the end of study period, respectively, but RW rats were able to run 50% longer than ABRW (P<0.05). ABRW maximal running duration increased steadily until week 4 while RW rats reached a plateau after 2 weeks; that both groups recorded similar maximal running duration during light cycle over the study period (P>0.05). ABRW rats ran for shorter periods (running bouts) than RW rats during the whole study period (Figure 6.11), with their total and mean dark cycle running duration increasing at about 50% the rate of RW rats (Figures 6.12 and 6.17). However, both groups light cycle running duration was similar during the exercise period (Figure 6.22). Mode of running duration was similar between the groups, similarly during dark and light cycles, although ABRW showed an upward trend in light cycle from third to fourth weeks (Figures 6.21 – 6.23).

Table 6.2: Running parameters of RW and ABRW rats of 4-week voluntary wheel running exercise.

Running parameters	Group	N	Week			
			1	2	3	4
Total distance (km)	RW	8	2.09±0.73	4.52±060	5.96±0.40	6.70±0.67
	ABRW	8	0.93±0.46	1.67±0.14	1.69±0.21	2.41±0.18
Dark cycle total distance (km)	RW	8	2.13±0.86	4.37±0.55	5.84±0.34	6.33±0.56
	ABRW	8	0.77±0.49	1.52±0.20	1.57±0.23	2.28±0.22
Light cycle total distance (km)	RW	8	0.13±0.09	0.16±0.08	0.12±0.10	0.18±0.13
	ABRW	8	0.17±0.07	0.19±0.13	0.13±0.12	0.13±0.08
Mean pulse velocity (m/s)	RW	8	0.66±0.08	0.77±0.02	0.82±0.02	0.84±0.01
	ABRW	8	0.53±0.14	0.69±0.02	0.75±0.02	0.78±0.01
Mode pulse velocity (m/s)	RW	8	0.70±0.10	0.82±0.08	0.81±0.06	0.83±0.10
	ABRW	8	0.51±0.25	0.82±0.03	0.83±0.11	0.90±0.09
Median Pulse velocity (m/s)	RW	8	0.70±0.09	0.82±0.03	0.88±0.02	0.89±0.01
	ABRW	8	0.55±0.17	0.76±0.03	0.83±0.02	0.86±0.02
Dark Mean Pulse velocity (m/s)	RW	8	0.67±0.09	0.78±0.02	0.83±0.01	0.84±0.01
	ABRW	8	0.52±0.17	0.71±0.03	0.76±0.01	0.79±0.01
Light mean pulse velocity (m/s)	RW	8	0.48±0.10	0.54±0.04	0.52±0.09	0.56±0.10
	ABRW	8	0.44±0.07	0.51±0.05	0.55±0.05	0.54±0.05
% time active (%)	RW	8	13.52±1.40	17.88±1.61	18.88±1.31	18.95±1.53
	ABRW	8	9.15±2.55	12.21±1.39	11.51±1.29	12.53±1.16
Max activity duration (min)	RW	8	18.30±5.29	32.75±7.36	33.4±6.59	33.19±3.24
	ABRW	8	11.82±4.41	17.11±1.38	16.17±2.03	22.64±2.39
Bouts of activity	RW	8	34±4	30±5	29±2	28±2
	ABRW	8	28±7	30±5	28±3	28±3
Mean of activity duration (min)	RW	8	5.62±1.31	8.63±1.27	9.14±0.76	9.47±0.37
	ABRW	8	4.11±0.89	4.99±0.28	5.09±0.48	6.10±0.40
Mode of activity duration (min)	RW	8	2.30±0.35	2.23±0.20	3.29±1.94	2.44±0.28
	ABRW	8	2.29±0.29	2.23±0.26	2.15±0.15	2.25±0.18
Dark % time active (%)	RW	8	23.86±3.29	32.66±2.01	35.25±1.88	35.06±3.73
	ABRW	8	13.52±6.97	19.89±1.54	20.38±1.76	22.14±1.29
Dark Bouts of activity	RW	8	29±4	26±3	25±1	25±3
	ABRW	8	20±9	24.37±2	24±2	24±2
Dark max activity duration (min)	RW	8	17.81±5.61	32.56±7.49	33.34±6.65	32.74±3.33
	ABRW	8	10.56±6.10	16.15±1.69	16.08±2.02	22.39±2.45
Dark mean activity duration (min)	RW	8	5.82±1.43	9.27±1.32	9.78±0.91	9.75±0.29

Dark mode activity duration (min)	ABRW	8	4.08±1.13	5.11±0.33	5.20±0.50	6.41±0.33
	RW	8	2.41±0.28	2.22±0.27	4.69±3.36	2.36±0.18
Light % time active (%)	ABRW	8	2.36±0.28	2.36±0.16	2.19±0.17	2.41±0.41
	RW	8	3.11±1.67	3.08±1.41	2.47±1.27	2.82±1.95
Light Bouts of activity	ABRW	8	4.74±2.16	4.33±2.21	2.61±1.75	2.91±1.55
	RW	8	5±2	4±2	3±2	3±2
Light max activity duration (min)	ABRW	8	8±4	6±3	4±2	4±2
	RW	8	6.41±3.21	7.83±2.25	6.80±3.39	7.99±4.19
Light mean activity duration (min)	ABRW	8	6.78±1.61	8.78±2.81	6.58±2.88	6.34±1.94
	RW	8	3.82±0.96	4.62±0.80	4.43±0.81	5.53±1.73
Light mode activity duration (min)	ABRW	8	3.75±0.66	4.10±0.51	3.96±0.87	3.84±0.66
	RW	8	2.47±0.42	2.49±0.66	3.41±0.93	4.38±2.28
	ABRW	8	2.45±0.44	2.23±0.25	2.76±1.06	2.26±0.51

6.3.3.1 Statistical analysis

6.3.3.1.1 Total daily running distance per week

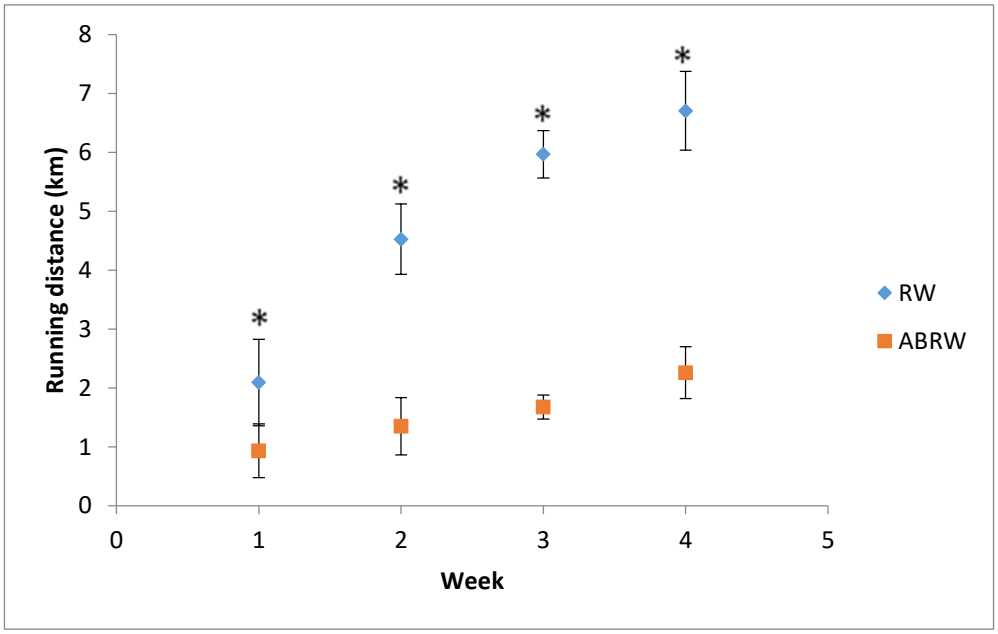

Figure 6.1: Total daily running distance per week of RW and ABRW rats of 4-week voluntary wheel running. *P<0.05.

6.3.3.1.2 Dark cycle total daily running distance per week

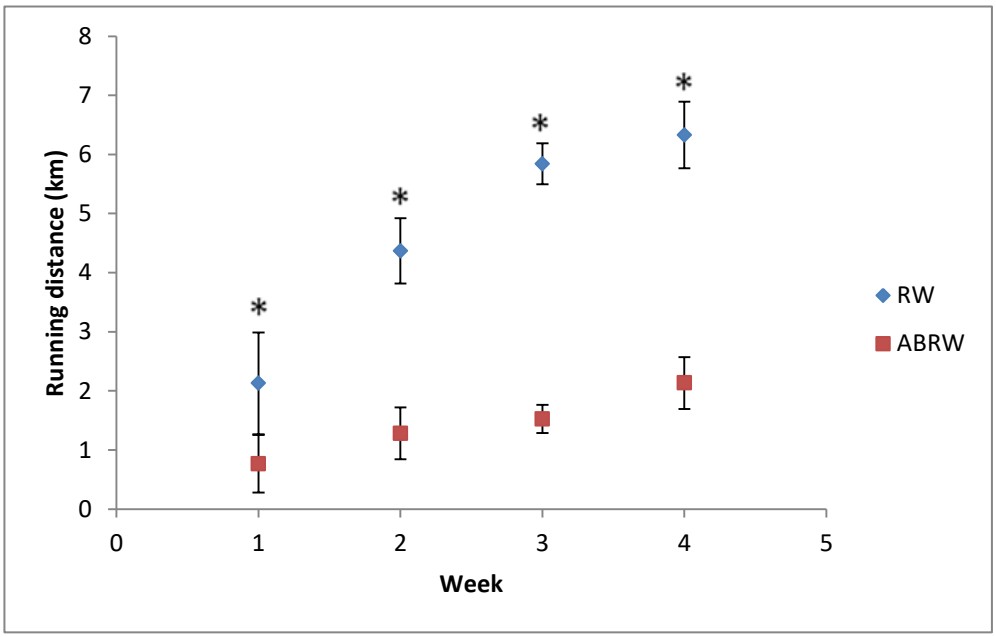

Figure 6.2: Dark cycle total running distance per week of RW and ABRW rats of 4-week voluntary wheel running exercise. *P<0.05.

6.3.3.1.3 Light running distance

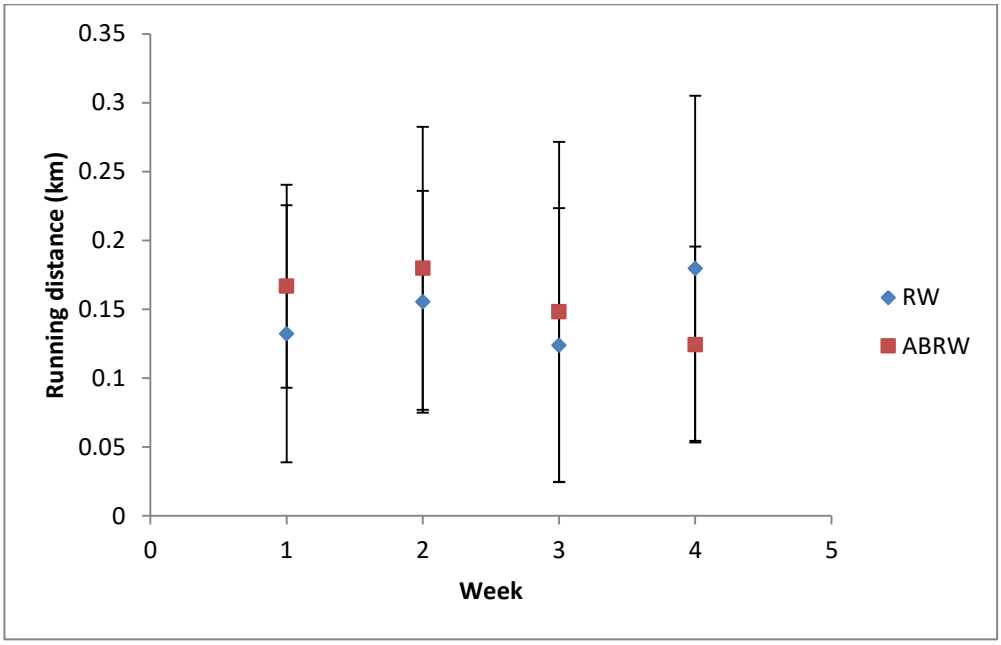

Figure 6.3: Light cycle total running distance per week of RW and ABRW rats of 4-week voluntary wheel running exercise (n.s.).

6.3.3.1.4 Mean running velocity

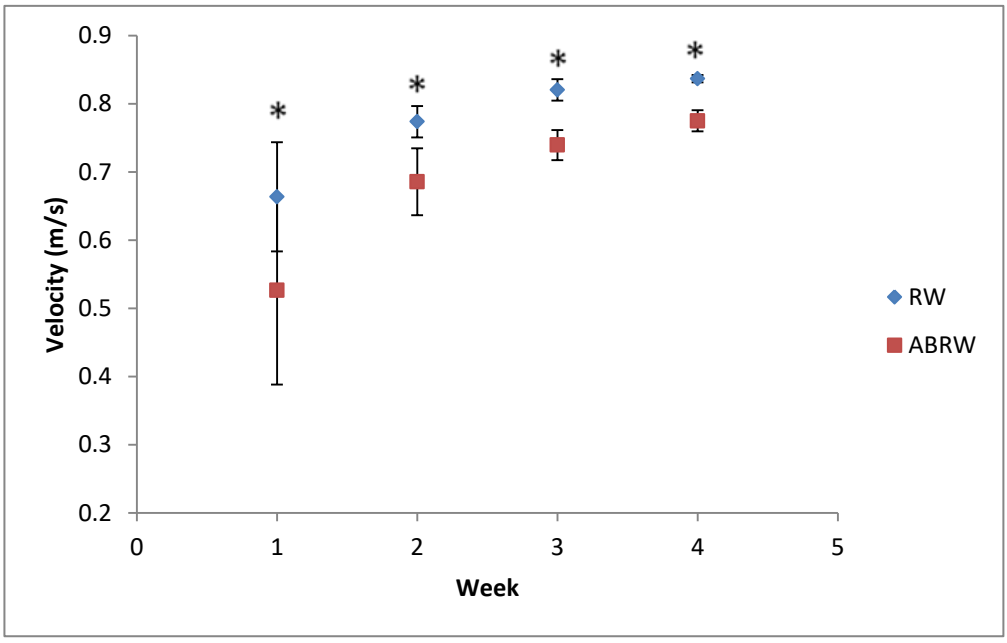

Figure 6.4: Mean running velocity of RW and ABRW rats of 4-week voluntary wheel running exercise. *P<0.05.

6.3.3.1.5 Mode running velocity

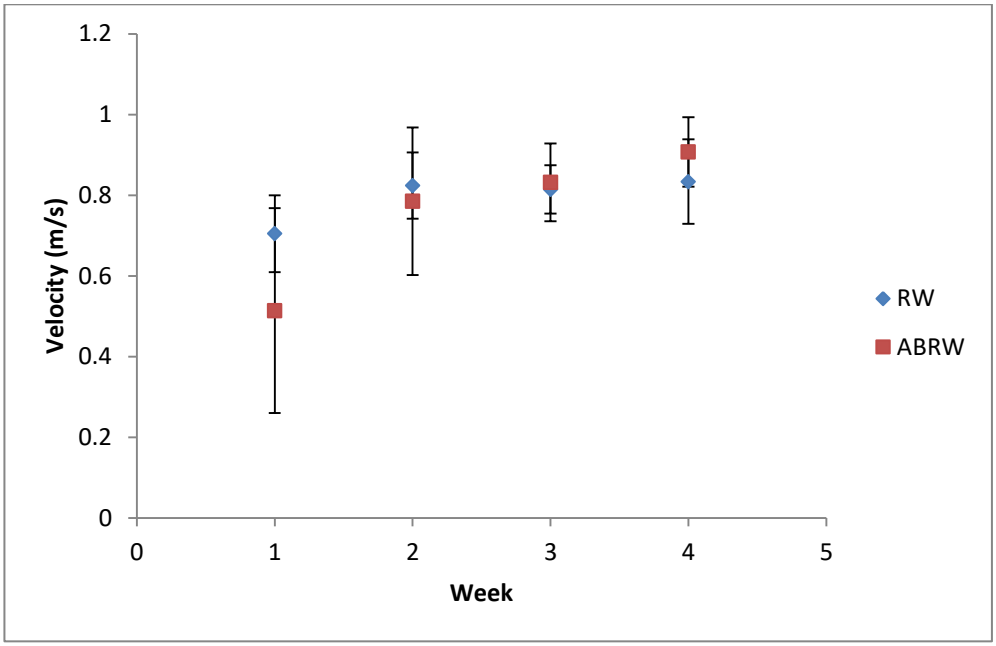

Figure 6.5: Mode running velocity of RW and ABRW rats of 4-week voluntary wheel running exercise (n.s.).

6.3.3.1.6 Median running velocity

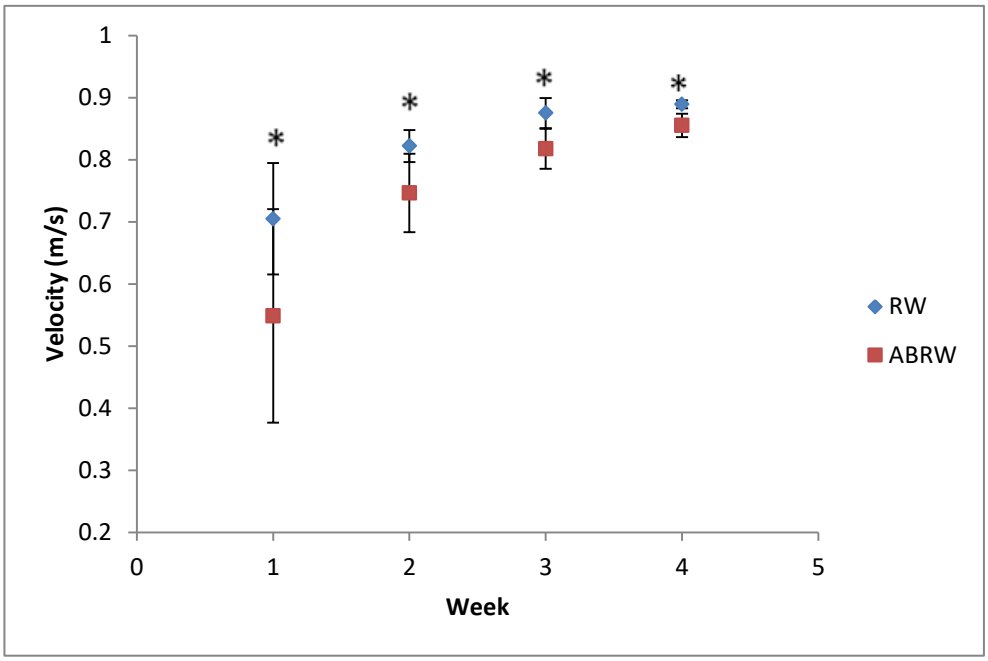

Figure 6.6: Median running velocity of RW and ABRW rats of 4-week voluntary wheel running exercise. *P<0.05.

