The role of the microcirculation in skeletal muscle function and plasticity

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ABSTRACT

The skeletal muscle microcirculation is crucial for the delivery of oxygen and nutrients, and the removal of waste products, but the importance of capillarisation for skeletal muscle performance and hypertrophy is yet to be fully elucidated. Therefore, the aim of the thesis was to assess, in rodents and humans, the role of capillarisation in skeletal muscle fatigue resistance and hypertrophy in health, disease (chronic heart failure (CHF) in particular) and ageing and to determine to what extent baseline muscle mass affects the hypertrophic response.

Through the use of microsphere injection to block up to 70% of capillaries in the *m. extensor digitorum longus* (EDL) it was shown that functional capillary density is positively related to the fatigue resistance of a muscle. The reduction in fatigue resistance as a consequence of unbiased blockage of capillaries can be overcome by overload-induced angiogenesis, and in the case of the rats with compensatory cardiac hypertrophy (a model for hypertension and early chronic heart failure (CHF)) by endurance exercise. CHF reduces functional capillary density in muscle and impairs the hypertrophic response to overload. Given the inverse relationship between fibre cross-sectional area (FCSA) and oxidative capacity, it was expected that the FCSA of highly-resistance trained men would decrease as their oxidative capacity increased with endurance training, with even greater reductions in FCSA in old resistance-trained men. This was, however, not the case probably because the endurance exercise-induced angiogenesis reduced intercapillary distances, facilitating the oxygen delivery via diffusion to the increased number of mitochondria in the muscle fibres. In mice it was seen that the inclusion of hypertrophic and endurance stimuli did not blunt the adaptations to either modality and that baseline muscle mass was not predictive of hypertrophic response in young mice. It was shown, however that old mice demonstrated less hypertrophy, which was associated with an impaired overload-induced angiogenesis.

In conclusion, the microcirculation plays a crucial role in skeletal muscle fatigue resistance and is important in reducing diffusion distances with hypertrophy. As such, it appears to be a useful therapeutic target to maintain muscle function and enhance muscle responses to rehabilitation in disease and old age.

THE ROLE OF THE MICROCIRCULATION IN MUSCLE FUNCTION AND PLASTICITY

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ABSTRACT

It is widely acknowledged that maintenance of muscle, size, strength and endurance is necessary for quality of life and the role that skeletal muscle microcirculation plays in muscle health is becoming increasingly clear. Here we discuss the role that skeletal muscle microcirculation plays in muscle function and plasticity. Besides the density of the capillary network, also the distribution of capillaries is crucial for adequate muscle oxygenation. While capillaries are important for oxygen delivery, the capillary supply to a fibre is related to fibre size rather than oxidative capacity. This link between fibre size and capillary supply is also reflected by the similar time course of hypertrophy and angiogenesis, and the cross-talk between capillaries and satellite cells. A dense vascular network may in fact be more important for a swift repair of muscle damage than the abundance of satellite cells and a lower capillary density may also attenuate the hypertrophic response. Capillary rarefaction does not only occur during ageing, but also during conditions as chronic heart failure, where endothelial apoptosis has been reported to precede muscle atrophy. It has been suggested that capillary rarefaction precedes sarcopaenia. If so, stimulation of angiogenesis by for instance endurance training before a hypertrophic stimulus may enhance the hypertrophic response. The microcirculation may thus well be a little-explored target to improve muscle function and the success of rehabilitation programmes during ageing and chronic diseases.

INTRODUCTION

Skeletal muscle comprises almost 40% of the human body. They contain a dense capillary network that serves to deliver oxygen and nutrients, and remove waste products and heat from the skeletal muscle cells. This becomes particularly important during exercise, where the metabolic rate can increase 30-50-fold (Payne and Bearden, 2006) with a commensurate increase in oxygen demand that is realised by increased blood flow and capillary recruitment characterised by elevated red blood cell flux and velocity (Poole, 2004) as seen already now 100 years ago by August Krogh (Krogh, 1919). The increased red blood cell (RBC) flux and velocity enhances oxygen diffusion from the capillary to the mitochondria in the muscle fibres (Poole, 2004). August Krogh also noted that in skeletal muscle the capillaries largely run parallel to the muscle fibres, an orientation that helped the development of his model of tissue oxygenation (Krogh, 1919). While this is the case at longer sarcomere lengths, at shorter sarcomere length, the capillaries become more tortuous, resulting in a larger contact area between the capillaries and the muscle fibres that facilitates oxygen diffusion (Poole, 2004). Capillaries are arranged in microvascular units, defined by the terminal arteriole and the 10-20 capillaries it feeds, and it is in the arterioles that the perfusion of the downstream capillaries is regulated via vasomotion (Wagenmakers et al., 2016). The capillaries drain into collecting venules that merge into collecting veins (Hudlicka, 2011, Korthuis, 2011).

Blood flow

The increased metabolic rate during muscle contraction is accompanied by an up to 100-fold rise in blood flow (Poole et al., 2011). A contracting muscle is, however, in a paradoxical situation as with increasing force not only the oxygen demand rises, but also the intramuscular pressure, and even more so during shortening contractions (Degens et al., 1998), that will impede blood flow. The rise in intramuscular pressure, irrespective of the size of the muscle, can even cause a complete cessation of blood flow and result in the deoxygenation during sustained isometric contractions as low as 30% maximal force (de Ruiter et al., 2007). While during intermittent contractions the blood flow is enhanced during the relaxation phase, it has been reported that the cessation of flow during the contraction phase is such that increasing the duty cycle beyond 10% does not result in a further increase in blood flow (Degens et al., 1998).

CAPILLARISATION IN SKELETAL MUSCLE

Capillary density and muscle oxidative capacity

Given the importance of the capillary bed for the delivery of oxygen to the muscle, it is expected that highly oxidative muscles have a denser capillary network than highly glycolytic muscles. Particularly in rodents, there is a large difference in the fibre type composition of skeletal muscle, where for instance in the soleus muscle 87% and in the extensor digitorum longus (EDL) muscle only 2% of the fibres are type I (S) fibres (Armstrong and Phelps, 1984). The soleus muscle also has a higher oxidative capacity and capillary density (CD: the number of capillaries·mm^{-2} of muscle) than the EDL (Gray and Renkin, 1978, Egginton et al., 1988). Such differences in CD are even seen within a muscle, where for instance in the rat plantaris muscle the deep oxidative region has a higher CD than the more glycolytic superficial region of the muscle (Wust et al., 2009, Hudlicka, 2011). The link between oxidative metabolism and the density of the capillary network in the muscle is also reflected between muscles from different species. For instance, in the highly active flight muscles of the hummingbird with maximal respiration rates more than twice that found in mammals, capillaries make up a 2-6 times greater proportion of the muscle volume than that seen in mammalian hind limb muscles (Suarez et al., 1991). In fact, it has been suggested that in the humming bird flight muscles, the mitochondrial volume density and inner membrane density are near their theoretical upper limit to maximise respiratory capacity (Suarez et al., 1991).