6.3.3.1.7 Dark Mean running velocity

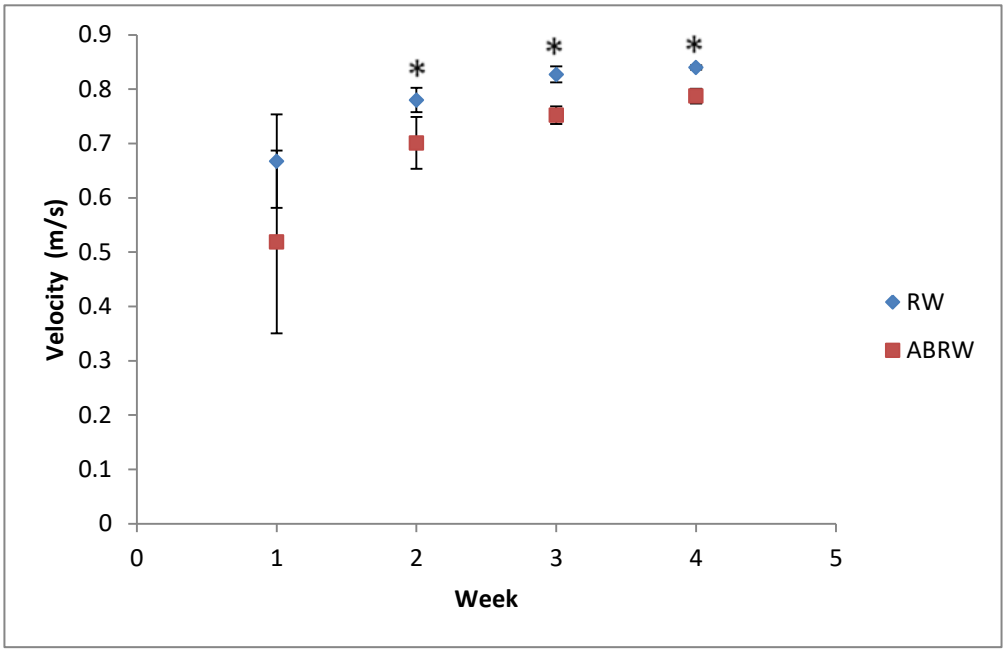

Figure 6.7: Dark mean running velocity of RW and ABRW rats of 4-week voluntary wheel running exercise. *P<0.05.

6.3.3.1.8 Light mean running velocity

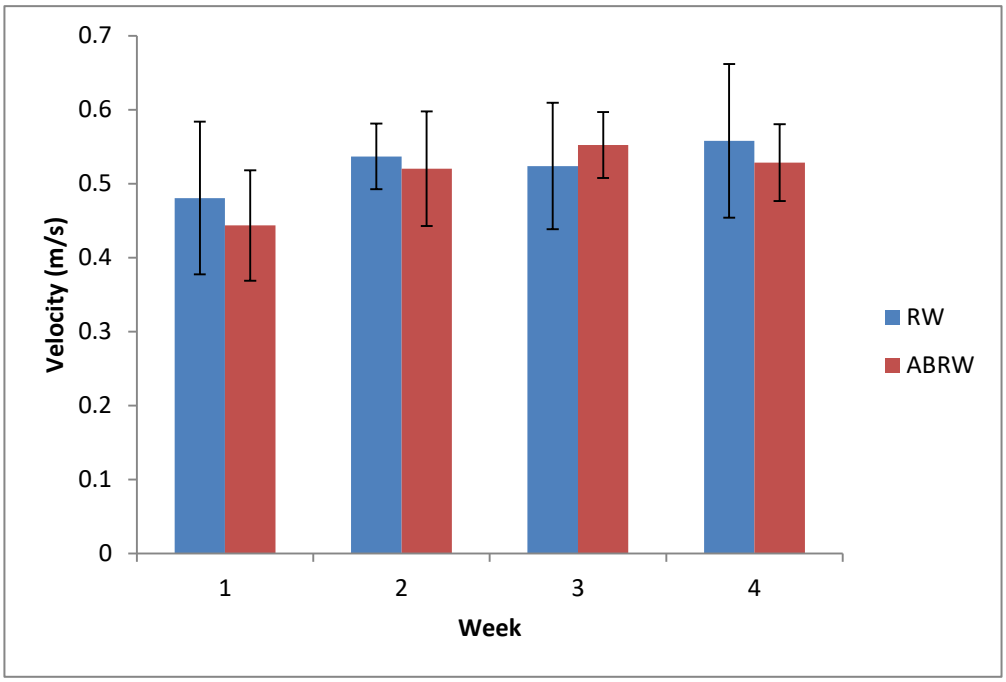

Figure 6.8: Light mean running velocity of RW and ABRW rats of 4-week voluntary wheel running exercise (n.s.).

6.3.3.1.9 Percentage time active

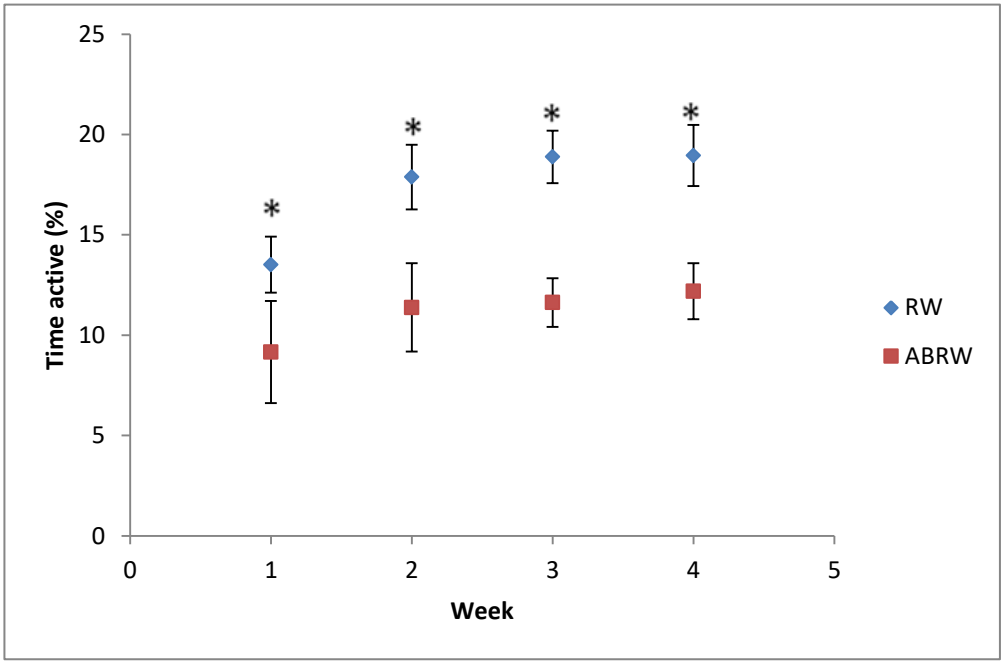

Figure 6.9: Percentage time active of RW and ABRW rats of 4-week voluntary wheel running exercise. *P<0.05.

6.3.3.1.10 Bouts of running activity

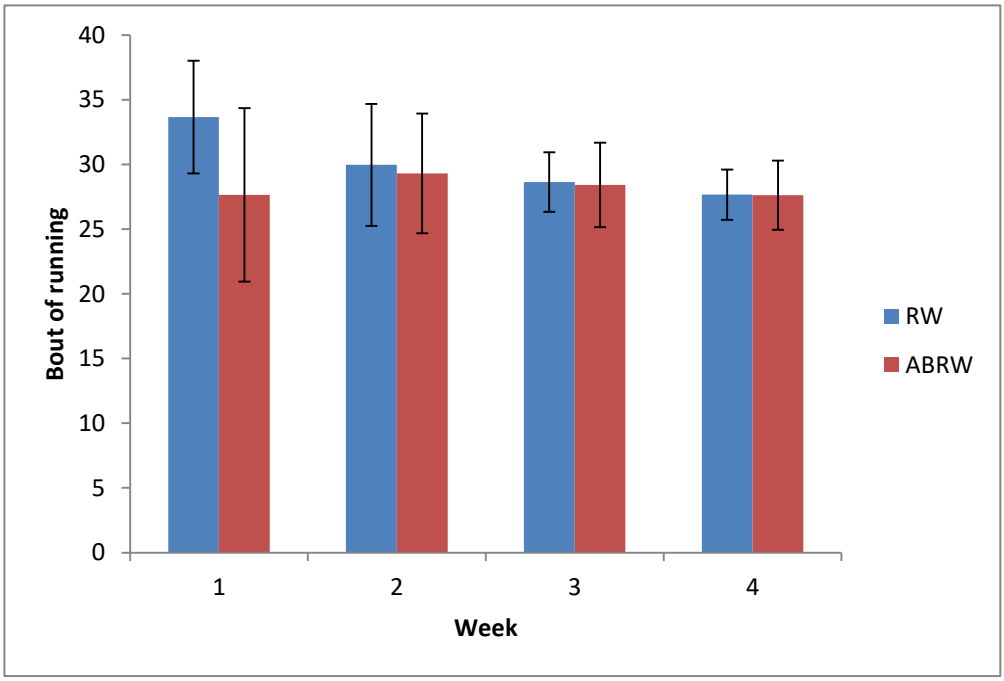

Figure 6.10: Bout of running activity of RW and ABRW rats of 4-week voluntary wheel running exercise (n.s.).

6.3.3.1.11 Maximal running duration

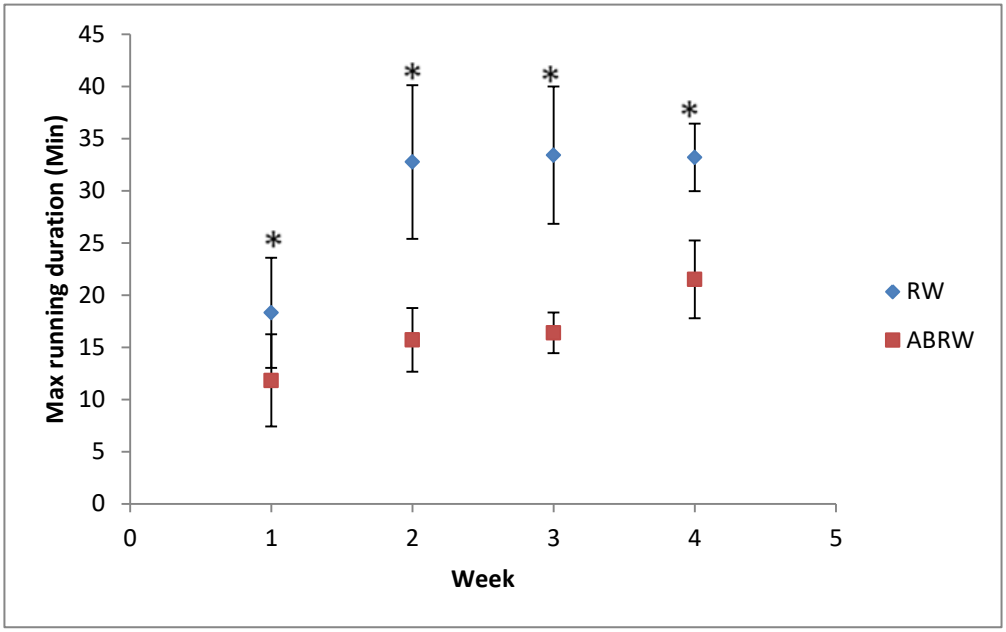

Figure 6.11: Maximal running duration of RW and ABRW rats of 4-week voluntary wheel running exercise. *P<0.05.

6.3.3.1.12 Mean of running duration

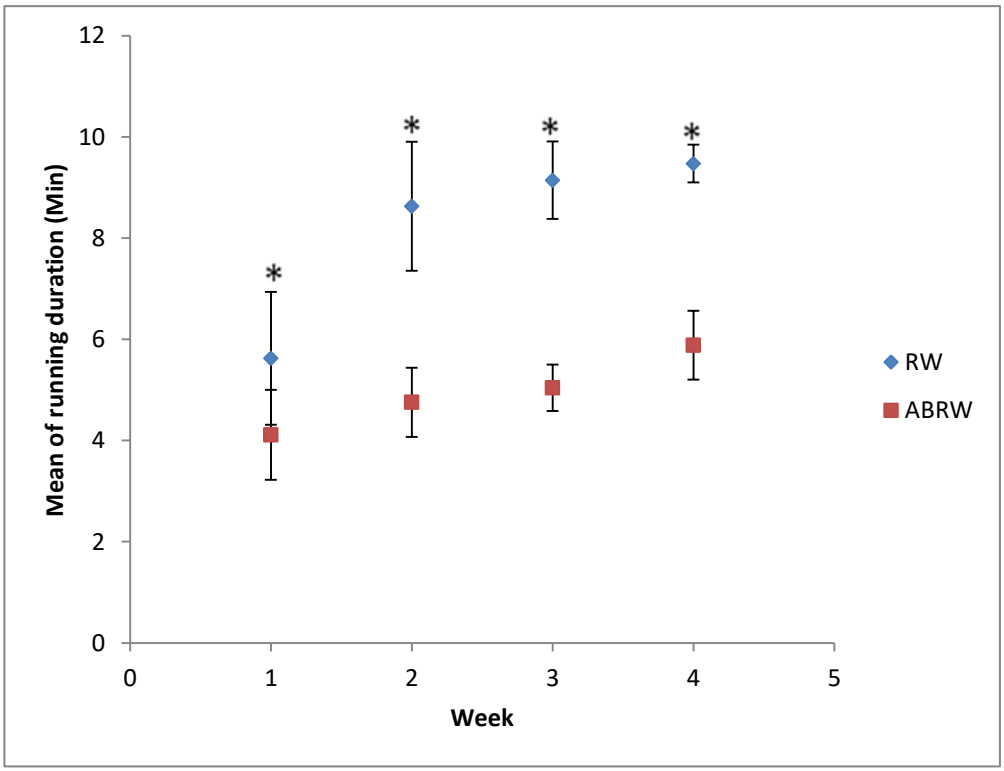

Figure 6.12: Mean of running duration of RW and ABRW rats of 4-week voluntary wheel running exercise. *P<0.05.

6.3.3.1.13 Mode of running duration

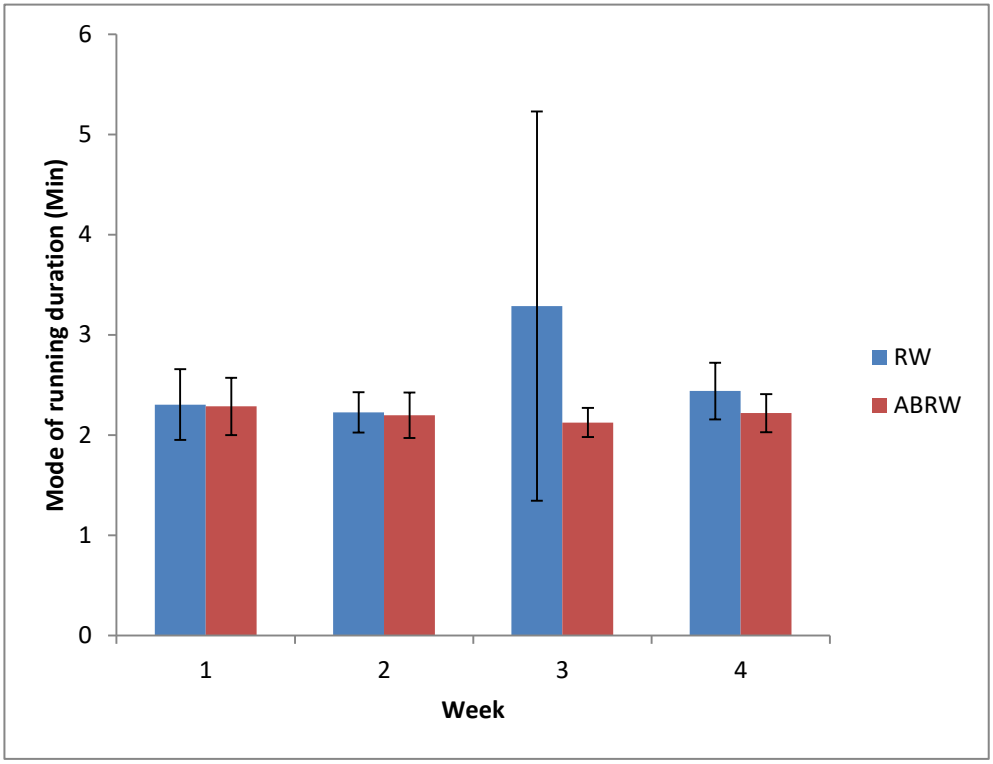

Figure 6.13: Mode of running duration of RW and ABRW rats of 4-week voluntary wheel running exercise (n.s.).

6.3.3.1.14 Dark percentage time active

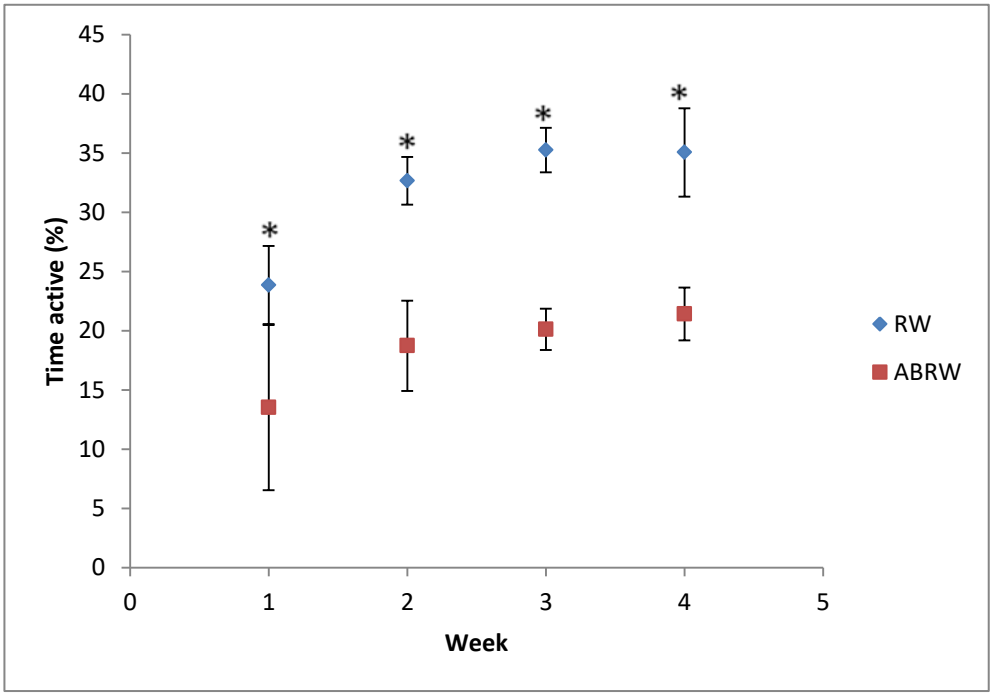

Figure 6.14: Dark percentage time active of RW and ABRW rats of 4-week voluntary wheel running exercise. *P<0.05.

6.3.3.1.15 Dark Bouts of running activity

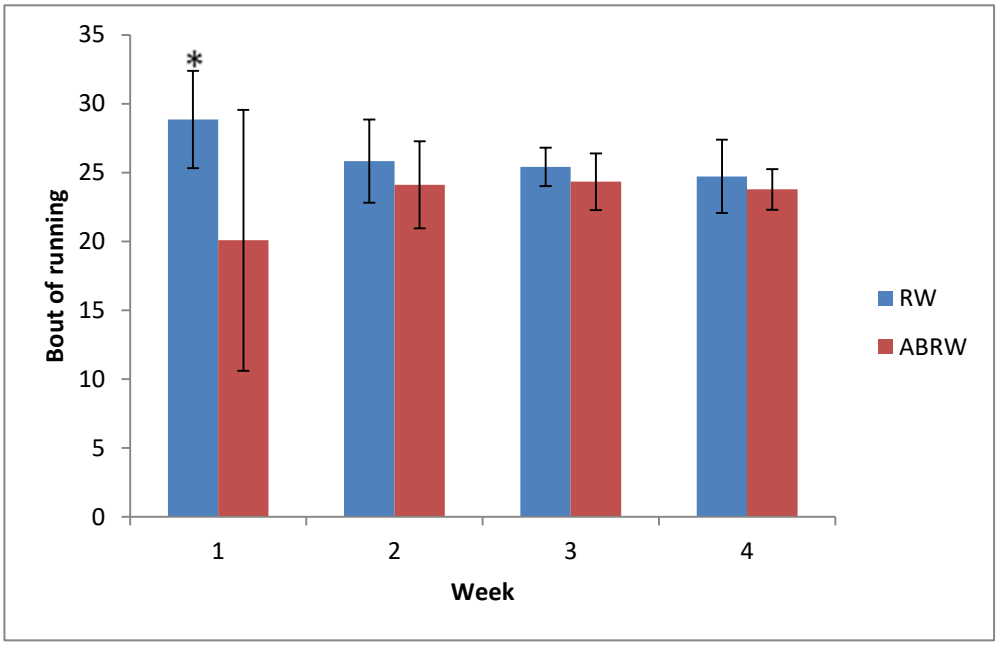

Figure 6.15: Dark bout of running activity of RW and ABRW rats of 4-week voluntary wheel running exercise. *P<0.05.

6.3.3.1.16 Dark maximal running duration

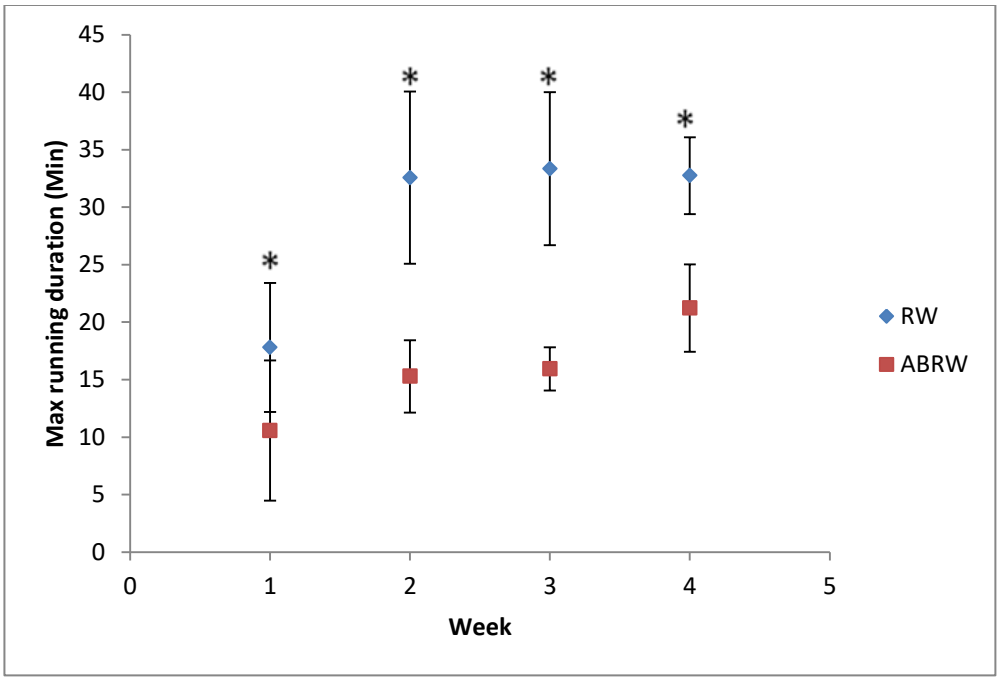

Figure 6.16: Dark maximal running duration of RW and ABRW rats of 4-week voluntary wheel running exercise. *P<0.05.

6.3.3.1.17 Dark mean running duration

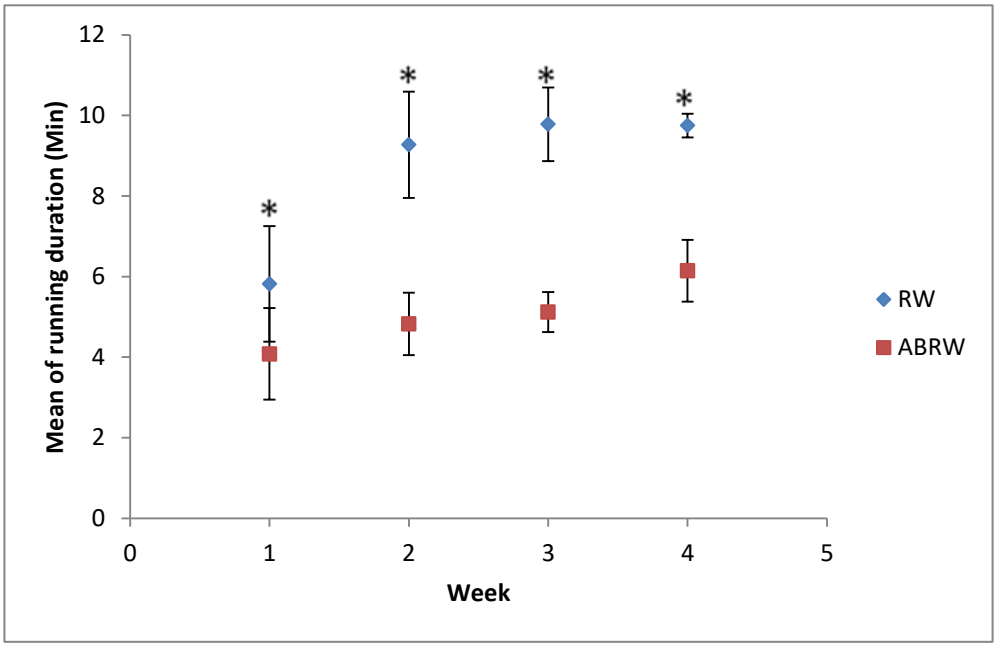

Figure 6.17: Dark mean running duration of RW and ABRW rats of 4-week voluntary wheel running exercise. *P<0.05.

6.3.3.1.18 Dark mode running duration

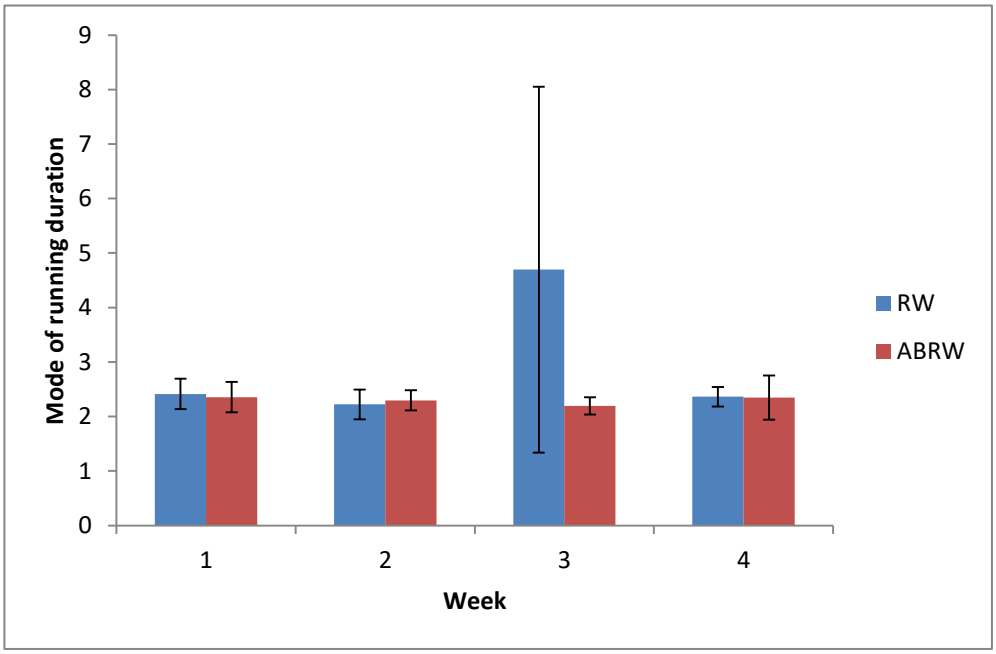

Figure 6.18: Dark mode of running duration of RW and ABRW rats of 4-week voluntary wheel running exercise (n.s.).

6.3.3.1.19 Light percentage time active

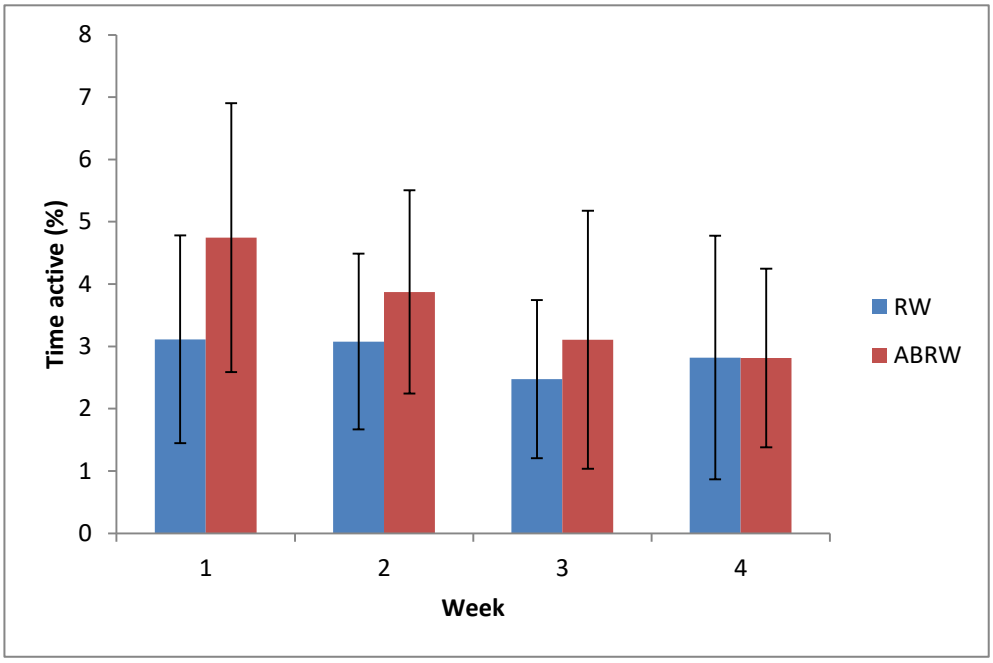

Figure 6.19: Light percentage time active of RW and ABRW rats of 4-week voluntary wheel running exercise (n.s.).

6.3.3.1.20 Light Bouts of running activity

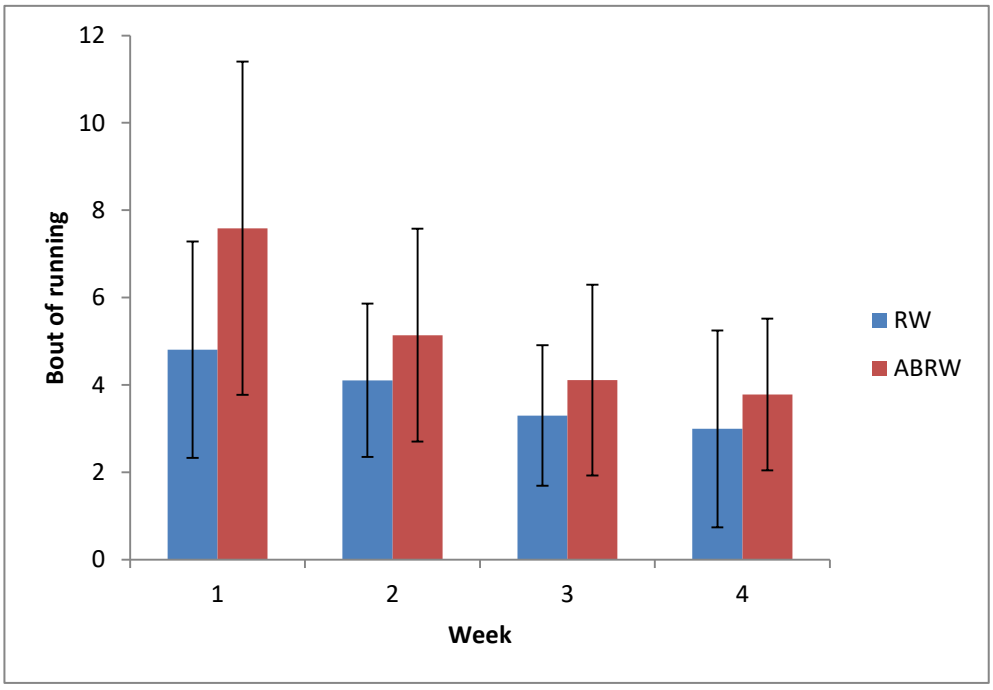

Figure 6.20: Light bout of running activity of RW and ABRW rats of 4-week voluntary wheel running exercise (n.s.).

6.3.3.1.21 Light maximal running duration

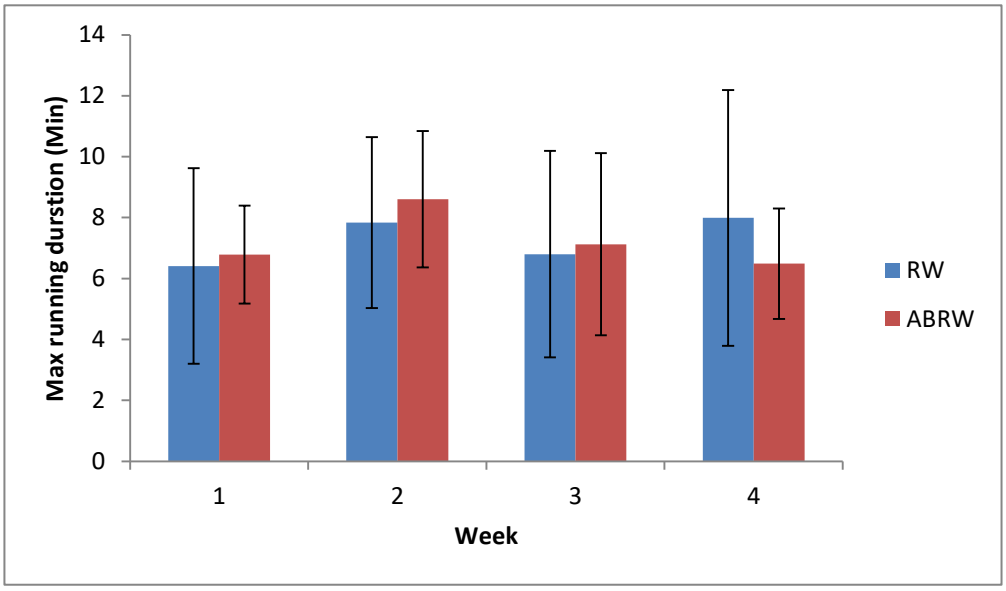

Figure 6.21: Light maximal running duration of RW and ABRW rats of 4-week voluntary wheel running exercise (n.s.).

6.3.3.1.22 Light mean running duration

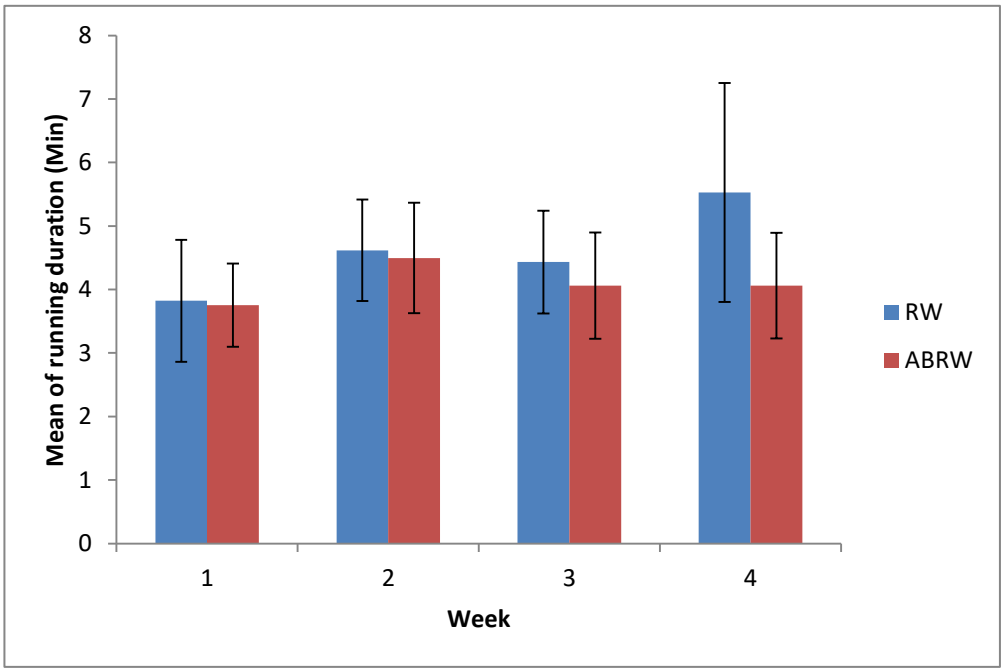

Figure 6.22: Light mean running duration of RW and ABRW rats of 4-week voluntary wheel running exercise (n.s.).

6.3.3.1.23 Light mode running duration

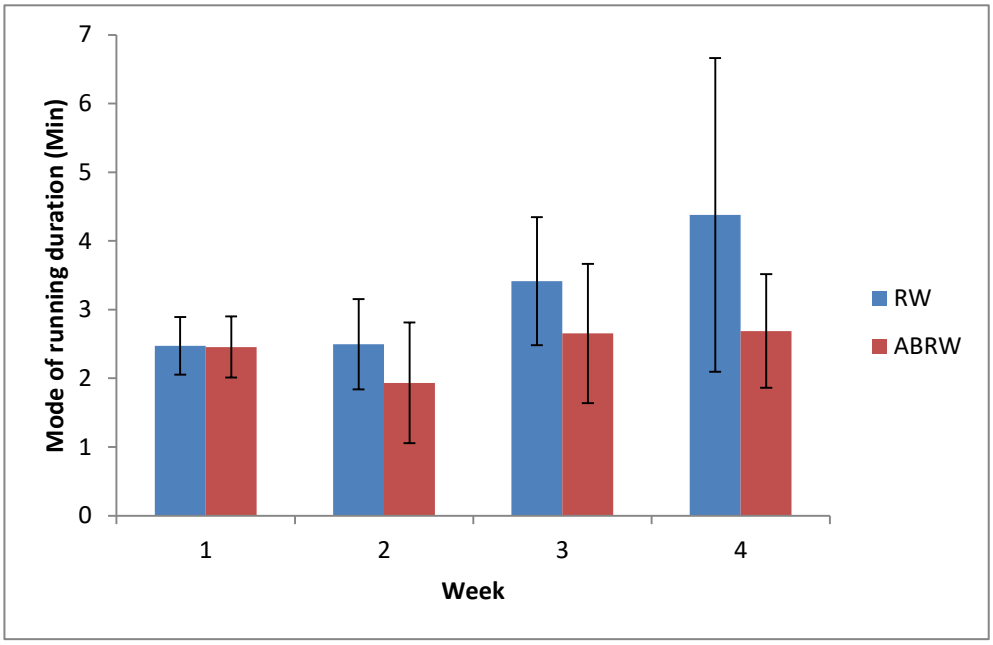

Figure 6.23: Light mode of running duration of RW and ABRW rats of 4-week voluntary wheel running exercise (n.s.).