The capillary supply to a muscle is highly adaptable to altered functional demands. One such adaptation is the increased CD in the flight muscle of pigeons after 2 months of cold exposure concomitant with an increase in mitochondrial density to meet the metabolic demands of shivering (Mathieu-Costello et al., 1998). Likewise, endurance athletes have a higher oxidative capacity and CD than sedentary people (Tesch et al., 1984, Saltin et al., 1977). Weightlifters and powerlifters, on the other hand, participate in activities that require a few maximal contractions that do not require the aerobic generation of ATP and therefore have a lower demand for oxygen delivery by the circulatory system. Accordingly, they have a lower CD than sedentary people and endurance athletes (Tesch et al., 1984). All these observations suggest that an important function of the capillary bed is the delivery of oxygen to the working mitochondria.

Distribution of capillaries

Most studies on muscle capillarisation report the CD and the capillary to fibre ratio (C:F). While these measures give a good reflection of the size of the capillary network in the muscle, they give no information of the distribution of the capillaries. Model calculations have shown that an increased heterogeneity of capillary spacing has a negative impact on muscle oxygenation (Piiper and Scheid, 1991, Turek et al., 1992, Degens et al., 2006b). Given the significance, it is somewhat surprising that the distribution of capillaries has received little attention. This may be partly attributable to the lack of techniques to obtain a measure of the heterogeneity of capillary spacing. The method of capillary domains (Fig. 1), where a capillary domain is defined as the area of tissue surrounding a capillary delineated from adjacent capillaries by equidistant boundaries, gives a quantitative measure of the heterogeneity of capillary spacing as the standard deviation of the logarithm of the domain areas (Hoofd et al., 1985). Using this method, it was found that the capillary distribution is more homogeneous in the oxidative soleus than the glycolytic EDL muscle, but no difference in slow and fast muscles of the eel was found, despite a 35-fold difference in CD (Egginton et al., 1988). It has been shown that the heterogeneity of capillary spacing correlates with the variation of fibre size (Degens et al., 2009b, Barnouin et al., 2017), which might be explicable by the morphological constraints of positioning the capillaries on the surface of the fibre only.

With a theoretical model of tissue oxygenation it was found that in rat soleus muscle with a typical CD, working at the anaerobic threshold (2/3 of maximum oxygen consumption), the impact of changes in the heterogeneity of capillary spacing on tissue oxygenation was more pronounced than the effects of redistribution of flow (Hoofd and Degens, 2009). Something similar was seen in heart muscle (Turek et al., 1992). An increased heterogeneity of the distribution of perfused capillaries has been seen in sepsis patients that in severe cases led, as predicted by the oxygenation models, to an impaired tissue oxygenation (Goldman et al., 2006, Walley, 1996). These observations provide evidence that the distribution of capillaries in the muscle is i) more important for a good tissue oxygenation than flow distribution and ii) that capillaries are not randomly arranged, but that their positioning is controlled to ensure adequate muscle oxygenation.