6.3.4 **Global angiogenic indices**

Aortic banding performed on Wistar rats (AB Control) caused fibre atrophy and absolute capillary rarefaction in EDL ($P \leq 0.05$; Table 6.3) associated with less movement activity, and 4 week voluntary wheel running exercise (ABRW) was able to restore muscle anatomy (MFA) similar to SC rats, maintaining both C:F and CD. Wheel running exercise could further increase muscle capillarity of EDL muscle along with fibre hypertrophy which lowered CD of the muscle, compared to AB control (Table 6.3).

Table 6.3: Global angiogenic indices of SC, AB control, RW and ABRW rats of 4-week voluntary wheel running exercise. Unshared letters denote statistical significance ($P \leq 0.05$) as determined by ANOVA with Tukey post-hoc-tests.

Group	N	C:F ratio	CD (mm^{-2})	MFA (μm^2)
SC	6	1.44 ± 0.04^{a}	666 ± 151^{e}	2322 ± 546^{g}
AB control	5	1.22 ± 0.20^{b}	576 ± 127^{f}	1575 ± 198^{h}
RW	7	1.79 ± 0.07^{c}	631 ± 34^{ef}	2966 ± 283^{i}
AB RW	8	1.51 ± 0.10^{d}	709 ± 46^{ef}	2227 ± 165^{g}

6.3.5 **Fibre type composition**

6.3.5.1 **Numerical and areal density**

Numerical density (N_N) and areal density (A_A) of all fibre types of RW rats were similar to SC value (P>0.05). AB control and ABRW rats showed similar trend in fibre type composition as N_N of both groups increased relative to SC and RW groups. However, A_A of type I and IIa fibres of AB control and ABRW significantly decreased (P≤0.05) to result in muscle atrophy that was compensated by an increase in type IIb fibre areal density (P≤0.05), Table 6.4, Figure 6.24 and Figure 6.25.

Table 6.4: Fibre type composition; numerical and areal density of fibre type I, IIa and IIb of SC, AB control, RW and ABRW rats following 4-week voluntary wheel running exercise.

Fibre composition	N	Group	Mean±SD
N_NTI	7	SC	0.022±0.01
	5	AB Control	0.052±0.02
	8	RW	0.028±0.01
	8	ABRW	0.046±0.02
N_NTIIA	7	SC	0.176±0.09
	5	AB Control	0.219±0.04
	8	RW	0.177±0.04
	8	ABRW	0.273±0.04
N_NTIIB	7	SC	0.808±0.09
	5	AB Control	0.745±0.04
	8	RW	0.802±0.04
	8	ABRW	0.689±0.04
A_ATI	7	SC	0.050±0.01
	5	AB Control	0.022±0.01
	8	RW	0.053±0.01
	8	ABRW	0.024±0.01
A_ATIIA	7	SC	0.291±0.10
	5	AB Control	0.111±0.03
	8	RW	0.293±0.03
	8	ABRW	0.163±0.03
A_ATIIB	7	SC	0.671±0.10
	5	AB Control	0.873±0.03
	8	RW	0.666±0.03
	8	ABRW	0.817±0.03

6.3.5.2 **Statistical analysis**

Numerical density, N_N

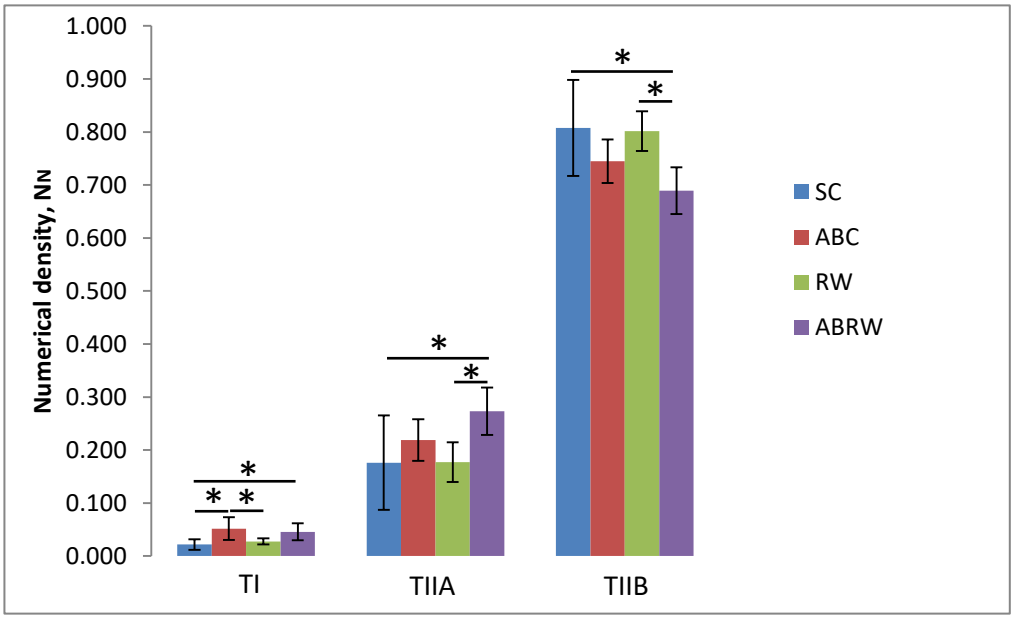

Figure 6.24: Numerical density (N_N) of fibre type I, IIa and IIb of SC, AB control, RW and ABRW rats following 4-week voluntary wheel running exercise, *$P \leq 0.05$.

Areal density, A_A

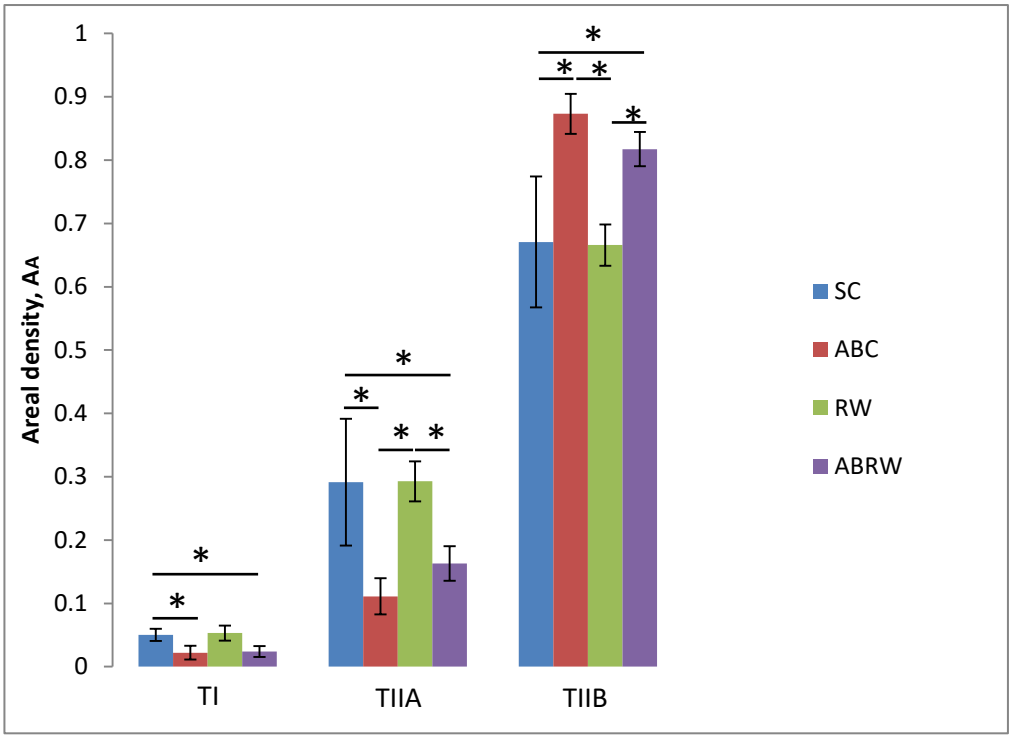

Figure 6.25: Areal density (A_A) of fibre type I, IIa and IIb of SC, AB control, RW and ABRW rats following 4-week voluntary wheel running exercise,*$P \leq 0.05$.

6.3.6 **Capillary domain area, CDA**

6.3.6.1 **CDA frequency distribution**

The frequency distribution of AB control group showed a lower proportion of domain area between 1000-1500 µm², while ABRW rats showed relatively higher proportion of larger CDA (500-2000 µm²) than AB Control, and similar distribution to SC and RW groups for all class of CDA (Figure 6.26).

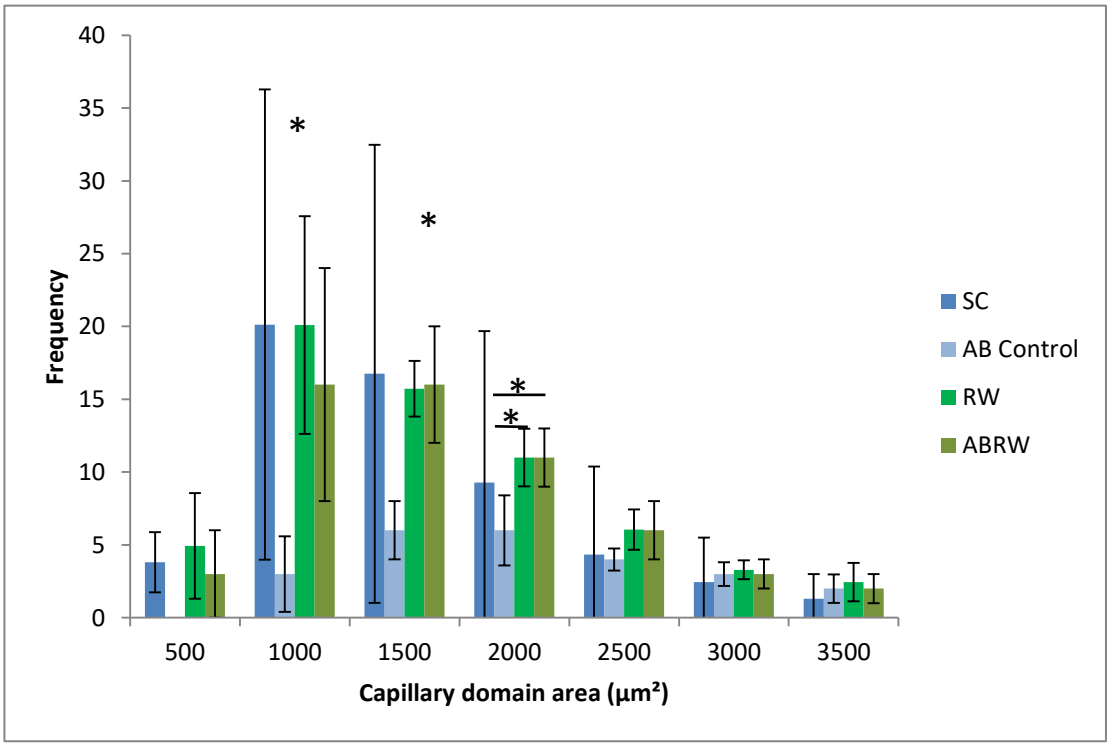

Figure 6.26: Frequency distribution of capillary domain area (CDA) of SC, AB Control, RW and ABRW. One-way ANOVA: the mean difference is significant compared to AB control at 1000 and 1500 CDA, and RW and ABRW CDA frequency were higher than AB Control for CDA range 2000-2500, *P<0.05.

6.3.6.2 **Heterogeneity of CDA, LogSD**

Distribution of supply area in SC, AB Control and ABRW showed less heterogeneous capillary spacing than RW ($P \leq 0.05$), while heterogeneity of CDA in ABRW was similar to SC and AB Control groups ($P > 0.05$; Figure 6.27).

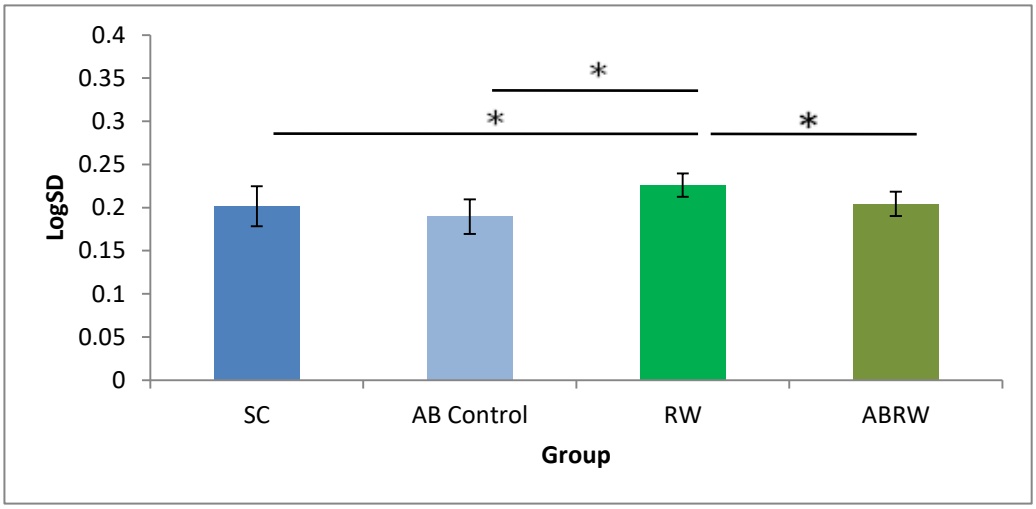

Figure 6.27: Heterogeneity of capillary domain area between SC, AB control, RW and ABRW. One-way ANOVA: the mean difference of CDA heterogeneity was significant between RW and SC, RW and AB Control, and RW and ABRW (*$P < 0.05$).

Local capillarity

LCFR of ABRW rats (1.14±0.20) was improved (*vs*. AB control: 0.94±0.13, $P < 0.05$) as exercise enhanced local capillary supply of EDL muscle in banded aortic rats to restore capillary supply to a similar level of SC rats (1.16±0.17, $P > 0.05$) but lower than RW rats (1.45± 0.13, $P < 0.05$). The relative increase of LCFR in ABRW was 21% greater than in AB Control rats. Wheel running exercise for 4 weeks further increased LCFR to 1.45±0.13 to support chronic running activity. ABRW rats maintained their LCD, which was similar to SC and RW rats ($P > 0.05$), while AB Control rats showed significant lower LCD compared to ABRW ($P < 0.05$).

6.3.6.3 Local capillary to fibre ratio, LCFR

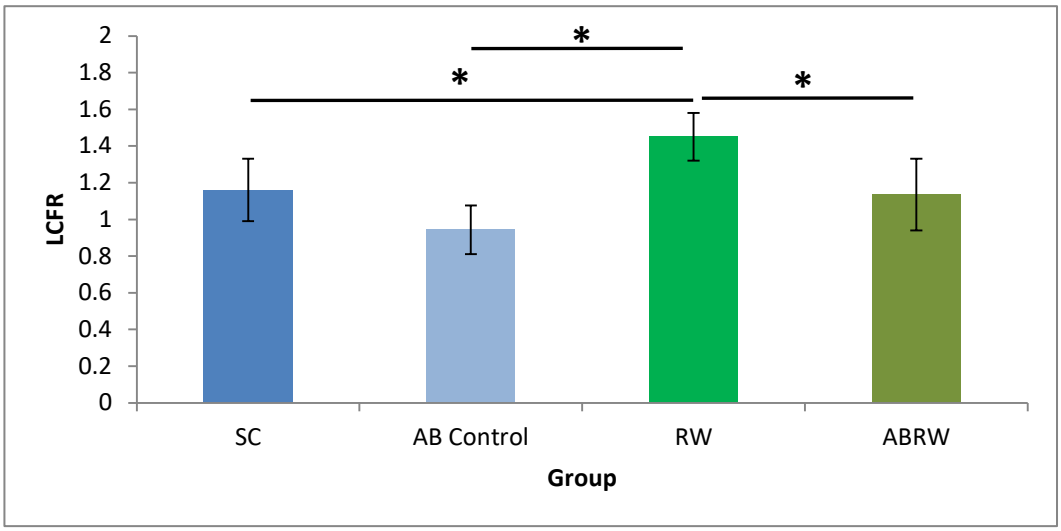

Figure 6.28: Local capillary to fibre ratio (LCFR) of SC, AB Control, RW and ABRW rats. *One-way ANOVA: mean difference of LCFR are significant between SC, AB Control and ABRW *vs.* RW (*$P \leq 0.05$).

6.3.6.4 **Local capillary density, CD**

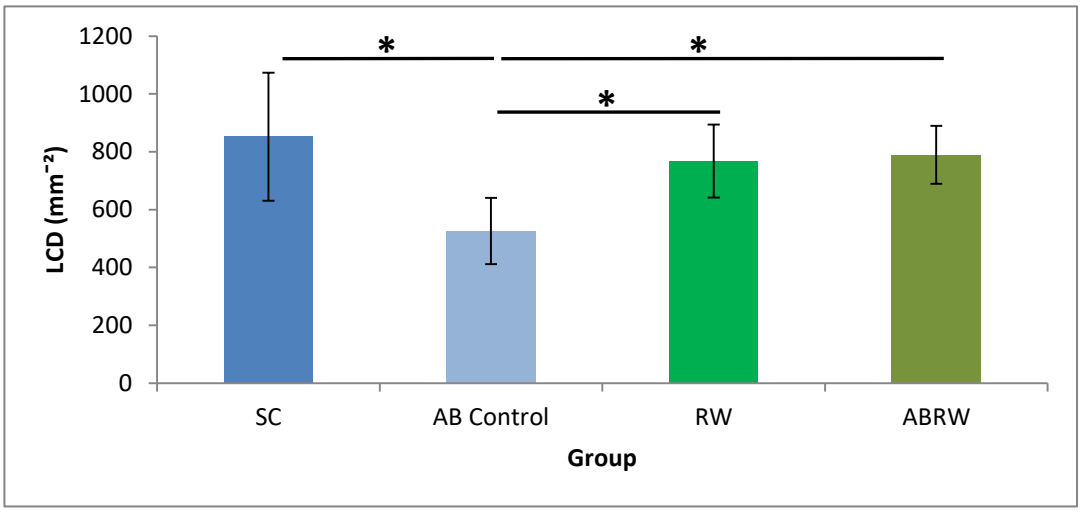

Figure 6.29: Local capillary density (LCD) of SC, AB Control, RW and ABRW rats. One-way ANOVA: mean difference of LCD are significant between SC, RW and ABRW *vs*. AB Control (*$P \leq 0.05$).