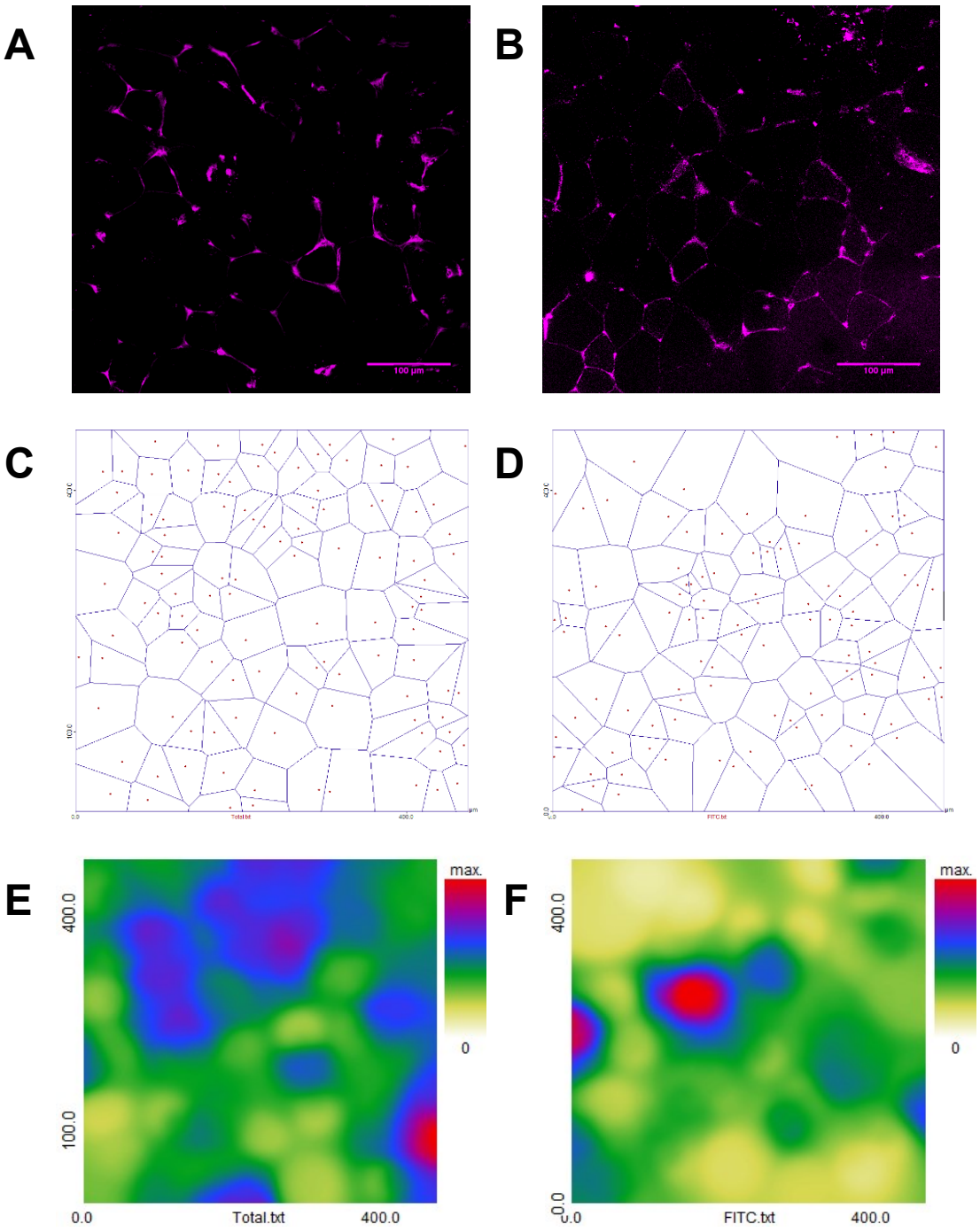

Figure 1. A) and B) images of rat skeletal muscle stained for capillaries with fluorescently-labelled lectin. Both images have the same capillary density but a different heterogeneity of capillary spacing. In image A the logarithmic standard deviation of the domain radii (log_RSD) is 0.093 and in B it is 0.121. Capillary domains (estimates of oxygen supply areas) for A and B are shown in C) and D), respectively. E) and F) are heat maps for capillary distribution and give a rough indication of the distribution of oxygen partial pressure in the tissue.

Capillarisation and muscle function

Evidence for the significance of the capillary network for muscle fatigue resistance comes from the observation that the increased fatigue resistance of chronically stimulated muscles was associated with an elevated CD (Lieber, 1986, Pette and Vrbova, 1992, Hudlicka et al., 1977). As the blood flow and oxidative capacity were also increased, it is difficult to disentangle to what extent the increased fatigue resistance in chronically stimulated muscles is attributable to an increase in oxidative capacity, blood flow and/or CD. However, the improved fatigue resistance in overloaded rat EDL (Egginton et al., 1998) and mouse plantaris muscles (Ballak et al., 2016) was accompanied by an increased CD, but without concomitant increases in blood flow or oxidative capacity, respectively. These observations indicate that the capillary network plays an important role in muscle fatigue resistance. This is further supported by an elegant study, in which a reduction in the number of functional capillaries through microsphere-induced arteriole occlusion resulted in a significant decline in performance of the isolated rat heart (Hauton et al., 2015).

Impact on metabolism

Skeletal muscle is the major storage tissue for glucose (in the form of glycogen) that is delivered to the muscle via the capillary blood (Jensen et al., 2011). The elevated post-prandial circulating glucose concentration induces the release of insulin from the pancreas. The vasodilating effect of insulin in turn causes a modest increase in skeletal muscle blood flow that enhances the transport of glucose into the muscle tissue (Vincent et al., 2006, Wagenmakers et al., 2016). As the network responsible for the transfer of substances between the circulatory system and skeletal muscle, capillaries are crucial for muscle glucose uptake. In line with this, a low skeletal muscle CD was associated with glucose intolerance and insulin resistance in both young and old humans (Snijders et al., 2017b, Landers-Ramos and Prior, 2018, Groen et al., 2014, Lillioja et al., 1987) and stroke patients (Prior et al., 2009). Moreover, an increased CD after training improved insulin sensitivity and glucose tolerance (Landers-Ramos and Prior, 2018, Prior et al., 2015). The observation that acute occlusion of arterioles and downstream capillaries with 15-μm microspheres, without a change in blood flow, resulted in a significant reduction in insulin-mediated glucose uptake (Vollus et al., 2007) indicates that the capillary bed is perhaps more important for glucose tolerance than blood flow. Therefore, a diminished CD in

disease or due to sedentary behaviour may thus contribute to the impaired insulin sensitivity and glucose intolerance in these conditions, which may be further aggravated by systemic inflammation and accumulation of fatty acid metabolites that both impair activation of the insulin signalling cascade (Wagenmakers et al., 2016).

SIZE PRINCIPLE IN RELATION TO CAPILLARY SUPPLY TO A FIBRE

The size principle

The size principle is defined as the inverse relationship between the oxidative capacity and the cross-sectional area of a fibre that is explained by diffusion limitations (van der Laarse et al., 1998). This diffusion limitation would limit fibre size. There is, however, a metabolic advantage of having large fibres, where for instance the cost of Na^+/K^+-ATPase function was twice as large in muscles with small fibres, with a 2-fold higher surface to volume ratio, than that in muscles with large fibres (Jimenez et al., 2011). Such observations have led to the concept of an 'optimal fibre size', which suggests that in larger fibres there is a trade-off between 'diffusion constraints' and 'metabolic cost savings' (Johnston et al., 2006). In fish muscle, the diffusion constraint is overcome to some extent by intramyocyte lipid that increases the oxygen permeability of the tissue (Hoofd and Egginton, 1997), or flattened fibres that increase the surface to volume ratio (Johnston, 1982). Another adaptation is migration of mitochondria to the periphery of the fibre, as seen in the white fibres of sharks and rays (Kinsey et al., 2011). While advantageous in terms of shortening the diffusion distances for oxygen, it creates another problem, as the radial distance over which ATP has to diffuse from the mitochondria to the inner myofibrils is increased (Kinsey et al., 2011). The overcome this conundrum, the musculature of the blue crab displays a particularly interesting adaptation where their large aerobic fibres are subdivided into smaller well-perfused subdivisions to shorten the diffusion distances (Hardy et al., 2009).

In vertebrates, large muscle fibres are not subdivided, nor is there evidence of an increase in intramycocellular lipids and/or changes in fibre shape. Yet, there is evidence that also in vertebrates the inverse relationship between fibre size and oxidative capacity can be overcome. For example, oestrogen-related receptor gamma (Errγ) overexpression in myostatin null mice (which demonstrate a hypermuscular phenotype) resulted in an elevated oxidative capacity to similar levels to that found in fibres of wild type mice (Omairi et al., 2016). Also, in

overloaded mouse plantaris muscle the oxidative capacity was elevated (Ballak et al., 2016) and in both cases this was accompanied with an increased CD. These data thus suggest that the alleged trade-off between muscle fibre size and oxidative capacity can also be broken in mammalian muscle, among others by capillary proliferation.