6.3.7 **Estimated muscle function - Oxygen transport modelling (OTM)**

Under simulated resting conditions both RW and ABRW showed relatively greater mean oxygen tension (partial pressure) than SC rat, with the same (negligible) proportion of calculated hypoxia coverage. In the exercise state, trained muscles performed relatively better than SC indicated by lower hypoxia coverage 1.26% (RW) and 6.50% (ABRW) *vs.* 7.92% (SC).

6.3.7.1 **Resting state**

Table 6.5: Oxygen partial pressure and percentage of hypoxia of EDL of SC, RW and ABRW rats in the resting state.

Compartment	PO_2 (mmHg)				% Hypoxia			
	SC	AB control	RW	ABRW	SC	AB control	RW	ABRW
Interstitia	26.5±1.9	25.1±1.9	28.0±1.3	26.1±2.0	0	0	0	0
Type I	28.1±0.8	26.6±0.8	29.0±0.4	27.6±1.2	0	0	0	0
Type IIa	27.6±1.0	24.8±1.7	28.6±0.6	26.1±1.5	0	0	0	0
Type IIb	25.8±1.7	26.2±1.2	27.5±1.1	27.5±1.3	0	0	0	0
All fibres	26.0±1.7	25.0±1.7	27.7±1.1	26.4±1.5	0	0	0	0
Tissue	26.1±1.8	25.0±1.7	27.8±1.1	26.3±1.6	0	0	0	0

6.3.7.2 **Maximal exercise level**

Table 6.6: Oxygen partial pressure and percentage of hypoxia of EDL of SC, RW and ABRW rats during exercise.

Compartment	PO_2 (mmHg)				% Hypoxia			
	SC	AB control	RW	ABRW	SC	AB control	RW	ABRW
Interstitia	16.1±8.3	9.9±8.6	21.1±7.2	13.4±9.4	7.2	17.9	1.5	10.8
Type I	22.3±4.3	16.9±4.2	25.2±1.7	20.5±5.6	0	0	0	0.3
Type IIa	20.9±4.3	8.7±6.5	24.6±2.8	12.9±6.9	0	11.3	0	4.9
Type IIb	12.1±6.9	14.9±5.7	18.3±5.9	20.1±6.1	9.3	0	1.5	0
All fibres	13.3±7.3	9.6±6.8	19.5±5.9	14.4±7.3	7.9	9.7	1.2	4.0
Tissue	13.6±7.5	9.7±7.0	19.6±6.0	14.2±7.6	7.9	10.4	1.3	4.7

6.3.7.3 **OTM of ABRW EDL muscle:**

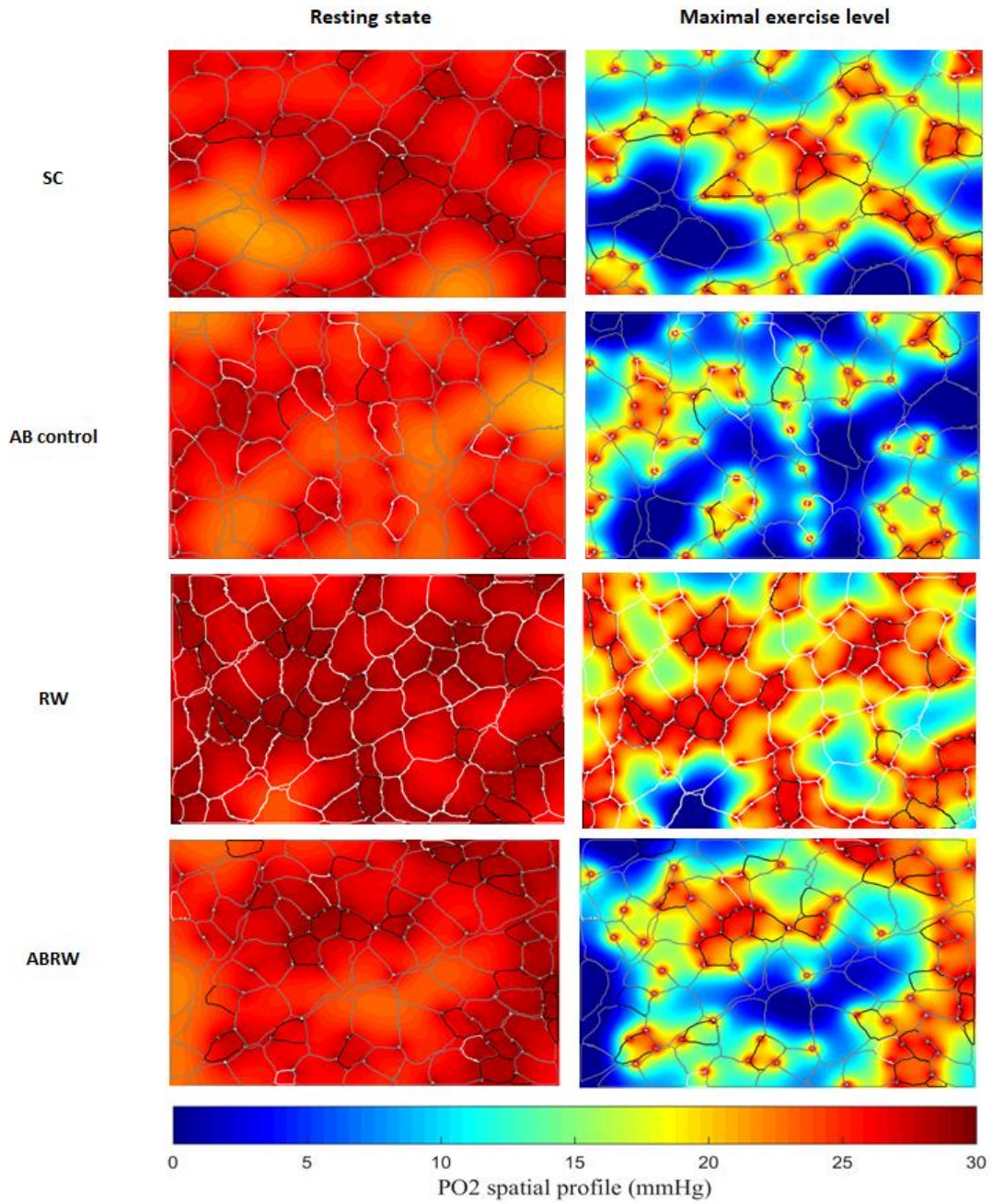

Figure 6.30: Estimated function of EDL of SC, AB control, RW and ABRW rats through mathematical modelling of muscle O_2 partial pressure, with O_2 consumption modelled at rest and during maximal exercise level. Note the relatively uniform oxygenation at rest, and the appearance of hypoxic regions at a high intensity exercise level.

6.3.8 **Cardiovascular effects of aortic banding**

Carotid blood pressure was higher after chronic banding compared to all unbanded groups (Table 6.7; $P<0.05$). Acute-AB animals had an intermediate carotid pressure that did not significantly differ from any other group. Tail blood pressure was elevated in RW ($P = 0.039$) and ABRW ($P = 0.016$) compared to Control (Table 6.7). Acute-AB had lower tail pressure than all other groups ($P<0.002$), and consequently a greater carotid:tail BP ratio ($P<0.001$). There were no significant differences in heart rate between groups. After controlling for the significant effect of body mass ($P = 0.005$) on heart mass using ANCOVA, there remained a significant effect of treatment ($P<0.001$) (Table 6.7). Control heart mass was lower than in all other groups ($P<0.05$); cardiac enlargement occurred in RW (compared to Control: $P = 0.028$), that was smaller than the compensatory hypertrophy in AB ($P = 0.005$) but similar to ABRW ($P = 0.904$); there was no difference between banded groups' heart mass (all n.s.).

Table 6.7: Cardiovascular response to aortic banding. Unshared letters denote statistical significance ($P<0.05$) as determined by ANOVA with post-*hoc* tests.

	Control	RW	Acute-AB	AB control	ABRW
Body mass (Mb; g)	254 ± 44 ^{ab}	316 ± 41 ^{c}	245 ± 40 ^{a}	304 ± 17 ^{bc}	305 ± 33 ^{bc}
Heart mass (% *M*b)	0.27 ± 0.04 ^{a}	0.32 ± 0.04 ^{b}	-	0.38 ± 0.04 ^{c}	0.35 ± 0.04 ^{bc}
BP (carotid; mmHg)	122 ± 3 ^{a}	124 ± 13 ^{a}	139 ± 12 ^{ab}	157 ± 25 ^{b}	148 ± 14 ^{b}
BP (tail; mmHg)	92 ± 7 ^{ab}	115 ± 11 ^{c}	58 ± 16 ^{d}	115 ± 21 ^{bc}	119 ± 12 ^{c}
Carotid:tail BP	1.33 ± 0.13 ^{a}	1.08 ± 0.11 ^{a}	2.54 ± 0.73 ^{b}	1.39 ± 0.22 ^{a}	1.25 ± 0.14 ^{a}
HR (bpm)	394 ± 43 ^{a}	383 ± 53 ^{a}	356 ± 68 ^{a}	359 ± 59 ^{a}	400 ± 43 ^{a}

6.3.9 **Muscle performance**

Fatigue index (FI) was lower compared to Control (FI = 0.49 ± 0.06) immediately after band application (Acute-AB: FI = 0.27 ± 0.10: $P<0.001$) and remained impaired after 4 weeks (AB: FI = 0.37 ± 0.05; $P = 0.039$, Table 6.8). FI was comparable to Control in ABRW (FI = 0.49 ± 0.09) $P>0.05$) and was significantly higher than AB ($P<0.05$).

Table 6.8: EDL fatigue resistance, isometric twitch performance and hindlimb blood flow. Unshared letters denote statistical significance ($P<0.05$) as determined by ANOVA with Tukey post-hoc-tests.

		Control	RW	Acute-AB	AB	ABRW
Fatigue index		0.49 ± 0.06 ^{a}	0.45 ± 0.05 ^{ab}	0.27 ± 0.10 ^{d}	0.37 ± 0.05 ^{bd}	0.49 ± 0.09 ^{a}
Femoral flow (ml/min)	rest	1.09 ± 0.22 ^{a}	1.32 ± 0.39 ^{a}	0.48 ± 0.21 ^{b}	1.23 ± 0.19 ^{a}	1.39 ± 0.32 ^{a}
	end-stimulation	1.74 ± 0.23 ^{ab}	2.07 ± 0.81 ^{a}	0.96 ± 0.48 ^{b}	1.80 ± 0.46 ^{ab}	2.13 ± 0.42 ^{a}

6.3.10 **Effects of aortic banding and exercise volume on muscle capillarity**

A two-way between groups analysis of variance (2-way ANOVA) was conducted to explore the impact of banding application and exercise volume on muscle capillarity (C:F). Rats were divided into three groups according to their running distance (low: 0-2 km/day; moderate: 3-7 km/day; high: 8 and above km/day). The interaction effect between banding application and exercise volume was not statistically significant, F (1, 7) = 1.214, *P* = 0.307. There was a statistically significant main effect for exercise volume, F (2, 7) = 8.320, *P*=0.014, the effect size was large (partial eta squared=0.704). Post-hoc comparisons using the Tukey HSD test indicated that the mean C:F for moderate running distance (M = 5.31, SD =1.06) was significantly different from the low running distance (M = 1.24, SD = 0.21). The high running distance group did not differ significantly from either of the other groups. The main effect for aortic banding application, F (1, 7) = 1.227, *P*= 0.305, did not reach statistical significance.

6.4 Discussion

6.4.1 **Cardiac hypertrophy and effect of exercise on skeletal muscle mass**

The current study found that 4 week duration of aortic banding in Wistar rats resulted in 33% cardiac hypertrophy. Similarly, 8 weeks aortic constriction in Sprague Dawley rats led to 32% left ventricle (LV) hypertrophy (Morris, Fitzsimons, Baldwin, & Barnard, 1993) while male Wistar-Kyoto rats showed 43% (LV) hypertrophy after 4 weeks (Yang et al., 1992). The percentage of cardiac or LV hypertrophy seemed to be dependent on the degree of aortic constriction, which proportionally affected running wheel performance (van Deel et al., 2011). However, Levy *et al*., (1996) showed that LV hypertrophy was independent of the duration - 3 and 6 weeks aortic constriction yielded 41% and 37% heart enlargement, respectively (Levy et al., 1996). Running wheel performance in the current study was lower than a study using mice (van Deel et al., 2011), in which a similar clip diameter was applied to constrict the aorta, 0.51 and 0.52 mm respectively, invoking a greater extent of aortic stenosis due to differences in body mass. This resulted in higher running distance in the rat study (van Deel et al., 2011), demonstrating that a suitable interior diameter (ID) clips is crucial for a species to model aortic stenosis.

Similar to LRW rats in the earlier chapter, there was a very large variation in total running distance in ABRW rats, which likely contributed to a similar mean body mass to control rats. EDL body mass ratio of ABRW was not significantly different compared to SC and AB Control rats, indicating aortic banding application reduced running activity of ABRW rats due to muscle weakness rather than bulk. Heart body mass ratio in ABRW was ~10% lower than AB Control but higher than SC rats, reflecting a potential benefit of wheel running exercise to ameliorate inadequate cardiac output during compensatory cardiac hypertrophy, while a significant improvement may be observed if exercise volume is increased. For example, low intensity treadmill exercise for 15 weeks preserved LV function of aortic-banded miniature swine by attenuating fibrosis and collagen levels, and inhibiting mitochondrial dysfunction (Emter & Baines, 2010). Thus, changes in cardiac size may be accompanied by better quality of the myocardium leading to improved functional capacity.

6.4.2 **Wheel exercise improves microcirculation in aortic banded rats**

Higher running performance demonstrated by RW rats was correlated with a greater C:F, and reduction in capillarity in ABRW was associated with low running distance, as shown by percentage of active time and running speed. However, wheel exercise for 4 weeks in ABRW rats was able to prevent severe capillary rarefaction, such that C:F was similar to SC rats. In previous work, capillary rarefaction was associated with impaired exercise capacity in clinical (Gerovasili, 2009) and induced heart disease (Duscha et al., 1999; Nusz et al., 2003). Furthermore, relatively larger capillary domain areas (CDA) in AB Control potentially reduced muscle performance due to diffusion limitations on oxygen delivery. Exercise intervention (RW and ABRW) shifted the distribution to the left, leading to a relatively higher frequency of small CDA and thus an enhanced local capillary supply (LCD). This reduction in intercapillary distance and area of tissue supplied by an individual capillary will improve oxygen supply to working tissue (Al-Shammari et al., 2019). The rapid upregulation of anti-angiogenic reactive oxygen species (Ungvari, Csiszar, Kaminski, Wolin, & Koller, 2004) and inflammatory cytokines (Agnoletti, 1999; Sun, 2007) during development of cardiac dysfunction cause the progression of microvascular rarefaction. Improvement in the microcirculation of ABRW rats due to exercise training may be due to stimulated production of angiogenic factors or reduction in anti-angiogenic factors to attenuate capillary rarefaction.

2-way ANOVA analysis showed that application of clips to constrict the abdominal aorta did not reduce running performance of the ABRW rats. Therefore, modest muscle capillary expansion in ABRW group was stimulated by voluntary wheel running activity which the rate relatively lower than RW rats due to limited blood supply caused by aortic stenosis.

6.4.3 **Fibre type composition**

Shifts in skeletal muscle fibre type (Type I to Type IIA fibre types) and muscle atrophy (due to Type IIA fibre atrophy) may develop with CHF, and are also associated with exercise intolerance (Carvalho et al., 2003; De Sousa et al., 2002; Mancini, 1992). The current observed changes in AB control and ABRW fibre composition, where numerical density of slow and fast oxidative fibres was higher, indicates this model of CHF also induces fibre type transformation. In addition, lower MFA induced a decreased areal density of aerobic fibre types, which may reduce functional capacity of the muscle by lowering oxidative capacity. Reduced MFA may be linked to reduced muscle mass which attenuates mammalian muscle force generation (Fleg & Lakatta, 1988; Frontera et al., 1988; Shephard et al., 1988), as

force production is proportional to cross sectional area. This was seen in aortic banded rats after 3 weeks that caused a 40% cardiac enlargement which impaired functional capacity in only slow twitch soleus muscle (Levy et al., 1996).

6.4.4 **CDA frequency distribution and heterogeneity**

Aerobic exercise after banding (ABRW) preserved capillary domain area distribution, thereby maintaining tissue oxygenation to a similar level to normal muscle, SC. Despite an attenuated microvascular network in banded rats, LogSD of CDA was maintained in AB Control and ABRW rats, suggesting that heterogeneity of functional and anatomical capillary spacing was unaffected in this early phase of CCH and hypertension, preventing further declines in oxygen transport capacity (Al-Shammari, Gaffney, & Egginton, 2014; Degens et al., 2006). This implies a high degree of regulation in position of capillaries, either lost or gained, to minimise gross disturbance in muscle oxygenation.

6.4.5 **Local capillarity and oxygen transport modelling**

4 week voluntary wheel running exercise increased local oxygen supply of ABRW rats by enhancing their LCD, i.e. local capillary supply capacity normalised for differences in fibre size. It was noted that aortic banding application could reduce local blood supply as LCD was significantly reduced in AB Control accompanying muscle atrophy, indicating true capillary rarefaction took placed by week 4 due to restricted muscle activity (Mancini et al., 1992; Minotti, Christoph, & Massie, 1992).

The mild expansion in microcirculation of ABRW rats led to improved oxygenation thus enhancing skeletal muscle function during exercise. OTM analysis showed that PO_2 of ABRW muscle was nearly 50% higher than SC, and hypoxia percentage was relatively lower than SC (6.50% *vs.* 7.92%, respectively).

i) Aortic banding application and its effect on central and peripheral blood pressure

Left ventricular pressure overload developed following surgically-induced abdominal aortic constriction, which resulted in CCH due to chronic hypertension above the stenosis (Cornelussen et al., 1994; E. De Sousa, 2002; Degens, de Brouwer, et al., 2006; Levy et al., 1996; Ogoh, Hirai, Nohara, & Taguchi, 2002). Interestingly, the degree of hypertension increased over time in banded animals, with an intermediate carotid pressure in the acute phase. In contrast, there was an acute reduction in tail blood pressure that occurred immediately following band application that subsequently recovered upon 4-week wheel exercise training, indicating that homeostatic mechanisms to maintain peripheral blood

pressure was not adversely affected by CCH (Ungvari et al., 2004). Hindlimb muscle function measurement was therefore performed under normotensive conditions. An adaptive cardiac enlargement was evident in wheel exercise rats (RW) that did not significantly differ in magnitude from the compensatory hypertrophy (ABRW), indicating the potential for contributions of adaptive (Moreira-Gonçalves, 2015) and pathological (Grossman, Jones, & McLaurin, 1975) stimuli to cardiac growth.

ii) Effect of exercise on muscle performance

A combination of aortic banding and treatment (wheel exercise) showed enhanced muscle performance compared to AB control, reflecting plasticity of skeletal muscle to both local and systemic influences. Aortic constriction exhibited a reduction in muscle performance among acute AB rats. However, they were otherwise healthy, indicating neither muscle tissue nor microcirculation dysfunction determined this impairment. Instead, decreased blood supply caused impaired fatigue resistance. This is consistent with attenuated ischaemic muscle performance due to feed artery stenosis (Fulgenzi et al., 1998; Murthy, Hargens, Lehman, & Rempel, 2001). A 25% reduction in FI of AB control rats compared to control revealed the detrimental systemic effect of CCH on muscle performance (Levy et al., 1996) that might be due to capillary rarefaction and muscle atrophy (Ogoh et al., 2002).