At first glance, it seems that the violation of the size principle is made possible by a concomitant increase in capillary supply to a fibre, suggesting that the oxidative capacity of, and the capillary supply to, a fibre are linked. However, a study of human vastus lateralis and soleus muscle showed that the capillary supply to a fibre is not determined by its oxidative capacity, but rather by muscle fibre cross-sectional area (Bosutti et al., 2015). Together with the observation that long-term high frequency electrical stimulation of glycolytic fibres led to an increased capillary supply without an increase in oxidative capacity (Egginton and Hudlicka, 2000), this suggests that oxidative capacity is not a determinant of capillary supply to a fibre and that capillarisation may play a more important role in metabolite removal than in substrate delivery. One explanation for the absence of a relationship between capillary supply to and oxidative capacity of a fibre is that even in the face of large variations in oxygen pressures in the tissue, the myoglobin saturation is, due to the shape of the myoglobin dissociation curve, rather homogeneous throughout the working muscle tissue (Fig. 2). This would ensure an adequate oxygen supply to the working mitochondria, even at a low oxygen partial pressure.

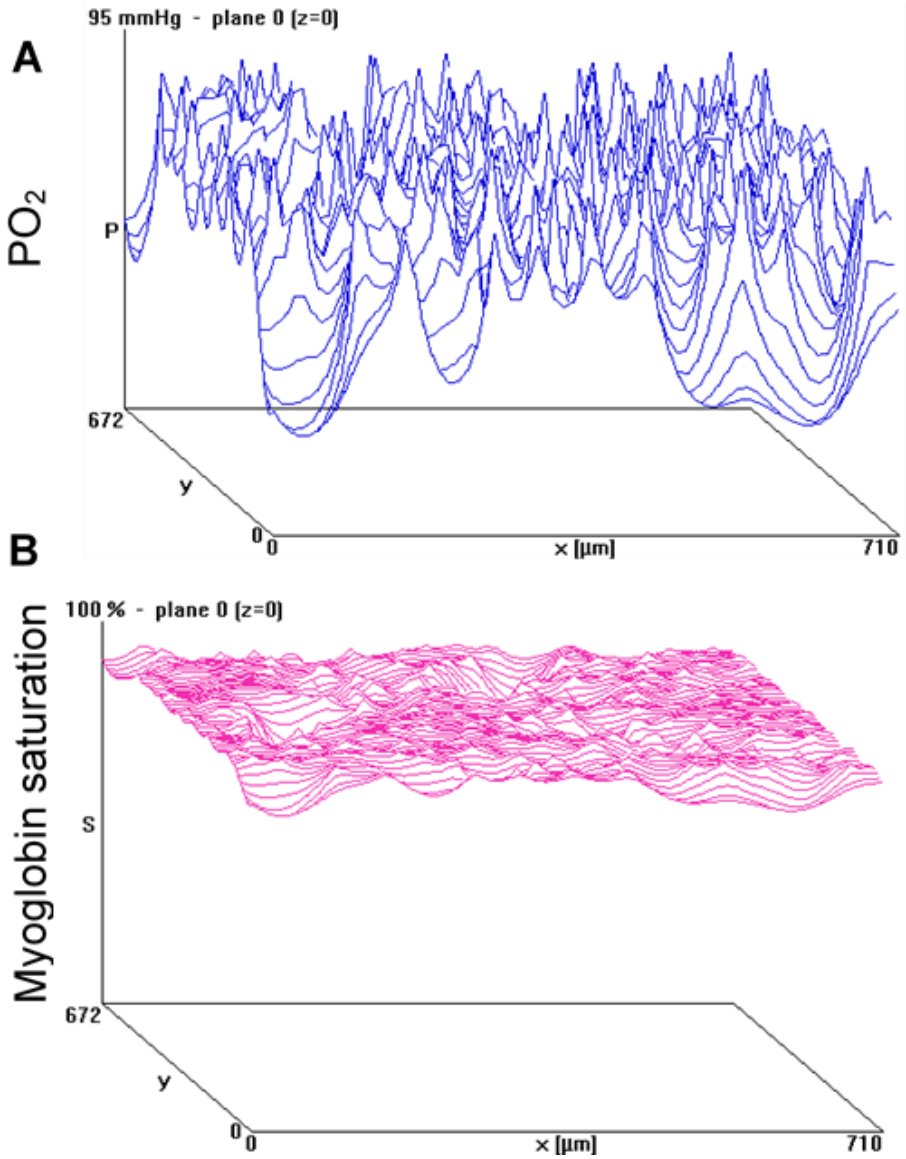

Figure 2. A) Oxygen partial pressure (PO2 in mmHg) and B) myoglobin saturation in a mouse soleus muscle working at maximal oxygen uptake, calculated using a mathematical model of tissue oxygenation (Hoofd 1995). Peaks in A illustrate capillary PO2 assumed to be 95 mmHg. Unpublished observations.

ADAPTATIONS OF THE CAPILLARY NETWORK

Muscle hypertrophy

Muscle has a remarkable ability to respond to altered functional demands. For instance, muscle hypertrophies in response to an overload stimulus, such as resistance exercise, and some models of compensatory hypertrophy even result in a doubling in muscle size (Degens, 2012). For the development of such hypertrophy the delivery of nutrients, including amino acids, is essential. The amino acids and

insulin facilitate hypertrophy via activation of mammalian target of rapamycin (mTOR) that stimulates protein synthesis through enhanced translation initiation and elongation (Fry and Rasmussen, 2011, Timmerman et al., 2010). Besides stimulating protein synthesis, insulin also enhances nutritive flow to the muscle (Fry and Rasmussen, 2011). Ultimately, the nutrients diffuse from the capillary blood into the muscle, and it is therefore likely that the density of the capillary network will have an impact on the development of hypertrophy. In addition, unlike in large fish muscle fibres, even at extreme levels of muscle hypertrophy mammalian skeletal muscle demonstrates a normal distribution of mitochondria (Chalmers et al., 1992). Without angiogenesis, the increase in fibre cross-sectional area pushes the capillaries apart, increasing the distance from the capillary to mitochondria in the interior of the fibre, thus putting a theoretical diffusion limitation on hypertrophy (Degens, 2012).

The increase in diffusion distances during hypertrophy is attenuated by capillary proliferation in both rodent (Degens et al., 1992, Egginton et al., 1998) and human muscle (Verdijk et al., 2016). Also, during the more than 8-fold increase in fibre size during maturational growth angiogenesis occurs (Ripoll et al., 1979, Degens et al., 2006b). However, during both maturational and overload-induced muscle fibre growth the capillary proliferation is proportionally less than the increase in fibre size, resulting in a decreased CD (Ripoll et al., 1979, Hudlicka et al., 1992, Degens et al., 1992). Yet, the heterogeneity of capillary spacing is maintained, both during maturational growth (Degens et al., 2006b) and overload-induced hypertrophy (Degens et al., 1992), and model calculations indicate that this is sufficient for adequate oxygenation (Degens et al., 2006b). This indicates that capillary proliferation is not random, but such that muscle oxygenation is preserved.

The number of capillaries supplying a fibre is positively related to fibre size in both human (Ahmed et al., 1997, Bosutti et al., 2015) and rodent muscles independent of fibre type (Degens et al., 1994b, Wust et al., 2009). This suggests that fibre growth and capillary proliferation are coupled. Perhaps an even stronger indication of such a coupling is the observation that capillary proliferation and fibre growth follow a similar time course in both overloaded rodent muscles (Plyley et al., 1998) and in human muscles subjected to a resistance training stimulus (Verdijk et al., 2016, Green et al., 1999, Holloway et al., 2018). Hypertrophy is accompanied by a similar amount of angiogenesis around each fibre type (Degens et al., 1994b).

Satellite cells (SCs) are thought to play an important role in muscle hypertrophy and regeneration (van der Meer et al., 2011). SCs are situated between the sarcolemma and basal lamina of a muscle fibre and act as a source of new myonuclei. Upon anabolic stimulation or muscle damage, SCs are activated and facilitate hypertrophy by fusing with an existing muscle fibre to donate a new myonucleus to maintain the nuclear domain size (van der Meer et al., 2011). In muscle damage following eccentric loading degeneration is characterised by the rupture and necrosis of muscle fibres and an ensuing inflammatory response. This is then followed by the activation, proliferation and/or differentiation of satellite cells that can fuse to form new muscle fibres, fuse to existing fibres to donate their nucleus to the fibre or return to quiescent state, depending on the up or down regulation of myogenic regulatory factors, therefore contributing to muscle fibre repair regeneration (Snijders et al. 2015). Interestingly, 82% and 68% of SCs in mouse tibialis anterior and human deltoid muscle, respectively, are within 5 μm of a capillary, and capillaries and SCs can reciprocally activate each other, probably via diffusion of secreted growth factors (Christov et al., 2007). Indeed, *in vitro* endothelial cells stimulate SC growth through secretion of growth factors including IGF, HGF and VEGF, and in muscle tissue it was seen that active SCs were located closer to capillaries than quiescent SC (Christov et al., 2007). This suggests that their ability to differentiate may be related to their proximity to capillaries and hence diffusion limitations. *Vice versa,* microvascular fragments develop smooth muscle sprouts when co-cultured with SCs but not when cultured on their own (Rhoads et al., 2009). That such a relationship may play a significant role *in vivo* is suggested by the observation that greater capillarisation in the vastus lateralis was associated with an amplified activation/expansion SC response and accelerated repair after muscle damage caused by eccentric contractions (Nederveen et al., 2018). Additionally, greater activation of SCs in response to a 16-week resistance exercise is complemented with an increase in C:F (Nederveen et al., 2017), and the blunted hypertrophic response in old mouse muscle was not associated by a reduced number of SCs, but by an attenuated overload-induced increase in CD (Ballak et al., 2016). In addition, regeneration after cardiotoxin-induced muscle damage was better in mouse muscles with a larger C:F, even if they had a lower number of SCs (Omairi et al., 2016). In this context, it is interesting to note that the distance between SCs and capillaries is larger in muscles from older than young-adult humans (Nederveen et al., 2016) and it is possible that the impaired hypertrophic response and

regenerative capacity in old age is due to diffusion limitations. These data indicate that there is significant cross-talk between capillaries and myosatellite cells. The coupling between fibre size and the capillary bed is, however, not limited to capillary – SC cross-talk, as also mature myofibres can release angiogenic growth factors, such as VEGF (Takahashi et al., 2002) and Follistatin-like I (Ouchi et al., 2008), that also stimulate muscle growth. In conclusion, there is significant evidence that capillaries play an important role in the hypertrophic response and muscle regeneration.

A denser capillary network not only resulted in a larger SC activation and expansion (Nederveen et al., 2018), but also a more pronounced hypertrophic response (Snijders et al., 2017a) to a resistance training programme. This may have implications for the design of rehabilitation programmes where stimulation of angiogenesis through a dedicated period of endurance training or muscle stimulation before starting a resistance-training programme could potentially improve the hypertrophic response. One way to explore this, is to precede a resistance training programme with an endurance training programme that has been shown in both human (Gavin et al., 2015) and rat muscle to induce angiogenesis (Kurosaka et al., 2012) and further reduce oxygen diffusion distances, thus potentially improving the hypertrophic response. An alternative approach could be to employ concurrent training. Concurrent training has traditionally been associated with the “interference effect” (Hickson, 1980) that was thought to compromise muscle hypertrophy through increased activation of AMPK through endurance training, which inhibits mTOR activation necessary for skeletal muscle hypertrophy from resistance training, recent research suggests that careful selection of training modalities could prevent this phenomenon and even augment increases in fibre size (Murach and Bagley, 2016).

As discussed above, there is a lose link between the capillary supply to a fibre and fibre size, and the time course of angiogenesis is similar to that of hypertrophy. Atrophy, however, is not associated with a concomitant proportional capillary rarefaction as reflected by the initial increase in CD after denervation (Paudyal et al., 2018, Degens et al., 2008). After longer periods of denervation further capillary loss occurs and even avascular regions may develop (Borisov et al., 2000). The early increase in CD may provide a window of opportunity for treatment of denervation-induced atrophy, where the elevated CD may be conducive for muscle growth and regeneration.

It thus appears that the relationship between capillary supply to and oxidative capacity of a fibre is maintained during muscle growth, whereas capillary rarefaction during atrophy is such that the CD is, at least transiently, elevated.