Voluntary wheel running prevented chronic functional impairment represented by ABRW rats, where FI was raised to control levels, demonstrating the beneficial effect of aerobic exercise in maintaining muscle performance, despite development of CCH. This, however, contrasts with findings that showed muscle performance enhancement in CHF patients following a resistance exercise therapy (Braith & Beck, 2008; Braith, 2005; Giuliano, Karahalios, Neil, Allen, & Levinger, 2017; Selig, 2004). This suggests that the self-selected exercise intensity level of ABRW rats was insufficient to increase exercise endurance capacity, but that it was sufficient to offset the pathological effect of CCH on impairment of skeletal muscle performance.

Kinematic data showed a significantly reduced running bout duration of ABRW rats, thus lowering the overall distance run; a similar observation was found in previous work using banded rats (De Sousa et al., 2002). Moreover, mild improvement in ABRW muscle capillarity in ABRW, +24% (*vs.* AB control), -7% (*vs*. SC) and -19% (*vs*. RW) C:F, accompanying Type IIa fibre atrophy (-80% A_A *vs*. RW) impaired muscle power generating capacity that resulted in declining locomotor performance, and lowered running velocity. White gastrocnemius of exercised CHF rats recorded a modest increase in CD (8%) which was not significant, following 10 weeks treadmill run at 60% $\dot{V}O_2$ max (Ranjbar et al., 2017).

In contrast, CHF mice performed swimming exercise for 8 weeks significantly enhanced CD of gastrocnemius (Medeiro, Vanzelli, Rosa, Irigoyen, & Brum, 2008), demonstrating that different exercise modalities resulted in different magnitudes of capillary adaptation in skeletal muscle.

However, the attenuated exercise performance restored EDL muscle function to normal baseline values, emphasising the potential of low intensity exercise in reversing pathological changes in skeletal muscle of those with CCH. Voluntary exercise capacity was demonstrated to reverse mitochondrial dysfunction and muscle metabolism abnormalities in clinical CHF (Adamopoulos, 1993; Hambrecht, 1995; Stratton, 1994) and an aortic coarctation model (De Sousa et al., 2002; Gomes, 2016). These results demonstrate that aerobic exercise generates a potent restorative effect on skeletal muscle structure and function at various phases of disease.

6.5 Conclusion

In summary, we have demonstrated that ABRW rats benefited from 4 week voluntary wheel running exercise which restored capillary supply by increasing global C:F while maintaining MFA and local LCD of EDL muscle. Aerobic exercise derived angiogenic stimuli likely contributed to the successful recovery of muscle performance. Therefore, improved aerobic capacity of EDL muscle of aortic-banded rats supports the hypothesis that skeletal muscle performance in CHF is primarily determined by peripheral factors, specifically the muscle microcirculation. The muscle performance (FI) of aortic banding rats was able to be restored by 4-week voluntary wheel running exercise. Aortic banding rats improved fatigue resistance by increasing femoral blood flow to match increased metabolic demand during running activity. Diminished muscle microcirculation in development of exercise intolerance during cardiac dysfunction underline how angiogenic restorative growth should be a key therapeutic target.

Chapter 7 : $\dot{V}O_{2\ peak}$ and running economy of local and systemic blood supply reduction in rat lower limb following 4-week voluntary wheel running exercise.

7.1 Introduction

Maximal oxygen consumption ($\dot{V}O_{2\ max}$) is measured to determine the limit of the cardiorespiratory system in transporting oxygen to working muscles. It is defined as the oxygen consumption attained during maximal exercise intensity level, and which does not rise despite additional increases in exercise workload (Hill & Lupton, 1923; Lighton, 2008; McArdle, Katch, & Katch, 1991). Respiratory exchange ratio (RER; amount of carbon dioxide produced / oxygen used in metabolism) of more than 1.1 or 1.15, and a heart rate within 15 beats per minute (BPM) of age-predicted maximal heart rate (i.e. 200-age), are additional indices used to confirm $\dot{V}O_{2\,max}$ (Ebbeling, Ward, Puleo, Widrick, & Rippe, 1991). The value is dependent on the ability of the blood transport system to supply oxygen and the efficiency of tissues to take up and utilise oxygen for energy production (Hartung, Krock, Crandall, Bisson, & Myhre, 1993). Measurement of this parameter ($\dot{V}O_{2\ max}$) has been widely performed both in human (Ferretti, 2014; Hawkins, Raven, Snell, Stray-Gundersen, & Levine, 2007; M. L. Pollock, 1973) and rodent (Davies, Packer, & Brooks, 1981; Gleeson & Baldwin, 1981; Lambert & Noakes, 1989, 1990) studies in determining effectiveness of an exercise regime in enhancing maximal aerobic capacity of exercised subjects or animals.

It has been showed by previous studies that $\dot{V}O_{2\ max}$ can be determined by performing a ramp incremental exercise test, using a treadmill equipped with a metabolic chamber (open mask) or flow-through respiratory mask that fitted on the subjects head (closed mask) (Withers, 1977). There were various ramp test protocols used to measure $\dot{V}O_{2\ max}$ with application of running stimulants such as electrical grid shock (Copp et al., 2009; Gleeson & Baldwin, 1981; Lambert & Noakes, 1990) necessary to obtain true $\dot{V}O_{2\,max}$ values in rats. In rodent studies using running wheel exercise, various training periods were used to determine its effect on $\dot{V}O_{2\,max}$. There were inconsistent findings on the effect of wheel exercise on $\dot{V}O_{2\,max}$ at comparable body mass (Lambert & Noakes, 1990; Lambert et al., 1996) due to group separation based on running distance capacity, which showed significant differences among studies. Use of a specific rat strain or defined exercise periods is required for consistency, as behavioural effects might influence the measurement and magnitude of $\dot{V}O_{2\,max}$ change (Lambert & Noakes, 1990; Lambert et al.,

1996), particularly evident in voluntary wheel exercise. The present study characterised the effects of these factors on aerobic capacity following chronic wheel running exercise.

Little information on $\dot{V}O_{2max}$ of the less aerobic (Wistar) strain of rat is available (Gordon et al., 2016), particularly in studying the effect of wheel exercise with local or systemic reduction in blood flow on hindlimb muscle performance or whole body aerobic capacity. The potent angiogenic stimulus invoked by exercise training *via* functional hyperaemia might trigger muscle capillary bed expansion (Degens, Veerkamp, Turek, Hoofd, & Binkhorst, 1993; Dunford, 2017; Mortensen, 2017; Waters et al., 2004). Subsequently, that could recover and/or enhance efficiency of oxygen utilisation, a key determinant of exercise intolerance, in pathological conditions characterised by poor peripheral microvascular supply (Hoier et al., 2013; Nusz et al., 2003; Schaufelberger et al., 1995).

Banning electrical shock use by the UK Home Office has made it difficult to obtain true $\dot{V}O_{2max}$ measurements, because no alternative effective running stimulant can be used to motivate rats to run at the higher treadmill speeds required. Use of compressed air or air gun may motive high speed running (Noble, Ho, & Dzialoszynski, 2006), but in the present study setting (using open mask respirometry) did not allow for its use as the compressed air (outside air/ commercial compressed air) would change the expired air composition which may lead to overestimation/underestimation of oxygen content. So, a change to source of compressed air was made i.e. sourcing metabolic chamber air that contains expired air, to minimise the measurement error. Moreover, a black box was put in front of rats to maximise running motivation, assuming the animals favour a dark surrounding for their nocturnal activity (Seo, 2014a).

Data from a preliminary study showed that $\dot{V}O_{2\ max}$ of RW rats could not be obtained, even when compressed air was directed to lower limbs and food reward have been applied. Application of compressed air (using expired air) only worked at lower speeds, whereas the animals tend to be at the back of chamber at higher speed. It was expected that in the present study, including unilateral ligation and aortic banding, rats would not reach their true $\dot{V}O_{2\ max}$ due to lack of a running motivator as effective as electrical grids to push rats running at higher speeds, so an alternative output was considered.

Some studies terminated maximal aerobic capacity testing prior to obtaining a true $\dot{V}O_{2\ max}$, e.g. in participants with exercise intolerance due to pain or fatigue (Noonan & Dean, 2000). It was then termed as $\dot{V}O_{2\ peak}$ to recognise the underestimation of $\dot{V}O_{2\ max}$

(Arena, Lavie, Milani, Myers, & Guazzi, 2010). Moreover, if inclusion criteria of $\dot{V}O_{2\,max}$ are not fulfilled, the highest $\dot{V}O_2$ rate obtained is considered as $\dot{V}O_{2\,peak}$ (Zeballos & Weisman, 1994). Therefore, due to difficulty in obtaining $\dot{V}O_{2\,max}$, an alternative cardiorespiratory fitness parameter (i.e. $\dot{V}O_{2\,peak}$) was used in the current study for assessing aerobic capacity.

Running economy (RE) is defined as energy expenditure for a particular running speed, at a submaximal running level, and expressed as submaximal $\dot{V}O_2$ rates at a given velocity (Costill, 1972; Daniels, 1985; Saunders, Pyne, Telford, & Hawley, 2004) with RER below 1 (Conley & Krahenbuhl, 1980; Saunders et al., 2004). It could be a useful indicator for endurance running performance, especially in differentiating long distance running performance (Pollock, 1977). It has been shown that enhancement in cardiorespiratory and metabolic efficiency result in improvement in oxygen use (Holloszy, Rennie, Hickson, Conlee, & Hagberg, 1977; Pattengale & Holloszy, 1967; Pollock, 1977). So, this measure may provide a meaningful aerobic capacity index in assessing the efficiency outcome of exercise intervention supported by the cardiorespiratory system.

Total running distance of RW rats in a preliminary study peaked after 4 weeks running in the wheels, which was associated with significant capillary expansion. Therefore, this study aimed to determine effect of 4-week voluntary wheel exercise on rat peak aerobic capacity ($\dot{V}O_{2\,peak}$) and RE (assessed from $\dot{V}O_{2\,submax}$) of running wheel (RW) rats and rat disease models with local (unilateral femoral artery ligation) or systemic (application of aortic banding) arterial stenosis resulting in lower limb muscle ischaemia, which mimics PAD disease.

This study aimed to determine $\dot{V}O_{2\,peak}$ and running economy (RE) of RW rats following 4 week voluntary wheel running exercise. Effects of the exercise regime on $\dot{V}O_{2\,peak}$ and running economy (RE) of unilateral femoral artery ligation and aortic banded rats on were determined and compared. We then hypothesised that: 1) 4-week voluntary wheel running exercise increases $\dot{V}O_{2\,peak}$ and RE of RW rats, partly supported by improved muscle capillarity. 2) 4-week voluntary wheel running exercise recovers exercise tolerance of LRW and ABRW rats, leading to improved post $\dot{V}O_{2\,peak}$, 3) Oxygen running cost of trained rats is improved relatively to their baseline value following 4-week voluntary wheel running exercise due to increased muscle oxidative capacity and a more efficient cardiovascular system.

7.2 Methods

7.2.1 Animals

7.2.1.1 Unilateral femoral-artery ligation

Refer to material and methods chapter 7.

7.2.1.2 Aortic banding

Refer to material and methods chapter 8

7.2.2 Wheel dimension and setting

Refer to general method

7.2.3 Treadmill familiarisation

Rats were familiarised with treadmill running (a dual lane treadmill, model 760303, Harvard Apparatus, Cambridge, UK) for 5-10 min/day for 5 days at 10% inclination (Copp et al., 2009) at 16-26 cm/s (Gleeson & Baldwin, 1981) prior to pre $\dot{V}O_{2\ peak}$ measurement, and all measurements were completed within 3 days. Weekly treadmill running familiarisation was performed on cage cleaning day (to minimise disturbance) when treadmill speed was increased up to 30-36cm/s along with increased body mass (Gleeson & Baldwin, 1981).

7.2.4 Flow respirometry system setting

The current study applied a pull mode flow system in flow-through respirometry, where expired air is pulled out of a metabolic chamber to gas analysers. Oxygen consumption and carbon dioxide production were measured using an oxygen analyser (S3A/II differential oxygen gas analyser: AEI Technologies, TX, USA) and carbon dioxide analyser (Sable Systems, Las Vegas, NV, USA). Surrounding air was pumped through a metabolic chamber at a flow rate of 10L/min. Subsampling of expired air was set at a flow rate of 200ml/min prior to passing through a Drierite® column to remove water vapour. The sampled air was first drawn into the carbon dioxide analyser, and then scrubbed by soda lime prior to the oxygen analysers (Copp et al., 2009; Lighton, 2008). Response time was 30 seconds for any change in oxygen composition. The metabolic chamber with a volume of 5.5 litres placed on the treadmill surface featured 2 inlets and an outlet. A tube was place at the back of the chamber which connected to a manual pump for generating compressed air. Layers of black tapes were put underneath the chamber lid to prevent animal tails from getting stuck and injury.

The oxygen analyser was switched on a day prior to use so that its temperature stabilised for accurate readings. A mixing fan was placed in the chamber (located on the chamber ceiling) set at moderate speed to promote mixing and avoid biased sampling. A black box was placed facing the running rats to encourage them to run at higher speed, with an assumption that nocturnal animals prefer to be in a dark area (Seo et al., 2014a). Both O_2 and CO_2 of expired air were measured to provide information required to compensate for gas interactions and determine the respiratory substrates consumed by determining respiratory exchange ratio (RER).

Setting of flow respirometry system:

Respirometry chamber on treadmill

↓

Mass flow meter

- Flow rate is set at 10 litres/min
- To collect and flow expired air out of metabolic chamber.

↓

Subsampling column

- Flow rate is controlled by flow meter that is set at 200 ml/min.
- Outside air (baseline) is sampled followed by calibration gas, room air and expired air by putting respective tube to a subsample column.

↓

Drierite column

- Water vapor in sampled air (Outside, room and expired air) is removed
- Calibration gas bypass this water vapor scrubber

↓

Carbon dioxide analyser

- Air flow rate is control by a flow meter at 200 ml/min.
- Carbon dioxide composition is read relative to baseline air (outside air) that signal is converted to electrical signal by sensor cell that is processed by readout/control unit.
- Delta change of carbon dioxide is displayed on computer screen and recorded.

↓

Carbon dioxide analyser scrubber

- It is soda lime that removes carbon dioxide from the sampled gas.

↓

Oxygen analyser

- Oxygen gas composition is read relative to baseline air (outside air). Flow rate is set at 200 ml/min.
- Air flows out to atmosphere.

7.2.5 **Respiratory chamber integrity**

Some parts of the respiratory chamber was sealed with tape (base of sides) to prevent room air from flowing into the chamber or expired air flowing out through moving treadmill belt. There were only 2 ports (incurrent air) and an outlet (excurrent air) on the chamber back and front sides, respectively, to ensure even air flow in the chamber. A nitrogen leak test was performed to check for leaking at varying treadmill speeds. This involved determining oxygen concentration at varying treadmill speeds, where a constant low nitrogen gas was contained in the chamber. Results showed that there was only a low degree of leakage, which did not adversely affect the measurements; 19.98, 19.95, 20.02, 20.01, 19.79 and 19.80% oxygen concentration at 32, 42, 47, 52, 57 and 62 cm/s, respectively.

7.2.6 **Running stimulant**

Other than using a black box and wrapping the chamber with black paper, rats were also fed with 0.5g cereal or chocolate (Wisløff, Helgerud, Kemi, & Ellingsen, 2001) as rewards after completing a treadmill running familiarisation or test to motivate them to run. Moreover, compressed expired air, directed to rats' legs and knocking the chamber with a hard tool were applied to motivate them to run at high speeds.

7.2.7 **Pre and post $\dot{V}O_{2\ peak}$ measurement**

Baseline, calibration gas (containing 20% oxygen and 1% carbon dioxide) and room air were sampled for reference. After body mass was recorded, each rat was placed in the respiratory chamber for 5 minutes to condition the animal and measure its resting $\dot{V}O_2$. The rat started running at 16cm/s (40-50% of $\dot{V}O_{2\ max}$) for 5 min at 10° inclination (Gleeson & Baldwin, 1981; Wisløff et al., 2001) or until rates of O_2 consumption reaches a plateau that served as a warm-up period. Oxygen consumption was measured at increasing treadmill speed increments of 6cm/s every 2 minutes (Wisløff et al., 2001). $\dot{V}O_{2\ peak}$ was determined when the $\dot{V}O_2$ was sustained for a minimum of 1 minute, or rats could no longer maintain their pace on the treadmill and the test was terminated. The analysers were again calibrated with calibration gas for drift correction after the test finished (Copp et al., 2009; Gleeson & Baldwin, 1981; Wisløff et al., 2001). Post $\dot{V}O_{2\ peak}$ was measured after 4 week voluntary wheel running exercise using the same protocol.

7.2.8 **Respiratory exchange ratio (RER)**

Ratio of $\dot{V}CO_2$ and $\dot{V}O_2$ rates were determined and the respiratory exchange ratio (RER) calculated, used as an indicator for exercising at submaximal level (RER <1; (Saunders et al., 2004)).

7.2.9 **Running economy**

$\dot{V}O_2$ rate at resting, 16, 21, 26, 31 and 41 cm/s at 10° inclination of post 4 weeks with or without wheel exercise were compared with pre or baseline value in which increased RE was indicated by lower $\dot{V}O_2$ rate at those particular running speeds.

7.2.10 **Exercise training**

Refer to methods in chapter 5 (unilateral femoral artery ligation) and 6 (aortic banding)

7.2.11 **Data analysis**

Changes (delta) in oxygen and carbon dioxide content of expired air relative to baseline value were recorded using LabChart programme. Data were extracted and drift correction in both calibration points was performed in which linear drift was assumed, and $\dot{V}O_{2\ peak}$ and RER were determined. Statistical analysis was performed using IBM SPSS v. 20. Normality test was performed on the data, where normally distributed data were analysed using independent- samples T-Test or One-Way ANOVA to compare mean values among the groups. Turkey post hoc analysis was further applied to determine specific pairwise differences between groups. *P* value ≤ 0.05 was considered as significant level. All results were presented as mean ±SD in table and graph forms.

7.3 Results

7.3.1 Unilateral femoral artery ligation study

7.3.1.1 Rat body mass characteristic and $\dot{V}O_{2\ peak}$

Initial and post study body mass were similar across the groups (P>0.05). All baseline pre $\dot{V}O_{2\ peak}$ measurements were taken prior to wheel exercise which showed similarity between LC, LRW and RW groups (82.9±5.7 *vs*. 82.3±12.5 *vs*. 85.9±10.1 ml/kg/min, respectively; Table 7.1, n.s.). After 4 weeks wheel running exercise, post $\dot{V}O_{2\ peak}$ of RW rats was unchanged relative to pre $\dot{V}O_{2\ peak}$, (P>0.05). A significantly lower exercise volume was performed by LRW rats; this decreased their post $\dot{V}O_{2\ peak}$ compared with baseline value (P<0.05), which was similar to LC rats (P>0.05). RER of all pre and post $\dot{V}O_{2\ peak}$ were lower than <1, confirming the animals ran at submaximal level (Copp et al., 2009).