Endurance exercise and chronic electrical stimulation

Prolonged endurance exercise training leads to an increase in CD. Based on observation in rodents, it has been suggested that the rate of angiogenesis is faster in response to large-volume low-intensity exercise than to low-volume high-intensity exercise (Olfert et al., 2016). Perhaps chronic low-frequency stimulation can be considered a proxy for large-volume low-intensity training and indeed does induce angiogenesis in as short a period as 2 days (Hudlicka et al., 1982) that may be due to increased sheer stress on the endothelial cells (Egginton et al., 2001, Olfert et al., 2016). Also in humans, training at both moderate and high intensities increases capillary supply to both type I and II fibres (Andersen and Henriksson, 1977, Jensen et al., 2004, Gavin et al., 2015).

Angiogenesis

Here we give a limited description of the process of angiogenesis and refer for further details to the review by Olfert et al. (2016). In short, the formation of new capillaries from existing blood vessels is known as angiogenesis and occurs in response to exercise and during wound repair. Two types of angiogenesis can be distinguished: splitting and sprouting (Olfert et al., 2016). Splitting angiogenesis, the longitudinal separation of capillaries, is thought to be a response to increased sheer stress to the lumen of capillaries due to hyperaemia (Olfert et al., 2016). Sprouting, the formation of capillaries from buds on pre-existing capillaries, occurs in response to stretch on the capillaries during conditions as overload (Zhou et al., 1998).

The increased blood flow during e.g. chronic electrical stimulation is attributable to vasodilation in response to nitric oxide (NO), prostacyclin, ATP and adenosine. The presence of NO in turn enhances expression of the vascular endothelial growth factor (VEGF), the most important angiogenic factor, which is involved in stimulation, proliferation and differentiation of endothelial cells and vascular smooth muscle cells (Benoit et al., 1999, Wagner, 2011, Egginton, 2009).

Angiogenesis through sprouting is realised by an increase in matrix metalloproteinases (MMPs), required for remodelling of the extracellular matrix, and elevated VEGF levels (Olfert et al., 2016). Indeed, acute resistance exercise

induces increases in VEGF and VEGF receptor 2 (VEGFR2/Flk-1) (Gavin et al., 2007). VEGF is also elevated in 2-week-overloaded rat muscles (Degens et al., 2003, Rivilis et al., 2002) to return to baseline levels at 28 days after induction of overload (Rivilis et al., 2002). While eNOS is not thought to play a role in rat models of overload-induced angiogenesis (Olfert et al., 2016), the resistance-exercise-induced angiogenesis in humans was associated with elevated eNOS and hypoxia inducible factor-α (HIF1-α) protein levels (Holloway et al., 2018). The discrepancy between the animal models and the human response may be related to elevated blood flow during resistance exercise that is likely more pronounced than the reported 3-fold increase in resting blood flow in overloaded muscles (Armstrong et al., 1986).

The significance of VEGF for both overload- and shear-stress-induced angiogenesis is further reflected by the observation that VEGF trapping abolished angiogenesis in both conditions (Williams et al., 2006a). Myofibre-derived VEGF, the main source of VEGF, is not essential for maintenance of the capillary bed as seen in mice with myofibre-specific conditional VEGF deletion (Knapp et al., 2016). Total VEGF deletion (Tang et al., 2004), however, does lead to capillary rarefaction, indicating that VEGF plays an essential role not only in angiogenesis, but also in maintenance of the capillary bed.

SPECIAL CONDITIONS

Chronic heart failure

The majority of chronic heart failure (CHF) cases are characterised by left ventricular systolic dysfunction and a reduced ejection fraction (Sullivan and Hawthorne, 1995). The muscle hypothesis of CHF suggests that much of the exercise intolerance of patients with CHF is related to changes in skeletal muscle, including fibre atrophy, an increased proportion of type II fibres, and a reduced oxidative capacity and C:F (Hirai et al., 2015, Rogers, 2001). The cause of these changes in skeletal muscle during CHF are not clear, but are at least partly attributable to disuse. In addition, left ventricular dysfunction is associated with a rise in catabolic factors, such as elevated tumour necrosis factor-α, and insulin resistance that blunts anabolism. Over time these conditions lead to skeletal muscle myopathy and fatigue. A shift to anaerobic metabolism due to disuse and poor oxygenation leads to an earlier onset of metabolite accumulation during exercise that enhances ventilation via the

ergoreflex. This in turn leads to excitation of the sympathetic system, which induces vasoconstriction aggravating the reduction in peripheral blood flow due to cardiac dysfunction and elevating the afterload that in turn further worsens left ventricular function (Coats, 1996, Rogers, 2001). In addition to the reduced peripheral blood blow resulting from cardiac dysfunction and sympathetic-induced vasoconstriction, the increased blood flow to the respiratory muscles due to heightened ventilation, further diminishes the cardiac output diverted to the periphery and hence perfusion of the locomotory muscles, all negatively impacting the exercise tolerance of patients with CHF.

In most CHF patients, the blood flow to the muscle during single-leg knee-extension exercise is not limited by cardiac output, yet it has been suggested that both impaired convective and diffusive oxygen transport impaired the lower oxygen consumption during single leg exercise in CHF patients (Esposito et al., 2010b). Although the authors concluded a lower diffusive oxygen transport played a role in their impaired oxygen consumption during single leg exercise, the C:F ratio was similar to that of controls (Esposito et al., 2010b), something also seen by others in humans (Niemeijer et al., 2018) and rodents (Degens et al., 2002). Most studies, however, do report a lower density of the capillary network in muscles of patients with CHF (Hirai et al., 2015, Poole et al., 2012) that was related to their lower maximal oxygen uptake (Duscha et al., 1999). Nevertheless, even in the absence of capillary rarefaction, diffusion limitations may be a consequence of reduced RBC flux and velocity, and an increased proportion of capillaries with intermittent RBC flux both at rest (Kindig et al., 1999) and during contractile activity (Richardson et al., 2003) as seen in rats with CHF. This may precipitate a mismatch between oxygen delivery and utilisation both at rest and during exercise (Hirai et al., 2015).

In an experimental rabbit model of heart failure induced by coronary artery ligation it was seen that vascular rarefaction and apoptosis progressed over time (Nusz et al., 2003). In rats with monocrotaline-induced CHF it was found that apoptosis in the endothelium preceded muscle apoptosis (Vescovo et al., 1998) and the authors suggested that loss of capillaries may lead to inadequate nutritional flow to the myofibres, inducing myofibre apoptosis. In addition, to meet the energy demands during exercise in the face of impaired oxygen delivery and diffusion problems due to capillary rarefaction, there is an increased reliance on anaerobic glycolysis and an earlier onset of muscle fatigue (Poole et al., 2012, Coats et al., 1994, Hirai et al.,

2015). These observations suggest that the microcirculation is a potential target to improve exercise tolerance in patients with CHF.