Table 7.1 : Pre and post body mass, $\dot{V}O_{2\ peak}$ and RER of 4-week voluntary wheel running exercise of LC, LRW and RW rats. Independent-samples T-Test: mean different is significant in LC and LRW rats between pre and post $\dot{V}O_{2\ peak}$ (*P<0.05). Unshared letters denote statistical significance (P≤0.05) in pre and post groups as determined by ANOVA with Tukey post-hoc-tests. Data presented as mean±SD.

Group	Pre				Post			
	N	Body mass (g)	$\dot{V}O_{2\ peak}$ (ml/kg/min)	RER	N	Body mass (g)	$\dot{V}O_{2\ peak}$ (ml/kg/min)	RER
LC	6	186±8	82.9±5.7[a]	0.79±0.09	6	311±11	69.6±5.2[*b]	0.79±0.06
LRW	4	204±3	82.3±12.5[a]	0.70±0.17	7	330±34	66.1±11.0[*b]	0.78±0.10
RW	13	187±19	85.9±10.1[a]	0.78±0.10	14	313±19	83.7±8.4[c]	0.79±0.10

The VO_2 peak is decreased after treadmill running for 4 weeks for LC and LRW groups, as these rats experienced limited blood supply (and hence reduced lower leg muscle function) resulting in attenuated capacity for performing treadmill running. Note that the VO_2 peak values and the highest VO_2 rate at submaximal exercise level were based on the (different) number of rats that were able to perform running activity on the measuring day (poor runners were still not running, even when measurements were repeated 24hours later).

7.3.1.2 **Running economy (RE) of LC, LRW and RW rats**

Rat running cost in LC rats was increased compared to their baseline value but the cost was similar at the highest speed of 31cm/s. All trained rats showed better efficiency in oxygen consumption (lower oxygen percentage) at resting state compared to their pre exercise value. RW rats benefited from wheel exercise, which resulted in a lower oxygen cost for running activity at those particular speeds. Similarly, the chronic exercise performed by LRW rats enhanced running economy by utilising lower proportion of oxygen.

A) LC group

Table 7.2: Running cost at pre and post 4 weeks ligation (LC). Independent-samples T Test: Oxygen running cost increased after 4 weeks ligation at resting and running speed up to 31 cm/s, **P*<0.05.

Speed (cm/s)	Pre		Post		*P*-Value	Additional oxygen consumption rate (%)
	N	$\dot{V}O_2$ rate (ml/kg/min)	N	$\dot{V}O_2$ rate (ml/kg/min)		
0 (Resting)	6	18.2±2.0	6	28.5±5.4	*0.004	36
16	6	45.1±3.8	5	61.7±6.0	*0.001	27
21	6	54.0±6.7	5	64.9±6.1	*0.020	17
26	6	59.7±4.9	5	65.8±3.2	*0.036	9
31	6	66.9±4.0	3	70.5±5.8	0.402	5

B) LRW group

Table 7.3 : Running cost of LRW at pre and post 4-week voluntary wheel running exercise. Independent-samples T-Test: Oxygen running cost of LRW decreased following 4-week wheel exercise, **P*<0.05.

Speed (cm/s)	Pre		Post		*P*-Value	Oxygen consumption rate reduction (%)
	N	$\dot{V}O_2$ rate (ml/kg/min)	N	$\dot{V}O_2$ rate (ml/kg/min)		
0 (Resting)	4	36.2±5.4	7	18.8±5.2	0.001*	48
16	4	59.5±4.3	7	50.8±10.4	0.149	14
21	4	67.1±3.7	7	55.3±10.1	0.023*	18
26	3	70.7±8.9	7	59.2±8.4	0.085	16
31	3	80.3±7.7	7	63.2±8.6	0.018*	21
36	2	92.9±4.3	4	73.0±5.6	0.012*	21

C) RW group

Table 7.4 : Running cost of RW rats at pre and post 4-week voluntary wheel running exercise. Independent-samples T Test: Oxygen running cost of RW significantly decreased following 4-week wheel exercise, *P*<0.05.

Speed (cm/s)	Pre		Post		*P*-Value	Oxygen consumption rate reduction (%)
	N	$\dot{V}O_2$ rate (ml/kg/min)	N	$\dot{V}O_2$ rate (ml/kg/min)		
0 (Resting)	4	36.9±8.1	7	22.1±5.7	0.006*	40
16	4	63.8±6.0	7	47.8±8.6	0.010*	25
21	4	70.9±7.2	7	55.9±7.8	0.012*	21
26	4	77.7±6.0	7	64.5±8.2	0.021*	17
31	4	84.5±6.4	6	67.2±2.7	0.008*	20
36	2	92.2±3.0	6	70.9±4.2	0.008*	23

7.3.2 **Aortic banding study**

7.3.2.1 **Rat body mass characteristic and $\dot{V}O_{2\ peak}$**

Pre and post body mass were similar between ABRW and RW rats (*P*>0.05). All pre $\dot{V}O_{2\ peak}$ measurements of ABRW and RW rats were performed prior to aortic banding application and/or wheel exercise which were similar, 80.5±7.6 *vs.* 85.9±10.1 ml/kg/min (*P*>0.05). Post $\dot{V}O_{2\ peak}$ of ABRW and RW rats was similar to their baseline $\dot{V}O_{2\ peak}$ (*P*>0.05). Wheel exercise for 4 weeks by ABRW rats improved their $\dot{V}O_{2\ peak}$ relative to pre $\dot{V}O_{2\ peak}$ value (80.5±7.6 *vs*. 75.6±5.0 ml/kg/min; *P*>0.05). RER of all pre and post $\dot{V}O_{2\ peak}$ of ABRW and RW rats were lower than <1, confirming the highest measured $\dot{V}O_2$ rates were $\dot{V}O_{2\ peak}$ (Conley & Krahenbuhl, 1980; Copp et al., 2009; Saunders et al., 2004).

Table 7.5: Pre and post body mass and $\dot{V}O_{2\ peak}$ of 4-week voluntary wheel running exercise of ABRW and RW rats. Independent-samples T-Test: mean $\dot{V}O_{2\ peak}$ is not significant in RW and ABRW rats between pre and post $\dot{V}O_{2\ peak}$ (*P*>0.05); mean of pre $\dot{V}O_{2\ peak}$ was similar between ABRW and RW (*P*>0.05), and post $\dot{V}O_{2\ peak}$ of ABRW was significantly lower than RW rats. Unshared letters denote statistical significance (P≤0.05). Data presented as mean±SD.

Group	Pre				Post			
	N	Body mass (g)	$\dot{V}O_{2\ peak}$ (ml/kg/min)	RER	N	Body mass (g)	$\dot{V}O_{2\ peak}$ (ml/kg/min)	RER
ABRW	8	191±19	80.5±7.6	0.80±0.21	5	291±15	75.6±5.0[a]	0.72±0.12
RW	13	187±19	85.9±10.1	0.78±0.10	14	313±19	83.7±8.4[b]	0.79±0.10

7.3.2.2 **Running cost of ABRW and RW rats**

RW rats benefited from wheel exercise that resulted in a lower oxygen cost for running activity at those particular speeds. In contrast, the exercise regime only maintained the oxygen consumption rate of ABRW at resting and all running speeds.

A) ABRW group

Table 7.6: Running cost of ABRW rats at pre and post 4-week voluntary wheel running exercise. Independent-samples T- Test: Oxygen running cost of post exercise was similar to baseline, *P*>0.05.

Speed (cm/s)	Pre		Post		*P*-Value
	N	$\dot{V}O_{2\ rate}$ (ml/kg/min)	N	$\dot{V}O_{2\ rate}$ (ml/kg/min)	
0 (Resting)	7	31.8±4.0	5	25.9±7.8	0.113
16	7	50.0±8.1	5	47.1±10.7	0.619
21	7	57.3±7.0	5	55.5±10.2	0.719
26	7	63.4±9.0	5	60.9±10.3	0.663
31	7	69.4±9.1	5	65.2±9.4	0.451
36	6	72.8±6.2	4	65.9±8.4	0.169
41	2	74.2±2.3	2	78.7±8.3	0.536
46	2	77.6±4.0	2	78.0±6.8	0.948

B) RW group

Table 7.7: Running cost of RW at pre and post 4-week voluntary wheel running exercise. Independent-samples T Test: Oxygen running cost of RW rats significantly decreased following 4-week voluntary wheel running exercise, **P*<0.05.

Speed (cm/s)	Pre		Post		*P*-Value	Oxygen consumption rate saving (%)
	N	$\dot{V}O_{2\ rate}$ (ml/kg/min)	N	$\dot{V}O_{2\ rate}$ (ml/kg/min)		
0 (Resting)	4	36.9±8.1	7	22.1±5.7	0.006*	40
16	4	63.8±6.0	7	47.8±8.6	0.010*	25
21	4	70.9±7.2	7	55.9±7.8	0.012*	21
26	4	77.7±6.0	7	64.5±8.2	0.021*	17
31	4	84.5±6.4	6	67.2±2.7	0.008*	20
36	2	92.2±3.0	6	70.9±4.2	0.008*	23

7.4 Discussion

Maximal oxygen consumption ($\dot{V}O_{2\ max}$) measurement was the initial parameter aimed for in determining rat whole body aerobic capacity. As described previously, two criteria need to be fulfilled for this to be accurate; a plateau (levelling off) of $\dot{V}O_2$ with increased exercise workload, and RER values of 1.1 - 1.15 or higher (Copp et al., 2009; Hill & Lupton, 1923; Lighton, 2008). In the present study, the majority of rats performed the ramp test adequately only at lower treadmill speeds, and stopped the test as they were unwilling to run at the highest treadmill speed (pre and post highest running speed were achieved at different points, with post value tending to be at a higher speed), which was considered as their peak running capacity, but with RER values lower than 1.

Alternatives to electrical grid use have been applied to increase rat running motivation which included a black box, black paper wrapping on chamber surface and compressed air. However, those approaches did not work well as the rats did not run consistently, particularly when reaching higher treadmill speeds (at 41cm/s and above). At such speeds, rat tails tend to get stuck at the base of chamber lid or the rats leant on the lid wall, even when compressed air had been applied, showing it was not powerful enough to be an effective stimulus to motivate running. Alternatively, the rats might be in an early phase of fatigue. To our knowledge, no study has ever managed to measure $\dot{V}O_{2\ max}$ without using electric shocks to encourage running whenever their tails accidentally touch the rear grid. All previous $\dot{V}O_{2\ max}$ measurements in rodent studies used electrical shock to motivate high speed running (Gleeson & Baldwin, 1981; Høydal, Wisløff, Kemi, & Ellingsen, 2007; Lambert & Noakes, 1990; Lambert et al., 1996; Meek, Lonquich, Hannon, & Garland, 2009; Sonne & Galbo, 1980). Therefore, $\dot{V}O_{2\ peak}$ was instead used as a parameter for assessing aerobic capacity relative to the baseline value of pre-training state. As it is a requirement for not using electrical shock in $\dot{V}O_{2\ max}$ measurement, other alternative and effective running stimulus should be considered so that $\dot{V}O_{2\ max}$ measurement can reliably be obtained.

The first part of this work addressed the question of whether 4-week voluntary wheel running exercise may maintain or enhance $\dot{V}O_{2\ peak}$ (relative to pre $\dot{V}O_{2\ peak}$) and improve running economy of unilateral femoral artery ligation. The main finding of this work was that $\dot{V}O_{2\ peak}$ of LC rats were relatively decreased compared to their pre $\dot{V}O_{2\ peak}$ following 4-week ligation. The reduced aerobic capacity in LC might be due to prolong attenuated blood supply to lower limb, thus reducing muscle endurance capacity as a result of reduced routine activity (hypokinesia). In contrast, the exercise programme maintained

post $\dot{V}O_{2\ peak}$ of RW rats relative to their baseline value. Such an unchanged aerobic capacity might be influenced by body mass, even though angiogenesis was observed, but points to moderate exercise as a possible preventative measure. However, measurements on sedentary Sprague-Dawley rats found a significant decrease in relative $\dot{V}O_{2\ peak}$ after 5 weeks training, and the reduced performance might be related with increasing body mass (Copp et al., 2009) that might be expected to reduce running capacity. Moreover, running distance variation in RW rats was great, ranging from 0.8 to 10.7 km/day; a similar finding was seen in previous studies that recorded up to 50% difference, even from the same generation of animals (Lambert & Noakes, 1989, 1990; Rodnick, Reaven, Haskell, Sims, & Mondon, 1989), which resulted in inhomogeneous mean outcome to the training effect and underestimate $\dot{V}O_{2\ peak}$.

It has been reported that voluntary wheel running performance was proportionally related to total running distance and oxidative enzyme capacity (Lambert et al., 1996; Lerman et al., 2002). Lower running intensity in wheel exercise than treadmill running, 17.3 *vs*. 32.0 m/min (57% of maximum flat treadmill running speed) (Lerman et al., 2002) resulted in a small increase (Lambert & Noakes, 1990) or similar (Lambert et al., 1996) in $\dot{V}O_{2\ max}$ values after 8 weeks of exercise training. Exercise intensity may therefore influence maximal aerobic capacity (Lambert et al., 1996), so the nearly 3-fold lower running volume of LRW than RW rats (4.82±0.60 *vs*. 1.76±0.29 km/day), would explain a significantly lower relative post $\dot{V}O_{2\ peak}$. Unilateral femoral ligated leg of LRW might limit wheel exercise activity that resulted in a low running distance that in turn caused insufficient pro-angiogenic stimulus for capillary enhancement, thus reducing oxygen supply to lower limb muscle, EDL (Bigard et al., 1991; Boutellier et al., 1982). This would then limit running performance as it indirectly reduced aerobic capacity (Lambert & Noakes, 1990; Mondon et al., 1985).

From a metabolic perspective, trained individuals are characterised by reduced $\dot{V}O_2$ rates at the same running speeds performed by sedentary controls (Thomas, Fernhall, & Granat, 1999) indicating improved running economy in the trained individuals for the same exercise workload, i.e. less oxygen is used by the former (good RE) than the latter (poor RE) (Thomas et al., 1999). RE is partly influenced by the efficiency of metabolic and cardiorespiratory systems (Bailey & Pate, 1991; Barnes & Kilding, 2015; Franch, Madsen, Djurhuus, & Pedersen, 1998) including a shift to oxidative fibre types (Kyrolainen, 2003; Reggiani, Bottinelli, & Stienen, 2000). Running economy of LC rats was impaired following 4 weeks unilateral femoral artery ligation, likely attributed to attenuated blood supply and

oxygen delivery leading to limited exercise activity; this would exert physiological stress on muscles of the ligated limb and cardiovascular system (SjØgaard, 1987).

The exercise regime increased running economy in RW rats, linked to enhanced blood supply due to capillary expansion (greater C:F) at the microcirculation level of trained rats, thus increasing oxygen delivery efficiency to mitochondria (Coyle, 1999; Waters et al., 2004). Increased running efficiency in RW rats may also be aided by Type I fibre hypertrophy, consistent with previous studies that associated a higher percentage of Type I (slow-oxidative) fibre with better RE (Bosco, 1987; Williams & Cavanagh, 1987). Moreover, endurance exercise attenuated lactate acid accumulation in the muscles (Favier, Constable, Chen, & Holloszy, 1986) and rate of liver and muscle glycogen depletion (Baldwin, Fitts, Booth, Winder, & Holloszy, 1975), which slowed glycogen depletion thus delaying fatigue and improving submaximal performance (Lambert et al., 1996). Endurance exercise for 4 weeks was able to improve RE in LRW rats facilitated by mild capillary growth (+11% *vs.* LC) accompanied by an increase in Type I fibre areal density to aid prolong muscle contractile activity (Bosco et al., 1987; Williams & Cavanagh, 1987).

The second part of this work examined the exercise effect on $\dot{V}O_{2\ peak}$ and RE in Wistar rats with systemic reduced blood supply (aortic banding application) in lower limb muscle. Post $\dot{V}O_{2\ peak}$ of ABRW rats was similar to their baseline value (80.5±7.6 *vs*. 75.6±5.0 ml/kg/min, $P>0.05$) but it was 10% lower than RW rats' value. This showed that mild capillary expansion, indicated by 24% higher C:F and 50% greater LCD compared to AB control, was likely able to prevent aerobic capacity of ABRW rats from deteriorating. Even though this study did not include AB control measurements, It was expected that $\dot{V}O_{2\ peak}$ of AB control rats might be lower than their pre-banded (baseline) value following 4 weeks aortic stenosis due to the prolonged limited blood supply (Fulgenzi, Graciotti, Collis, & Hudlicka, 1998; Murthy et al., 2001). Even though both LRW and ABRW rats produced comparable weekly running distances; 0.93±0.46 *vs*. 1.05±0.49, 1.67±0.14 *vs*. 1.61±0.22, 1.69±0.21 *vs*. 1.74±0.18 and 2.41±0.18 *vs*. 2.66±0.26 km, respectively, that the aerobic capacity of LRW was lower than ABRW might be attributed to a higher degree of vascular insufficiency. In contrast to RW rats, oxygen running cost of ABRW rats following 4 weeks wheel exercise was similar to their pre value, suggesting that a small degree of microcirculation expansion did not improve RE of ABRW rats.

7.4.1 **Determinants/limitations of $\dot{V}O_2$ peak**

There were three factors that may determine successful of $\dot{V}O_2$ peak measurement; respiratory chamber integrity, animal running capacity and an effective running stimulus. In this study, nitrogen leak test was performed to check for respiratory chamber potential of leakage during measuring oxygen and carbon dioxide content in sampled air- leak testing found similar oxygen contents in air sample at different treadmill speeds. This study found that rats running capacity and running stimulus were limitations in the measurement. Not all rats can run on the treadmill during first day of treadmill familiarisation but improved after completing 5-day familiarisation. All RW rats were able to run on the treadmill at lower speeds i.e. 31cm/s, showing treadmill running skill was obtained during familiarisation period. The rats refused to run at higher speeds as only 33% of them ran at speed of 36cm/s and above, even though their running activity was motivated by compressed air spray (directed to animal hindlimb).

Percentage of RW rats achieving high speed running (31 or 36 cm/s) was increased after 4-week voluntary wheel running exercise, 86% (*vs*. 29% of pre exercise), this indicated that wheel exercise further improved treadmill running capability, that likely increased chances of getting $\dot{V}O_2$ peak measurement. The running stimulus was also not working particularly at the highest recorded speed (36 cm/s) showing compressed air use was not as aversive as electrical shock in motivating rat running activity. This measurement could be improved if more aerobic strains such as Brown and Sprague Dawley rats be used. Other alternatives to electrical grid shock should be considered (without compromising oxygen or carbon dioxide content of expired air in the respiratory chamber) so that rats' running capacity can be pushed to maximal level and thus systemic aerobic capacity can be accurately measured.

7.5 Conclusion

In conclusion, $\dot{V}O_{2\ max}$ cannot be determined without using an aversive electrical grid shock that serves as a powerful and effective motivator for high speed running in rodent studies. 4-week voluntary wheel running exercise only maintained relative post $\dot{V}O_2$ peak of RW rats due to running distance variability that collectively underestimated exercise effects. Relative post $\dot{V}O_{2\ peak}$ of LRW was not improved while ABRW rats enhanced their $\dot{V}O_{2\ peak}$ relative to baseline values. The study period of 4 weeks was of an insufficient duration for these pathological models to recover their baseline level, or produce similar effect to RW group value. The 3-fold reduction in running distance performed by LRW and ABRW compared to RW rats suggested that this strain might not be suitable for PAD models

considering the duration needed to produce similar exercise effects as in RW rats, i.e. exercise volume may need to be tripled for the expected result. The exercise regime was able to enhance RE in RW and LRW that involved relatively reduced oxygen consumption, but the effect was not observed in ABRW rats. This suggests that moderate exercise therapy may be useful for attenuating adverse effects of local ischaemia on activity patterns, but that systemic reduction in hindlimb blood flow may require more intense intervention.

Chapter 8 : General discussion

Exercise intolerance is common in patients with limited blood supply, and hence poor oxygenation of lower limb muscles, due to narrowing of major arteries. The objective of the thesis was to elucidate the potential of less stressful exercise activity than normally used in animal models of cardiovascular disease, i.e. voluntary wheel running exercise (VWRE), to enhance aerobic capacity *via* capillary growth (angiogenesis) and transformation of fibre types towards a more oxidative composition. The benefit of this tolerable exercise was tested on two defined models of reduced blood flow to lower limb muscle, analysing the effects on the EDL as representative of a typical 'mixed' locomotor muscle. Applying immunohistochemistry staining in histological work led to the simultaneous identification of muscle fibre types and anatomical capillary locations. Moreover, modern analysis programmes (Dtect, Capillar and OTM) that used a tessellating polygon concept to quantify supply regions of individual vessels provided a more complete analytical approach than possible with the global parameters normally used to define muscle capillarity. This permitted exploration of local angiogenic indices (LCD and LCFR), thus providing a better understanding of the mechanisms of angiogenesis in wheel-trained skeletal muscle. Seven-week VWRE in a preliminary study showed the capacity of this form of exercise to stimulate muscle capillarity enhancement, and fibre shift towards fast oxidative fibres when energy supply was enhanced (in rats that consumed fructose). However, the running distance peaked following 4 weeks of exercise, which allowed a refinement in our approach that permitted a shorter intervention and thus avoided some of the complications with use of older animals (large body size, reduced physiological response etc.).

8.1 Angiogenesis and fibre type composition (Chapters 3 and 4)

It was shown that 4-week running wheel exercise is able to stimulate angiogenesis and change fibre type composition to generate a more aerobic phenotype, suitable for supporting endurance-type activity. Even though the exercise peaked at week four, where muscle capillarity in RW rats was significantly higher than SC rats, 11 and 30% (7- and 4-week study, respectively), the rate of angiogenesis was 19% higher in 7-week wheel exercise, indicating capillary growth was still progressing (after week 4) to adapt to chronic hyperaemia accompanied by relatively lower MFA than SC rats. It is suggested that 7 weeks may be the maximal duration required to complete the angiogenic process before anti-angiogenesis mechanisms become established to terminate capillary growth. That this does represent the peak is indicated by a similar C:F for both RW and RWF, even though exercise volume was greater in the latter group. With regards to CD, RW rats of both studies showed

similarity, but it increased in RWF group (in the 7-week study) as a result of fibre atrophy, i.e. C:F is a good index of angiogenesis, whereas CD is a good index of functional microvascular capacity but is influenced by other physical factors. This study confirmed previous findings that exercise-induced angiogenesis was preceded by improvement in C:F and followed by CD. This thesis added that along the angiogenesis process, there was a series of MFA changes i.e. fibre hypertrophy (by 4 weeks) and then atrophy (by 7 weeks), as body adaptation to increased metabolic demand included shifting from glycolytic to fast oxidative fibres. This might correspond to previous findings that revealed fibre hypertrophy following endurance exercise in humans that might be attributed to insufficient exercise volume, and therefore be consistent with a notion of physiological adaptation of muscle atrophy in endurance exercise (Andersen & Henriksson, 1977; Gollnick et al., 1972).

Moreover, it was noted that further increase in exercise volume changed fibre composition to a more oxidative type, indicated by a shift to a higher percentage of Type IIa (and corresponding lower percentage of Type IIb). Collectively, both studies enhanced muscle capillarity by a different magnitude. It is suggested, using this Wistar strain, that angiogenesis requires at least 7 weeks with energy supplementation (fructose intake) to further exercise volume so that muscle metabolic adaptation is optimised. However, 4 weeks training is sufficient to improve exercise capacity, in common with human studies that show significant angiogenesis between 4-6 weeks of endurance exercise training e.g. (Hoier & Hellsten, 2014).

8.2 Exercise-induced angiogenesis improved ischaemic muscle function (Chapter 5 and 6)

Given that pharmaceutical therapies for peripheral vascular disease has not proved to be very effective, and that exercise remains probably the most effective intervention (Anderson, 2004), we then sought to test the application of the voluntary training regime in alleviating some of the issues associated with ischaemic muscle. We investigated the potential for exercise-induced angiogenesis in improving exercise intolerance in ischaemic muscle developed by local and systemic arterial insufficiency, in order to establish whether the outcomes were specific to the origin of ischaemia. VWRE showed relatively similar effects on capillarisation, which was sufficient to restore attenuated muscle performance. Interestingly, both ischaemic rat models involved a similar magnitude of exercise volume, about 3-fold lower than average RW rats (2.66±0.26 and 2.41±0.18 vs. 6.70±0.67 km/day). Furthermore, both LRW and ABRW rats showed comparable values on other important running parameters, for instance running velocity (0.78±0.01 *vs*. 0.78±0.01 cm/s), relative

time active (13.02±1.27 *vs*. 12.53±1.16 %), mean activity duration/ bout (6.28±0.41 *vs*. 6.10±0.40 min), and number of running activity bouts (27±4 *vs*. 28±3). The results suggest that the adaptive responses initiated by exercise were similar, irrespective of the origin of ischaemia. These data indicated that pathological rats (local and systemic ischaemia) performed the wheel exercise at a lower speed than controls, by which adaptation they were able to gradually progress to increase muscle performance capacity, indicated by increasing trend of running distance along the study period. Therefore, if the exercise volume is increased, it is expected greater effects could be observed in ischaemic muscle physiology and anatomy.

Initially reduced arterial blood flow to working muscle after banding application attenuated muscle performance (FI) of acute AB rats, both being lower than AB control animals. Less functional impairment in the latter group may indicate formation of vascular anastomoses to alleviate the consequences of arterial stenosis, even without additional mechanical stimuli (VWRE). This adaptation led to improved blood supply, indicated by increased femoral artery blood flow in AB controls. The same mechanism was suggested to occur in LRW rats, where improvement in blood supply to EDL after 4 weeks occurred, likely due to development of collateral anastomoses.

EDL muscle mass was similar between AB control and LC rats, and comparable to SC rats, showing that muscle mass was not affected following 4 weeks impaired blood flow in animals undertaking routine activity levels. It was suggested that reduced blood flow during the early stage of muscle ischaemia was adequately compensated by ameliorated femoral artery blood flow to support muscle function under resting conditions, but not at higher demands (exercise level) indicated by greater FI in AB control rats compared to acute AB rats, but significantly lower than ABRW rats.

8.3 $\dot{V}O_{2\ peak}$ and RE as aerobic capacity predictors (Chapter 7)

It has been shown by this study that $\dot{V}O_{2\ max}$ estimates could not be measured with sufficient accuracy or repeatability without using a conventional running motivator, electrical grid shock. To our knowledge, other approaches - for instance blasts of compressed air, food rewards following successful training, and wrapping the metabolic chamber with dark paper - were not as effective as electrical shocks to push rats to run at the necessary higher treadmill speeds. Therefore, $\dot{V}O_{2\ peak}$ and RE were used in assessing aerobic capacity - $\dot{V}O_{2\ peak}$ represented the highest $\dot{V}O_2$ rate obtained before rats terminated the ramp test, while RE indicated oxygen running cost at those particular speeds (greater efficiency = lower oxygen consumption). Improvement in whole body systems is important

for eliciting maximal performance, and enhancing $\dot{V}O_{2\ peak}$ and RE in this study followed enhancement in muscle capillarity and cardiovascular system, leading to greater aerobic capacity and improved efficiency in tissue oxygenation for energy production. A modest increase in muscle capillarity and relatively higher cardiac mass in ABRW contributed to greater $\dot{V}O_{2\ peak}$ relative to pre-exercise values. It is worth noting that increased Type IIa N_N did not contribute to increased muscle aerobic capacity as their A_A was significantly lower than other groups, showing that Type IIa fibre atrophy likely limited muscle functional capacity. Unilateral femoral-artery ligation rats improved RE with exercise, and this could be linked to a modest increase in muscle capillarity, and N_N and A_A of Type I fibres. Increase in Type I proportion in LRW rats was attributed to the lower running speed that resulted in recruitment of Type I fibres.

8.4 Summarised results of all studies

8.4.1 Exercise volume

Table 8.1: Exercise volume indicated by running distance performed by rats of pilot study, running wheel (RW), ligation running wheel (LRW) and aortic banding running wheel (ABRW). Exercise volume was similarly impaired after peripheral (ligation) and central (banding) limitations to muscle blood flow, and in both cases improved to the same extent following voluntary wheel exercise – thus demonstrating the efficacy of exercise as a viable therapeutic intervention for muscle ischaemia that is independent of origin.

	Exercise volume (km/day)			
	Week 1	Week 2	Week 3	Week 4
RW (Pilot study)	2.92±0.66	3.52±1.02	4.35±0.84	4.62±0.41
RW (4-week study)	2.09±0.73	4.52±0.60	5.96±0.40	6.70±0.67
LRW	1.05±0.49	1.61±0.22	1.74±0.18	2.66±0.26
ABRW	0.93±0.46	1.67±0.14	1.69±0.21	2.41±0.18

8.4.2 Angiogenic indices

Table 8.2: Gross and local angiogenic indices of EDL muscle of Wistar rats of pilot study, sedentary control (SC), ligation control (LC), aortic banding control (AB Control), running wheel (RW), ligation running wheel (LRW) and aortic banding running wheel (ABRW). Data indicate instances of both angiogenesis and capillary rarefaction (C:F), with variable CD influenced by MFA. Local capillarity indices, LCFR and LCD, in general show less striking changes compared to gross indices of capillarity emphasising the importance of an integrated response during muscle remodelling.

	C:F	CD (mm^{-2})	MFA (um^2)	LCFR	LCD (mm^{-2})
SC (Pilot study)	1.50±0.15	718±99	2229±358	-	-
RW (Pilot study)	1.95±0.06	784±73	2562±140	-	-
SC	1.62±0.01	773±39	2138±128	1.16±0.17	852±221
LC	1.33±0.07	614±86	2264±277	1.29±0.14	806±123
AB Control	1.22±0.20	576±127	1575±198	0.94±0.13	526±115
RW	1.79±0.07	631±34	2966±283	1.45±0.13	768±126
LRW	1.48±0.07	667±46	2319±297	1.38±0.26	886±132
ABRW	1.51±0.10	709±46	2227±165	1.14±0.20	789±101

8.4.3 **Muscle fibre composition**

Table 8.3: Numerical and areal density of EDL muscle of Wistar rats of pilot study, sedentary control (SC), ligation control (LC), aortic banding control (AB control), running wheel (RW), ligation running wheel (LRW) and aortic banding running wheel (ABRW). Data illustrates the complementary nature of changes in fibre composition, particularly towards a more oxidative phenotype, to those of capillarity in enhancing exercise tolerance.

	Numerical density (N_N)			Areal density (A_A)		
	TI	TIIa	TIIb	TI	TIIa	TIIb
SC (Pilot study)	0.080±0.02	0.281±0.05	0.672±0.05	-	-	-
RW(Pilot study)	0.040±0.01	0.400±0.04	0.582±0.04	-	-	-
SC	0.022±0.01	0.176±0.09	0.808±0.09	0.050±0.01	0.291±0.10	0.671±0.10
LC	0.020±0.01	0.213±0.04	0.772±0.04	0.043±0.01	0.346±0.04	0.623±0.05
AB control	0.052±0.02	0.219±0.04	0.745±0.04	0.022±0.01	0.111±0.03	0.873±0.03
RW	0.028±0.02	0.177±0.04	0.802±0.04	0.053±0.01	0.293±0.03	0.666±0.03
LRW	0.028±0.01	0.163±0.02	0.818±0.02	0.056±0.01	0.280±0.03	0.682±0.03
ABRW	0.046±0.02	0.273±0.04	0.689±0.04	0.024±0.01	0.163±0.03	0.817±0.03

8.4.4 **$\dot{V}O_2$ peak and fatigue index (FI)**

Table 8.4: Pre and post $\dot{V}O_2$ peak and fatigue index (FI) of Wistar rats of sedentary control (SC), ligation control (LC), aortic banding control (AB control), running wheel (RW), ligation running wheel (LRW) and aortic banding running wheel (ABRW). While moderate exercise proved inadequate to markedly affect cardiorespiratory fitness ($\dot{V}O_2$, FI, and RE) in intact animals (RW), it did ameliorate the deleterious effects of muscle ischaemia.

Group	Pre-exercise $\dot{V}O_2$ peak (ml/kg/min)	Post-exercise $\dot{V}O_2$ peak (ml/kg/min)	Fatigue index (FI)	Running economy ($\dot{V}O_2$ at submaximal exercise post-exercise)
SC	-	-	0.49±0.06	-
LC	82.9±5.7	69.6±5.2	0.38±0.05	70.5±5.8
AB Control	-	-	0.37±0.05	-
RW	85.9±10.1	84.0±9.3	0.45±0.05	70.9±4.2
LRW	82.3±12.5	66.1±11.0	0.46±0.05	73.0±5.6
ABRW	80.5±7.6	75.6±5.0	0.49±0.09	78.0±6.8

8.5 Limitations of the current study

The limitations inherent in published studies of inbred strains selected for high aerobic capacity were reduced here by comparing an inbred strain (Wistar) with a recent derivative of a wild population (Brown). The results demonstrated an intrinsic difference in structural influences on peripheral oxygen transport, but there was insufficient time and resources to test whether these potential benefits for exercise performance are realised in terms of fatigue resistance etc.

Even though VWRE is less stressful muscle activity compared to other exercise modalities, e.g. treadmill running, it is not feasible to control exercise volume or intensity to ensure similar magnitude of exercise impact on the muscle under study, thus resulting in a large variation in exercise volume (running distance) among the experimental animals. Future studies using wheel exercise may be able to exploit the running distance variation by increasing sample size to accommodate different exercise volition, and improve the probability of investigating physiological differences between poor and good runners.

It was noted that $\dot{V}O_2$ max measurement was not possible without an aversive stimulus, i.e. electrical shock, as the application is banned by the UK. The alternative methods used here - application of a black box (placed in front of running rats), wrapping metabolic chamber with black paper and compressed air spray to rats' hind limb - were not able to sufficiently motivate rats to keep running on the treadmill, particularly at higher speeds.

There was a delay in β-oxidation reagent delivery by a supplier due to stock shortage and university closure during Covid lock-down. The β-oxidation assay needs to be performed to complete enzyme assays, which included key rate limiting enzymes (citrate synthase, phosphofructokinase and β-oxidation), to provide a more comprehensive information on the effect of 4-week VWRE on intermediary metabolism and likely energy sources.

8.6 Future work

1. A complementary aspect:

Muscle aerobic capacity is not only influenced by high capillarity and oxidative fibre composition. Greater capability to utilise oxygen is another aspect in expansion of whole muscle aerobic capacity that could be assessed through enzyme activity assays. Incomplete work in determining rate limiting enzymes activity following chronic wheel exercise involving citrate synthase (CS; a marker of mitochondrial oxidative capacity), phosphofructokinase (PFK; a rate-limiting enzyme in anaerobic glycolysis) and 3-hydroxy-acyl CoA dehydrogenase (HOAD; β-oxidation pathway capacity) enzymes will be finished at a later date. Part of oxidative enzyme (CS) activity finding is as follows:

A) Local ischaemia (unilateral femoral artery ligation rats)

Table 8.5: Citrate synthase activity of sedentary (SC), ligation control of ligated side (LC ligated), ligation control of non-ligated side (LC non-ligated), running wheel (RW), ligation running wheel of ligated side (LRW ligated) and ligation running wheel of non-ligated side (LRW non-ligated). Shared letters denote statistical significance (P<0.05) as determined by ANOVA with Tukey post-hoc tests.

Group	N	CS activity (IU/mg)
SC	7	0.59±0.06[a]
LC (ligated)	5	0.45±0.06[d]
LC (non-ligated)	7	0.59±0.15b[b]
RW	14	0.62±0.07[c]
LRW (ligated)	5	0.42±0.07[abc]
LRW (non-ligated)	6	0.52±0.04

Briefly, wheel running exercise did not increase CS activity in RW rats. Exercise intolerant rats which performed mild exercise (LRW ligated) activity imposed very little physiological stress to enhance oxidative phosphorylation as CS was not significantly different compared to LC control. This suggests a consistent response in elements responsible for peripheral O_2 transport; both supply (capillarity) and demand (enzyme activity).

B) Systemic ischaemia (aortic banded rats)

Table 8.6: Citrate synthase activity of sedentary control (SC), aortic banding control (AB Control), running wheel (RW) and aortic banding running wheel (ABRW) rats. Unshared letters denote statistical significance (P<0.05) as determined by ANOVA with Tukey post-hoc tests.

Group	N	CS activity (IU/mg)
SC	7	0.59±0.06[ab]
AB control	6	0.48±0.09[b]
RW	14	0.62±0.07[a]
ABRW	8	0.50±0.12[b]

Aortic banding application reduced by 23% CS enzyme activity compared to SC. Exercise training for 4 weeks (RW) did not change the activity, but that might due to collectively insufficient training effort. The CS activity of ABRW was similar to AB control group, indicating that a very low exercise activity due to limited exercise capacity was insufficient to increase CS activity. Again, this indicates a similar response to muscle ischaemia, irrespective of the origin.

PFK activity has not been completely analysed and β-oxidation assay is still in progress that will be completed soon. These data will complement the histological profile of muscle remodelling, thus providing a better understanding on improvement in muscle aerobic capacity upon wheel running exercise and determine the extent of metabolic adjustment that is required to complement the anatomical changes observed.

2. Further analysis on TA and Sol muscles

It is useful to undertake further analysis using TA and Sol skeletal muscles which express different metabolic properties. Whether or not the exercise exerted a similar effect on TA as seen in EDL muscle considering both are fast muscle and on Sol muscle, characterised with mostly Type I fibres. The question as to how low intensity of running may induce differential effects in different ischaemic muscles would be answered from this analysis.

3. Sample size and exercise volume

Biological variation in exercise performance of rats may influence the reported average training effect. Therefore, it is worthwhile to increase sample size to increase data resolution. Also, exercise volume is crucial in determining the optimum muscle adaptation, particularly in disease models. Therefore, a future study needs to extend the study period or use other approaches (e.g. fructose consumption) to increase exercise volume so that an

optimum duration would be guaranteed, and therefore maximise the benefit of the exercise in stimulating angiogenesis to improve exercise tolerance in ischaemic muscle.

8.7 Conclusion

Collectively, exercise-induced angiogenesis is proven by this work to be useful in treating ischaemic muscles. This thesis provides additional insight into the role of angiogenesis in muscle performance restoration of ischaemic muscle, and voluntary wheel running exercise as a therapeutic intervention.

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