Exercise intolerance is a major cause of further inactivity in CHF patients, and improvements to oxygen delivery and diffusion capacity in the muscle likely improve exercise tolerance. Exercise training not only improves oxygen delivery to the muscle, but also the diffusion capacity that could partly be due to an increase in skeletal muscle CD (Hirai et al., 2015). Indeed, it has been observed in patients with CHF that 8 weeks of knee extensor training resulted in an increased mitochondrial volume density, C:F and number of capillaries around a fibre, indicating angiogenesis (Esposito et al., 2011). These training-induced changes led to both improved knee extension and whole-body exercise capacity without an improvement in maximal cardiac output, supporting the muscle hypothesis of exercise intolerance in CHF and the important role of the microcirculation in exercise performance in these patients. These are encouraging observations as they demonstrate that the microcirculation can be improved in CHF patients, for instance through small muscle training, to enhance whole body exercise capacity (Esposito et al., 2011, Hirai et al., 2015).

Ageing

During ageing, there is a progressive reduction in exercise capacity, which is evident in a reduction in maximal oxygen consumption (VO_2max). While largely due to a reduction in cardiac output, this decline in VO_2max is also attributed to age-related muscle atrophy (Degens, 1998, Fleg and Lakatta, 1988). In addition, there is a significant ageing-related reduction in C:F in type II fibres that showed atrophy, but neither atrophy nor a reduction in C:F in type I fibres (Barnouin et al., 2017). Interestingly, it has been reported that sarcopaenic older people had a lower C:F ratio than non-sarcopaenic people (Prior et al., 2016) and it has been suggested that capillary rarefaction may contribute to, and even precede, the age-related muscle atrophy and decline in exercise capacity (Prior et al., 2016, Larsson et al., 2019).

In many rodent studies that assess age-related change in the microcirculation no reductions in C:F are found, and in many cases even no atrophy (Faber et al., 2011, Larsson et al., 2019, Ballak et al., 2016). In one study even an increase in C:F was found (Davidson et al., 1999). While this increase may be due to the reduction in fibre number, which would increase C:F without capillary proliferation, current data

do not suggest that capillary rarefaction is involved in the onset of sarcopaenia in rodents. This conclusion is probably somewhat premature, as most of these studies are plagued by the use of a young control group that is not yet fully matured and an old group that is only in the initial stage of ageing. This is important as rodent muscles continue maturation throughout the first year of life (Maltin et al., 1989) and may only start to show signs of ageing after the age of 22 months (Lushaj et al., 2008), thus making comparisons akin to a 17-year-old with a 60-year-old person, that mask the effects of age. For example, the similar fibre size and C:F in the plantaris muscle of 5- and 25-month-old rats could falsely lead to the conclusion that 25-month-old rats do not yet show signs of ageing. Yet, the fibre size and C:F were lower in the muscles of 25- than 13-month-old rats (Degens et al., 1993a, Degens et al., 1994b, Larsson et al., 2019). Thus, to study the effects of ageing, it is important to choose a fully matured age as the young control group and animals that show at least early signs of ageing, such as atrophy and loss of force generating capacity, in the old group to make appropriate comparisons with human ageing (Ballak et al., 2014b).

In contrast to rodent studies, most human studies report an ageing-related muscle fibre atrophy and reduced C:F, of particularly type II fibres (Larsson et al., 2019, Barnouin et al., 2017). Overall, it appears that the reduction in C:F, indicating capillary rarefaction, is proportional to the decline in fibre size as reflected by the maintained CD (Parizkova et al., 1971, Larsson et al., 2019). The reduction in C:F during ageing may underestimate the real extent of capillary rarefaction if there is also a concomitant ageing-related loss of fibres, which without capillary loss would result in an increased C:F (Larsson et al., 2019). In fact, in a 12-year longitudinal study of sedentary men from age 65 to 77 reductions in C:F and CD were seen, without a decrease in fibre size, but a loss of overall muscle size, indicative indeed of a reduction in fibre number (Frontera et al., 2000). Therefore capillary loss during ageing is probably more significant than indicated by the reduction in C:F (Larsson et al., 2019).

In old rodent muscles the absence of significant ageing-related changes in C:F and CD in the face of reduced oxidative capacity suggests a capillary supply in relative excess to oxidative capacity (Hepple and Vogell, 2004). In human muscle, however, the maximal oxygen consumption supported by a capillary did not change with age (Barnouin et al., 2017), and even when a reduction in oxidative capacity was found, the CD was reduced (Coggan et al., 1992). This suggests that the superfluous

capillary supply in ageing rodents is not found in humans, or only occurs later during accelerated denervation with incomplete reinnervation (Larsson et al., 2019). This deserves further study, but the excessive capillary supply in relation to the oxidative capacity during the first 4 weeks after denervation in both the soleus (Degens et al., 2008) and gastrocnemius (Paudyal et al., 2018) muscles supports this proposition.

As previously stated, while C:F and CD are useful in determining capillary supply to skeletal muscle, the distribution of the capillaries in the tissue is also crucial for adequate muscle oxygenation (Degens et al., 2006b). While the impact of ageing on heterogeneity of capillary spacing has not been studied extensively, it has been reported that old rats exhibited an larger heterogeneity of capillary spacing than young rats, which was related to the larger variation in muscle fibre size (Degens et al., 2009b). Also in human muscle a relationship was found between the variation in fibre size and the heterogeneity of capillary spacing in a muscle, but neither the variation in fibre size nor the heterogeneity of capillary spacing differed between muscles from young-adult and older people (Barnouin et al., 2017). This suggests that not only angiogenesis, but also capillary rarefaction is a controlled process to ensure an adequate tissue oxygenation.

The impaired exercise-induced vasodilation and blood flow, even in endurance-trained people (Proctor et al., 1998, Hildebrandt et al., 2017), is to a large extent attributable to endothelial dysfunction. As VEGF expression is increased under conditions of elevated flow and hence higher sheer stress (Olfert et al., 2016, Milkiewicz et al., 2001) an impaired vasodilation may underlie the reduced VEGF expression in old age and the slow but progressive loss of capillaries (Larsson et al., 2019). As discussed above, conditional deletion of VEGF led to capillary rarefaction and apoptosis (Tang et al., 2004), indicating the importance of VEGF for maintenance of the capillary bed and protection against apoptosis. Interestingly, the large majority of apoptotic nuclei in old mouse muscles belonged to endothelial cells and a large number of SCs (Wang et al., 2014). This resembles the situation in experimental heart failure in rodents where apoptosis of endothelial cells preceded muscle atrophy (Vescovo et al., 1998). In addition to lower VEGF expression in muscles of the elderly, endothelial cells have been shown to become desensitised to VEGF due to the loss of NAD+ dependent sirtuin deacylase 1 (SIRT1) activity in mouse endothelial cells (Das et al., 2018). Accordingly, treatment with an NAD+ booster, nicotinamide mononucleotide (NMN), that activated SIRT-1 led to enhanced VEGF sensitivity, stimulated angiogenesis and improved exercise

capacity in old mice (Das et al., 2018). Dietary restriction also leads to angiogenesis via SIRT1 activation (Das et al., 2018, Longchamp et al., 2018). Such changes and the reduction in the VEGF receptor Flk-1 may underlie the attenuated angiogenic response and hypertrophy in overloaded muscles from old mice (Ballak et al., 2016).

In addition to an attenuated exercise-induced vasodilation also the vasodilatory response to insulin is diminished (Fry and Rasmussen, 2011). The endothelial dysfunction and capillary rarefaction may contribute to the ageing-related decline in muscle size and strength as it may hamper the delivery of amino acids to the muscle (Timmerman et al., 2010, Groen et al., 2014, Fry and Rasmussen, 2011) and contribute to the anabolic resistance in old age (Rennie, 2009).

In addition to a reduction in SC content in type II fibres, the distance between type II fibre associated SCs and capillaries is greater in the muscle of older than in those from young men (Verdijk et al., 2007, Nederveen et al., 2016). This greater distance between capillaries and SCs is associated with an impaired SC function in old age (Snijders et al., 2014). Evidence for the importance of the proximity of capillaries to SCs comes from the observation that SC activation after damaging exercise is better when capillaries and SCs are in close proximity (Nederveen et al., 2018). Thus, the increased distance between SCs and capillaries in ageing (Nederveen et al., 2016) could be instrumental in impaired muscle health. Adequate type II fibre capillarisation was found to be crucial in increasing SC content and muscle fibre size in elderly men subjected to resistance training (Snijders et al., 2017a). A recent study by Snijders et al. (2019) found that adhering to a 12-week program of exercise training in healthy older men led to an increase in skeletal muscle capillarisation, which correlated with increased SC content at 24h post exercise. This shows that prolonged exercise training can increase the number of capillaries per type II fibre, which is associated with improved SC and hypertrophic responses, providing further evidence for the role of capillaries in hypertrophy.

Part of the decrement in muscle mass and function is attributable to disuse (Degens and Alway, 2006). Master athletes maintain high levels of physical activity (Hannam et al., 2017) and are a useful model to disentangle the effects of disuse from those of ageing *per se* (Rittweger et al., 2004). It appears that regular physical activity can largely attenuate or even prevent ageing-related changes in skeletal muscle microcirculation. For instance, the C:F was higher, fibre size larger and oxidative capacity similar in activity-matched older than young endurance athletes (Coggan

et al., 1990), and in master cyclists there was no significant ageing-related reduction in fibre size and C:F (Pollock et al., 2018). Even in previously sedentary older people endurance and strength training can induce angiogenesis (Hepple et al., 1997, Holloway et al., 2018, Snijders et al., 2019). These observations indicate that indeed a large part of the decrement in the density of the capillary network is attributable to disuse. One thing to consider, however, is that the hypertrophy and SC activation in response to resistance exercise has been reported to be less in older people with a lower CD (Snijders et al., 2017a). The implication is that benefits of resistance exercise in older people may be helped if preceded or performed concurrently with endurance exercise, which is a potent stimulator of angiogenesis inducing also an increase in CD (Hepple et al., 1997).

Obesity and diabetes

In addition to many other detrimental health outcomes, greater adiposity is associated with lower capillary density (Lillioja et al., 1987). As mentioned above, this is linked to glucose intolerance and insulin resistance (Lillioja et al. 1987; Snijders et al. 2017b). VEGF and VEGF receptor expression in obese individuals is similar to healthy controls however (Gavin et al., 2005). The angiogenic response to endurance exercise training also seems to be preserved in obesity; lower C:F found in obese rats was increased to that of control animals after 10 weeks of swimming training (Gomes et al., 2017) and 6 weeks of treadmill running led to increased skeletal muscle capillary density in obese rats (Torgan et al., 1989).

Type II diabetes is associated with a reduction in CD which may negatively impact the oxygen exchange from capillary to fibre and contribute to complications of the disease (Mathieu-Costello et al. 2003; Olfert et al., 2016). As such, prevention of microvascular deterioration may improve outcomes for people with type II diabetes. An acute study found that the angiogenic response to exercise does not seem to be maintained in diabetes; in contrast to healthy mice, diabetic mice demonstrated no increase in proangiogenic factors in muscle with a single bout of exercise (Kivela et al., 2008). However, capillary volume and proangiogenic factors were increased after 3 weeks of a low intensity training programme in a diabetic rat model (Kondo et al. 2015). This promising outcome from exercise intervention prompts further study into microvascular adaptation to training in human diabetic patients.

CONCLUDING REMARKS

Not only the quantity, but also the distribution of capillaries is important for adequate muscle oxygenation. Interestingly, the main determinant of capillary supply to a fibre is not the oxidative capacity, but rather the size of the fibre. Indeed, muscle hypertrophy and angiogenesis follow a similar time course, and the attenuated hypertrophy in old age is at least partly attributable to a diminished density of the capillary network and impaired angiogenesis. Endothelial dysfunction and capillary rarefaction most likely contribute to the exercise intolerance, may precede muscle atrophy and a greater reliance on glycolytic metabolism during CHF and ageing. Therefore, we suggest that the microcirculation is an important target for rehabilitation in CHF and combating sarcopaenia.

A discussion of human and animal models

The main benefit of human models in research is the extent that findings can be generalised to human interventions. However, due to genetic diversity larger numbers of participants may be required for a study to reach sufficient statistical power. One advantage of animal models is that they are more homogenous than human subjects which increases the sensitivity and reproducibility of experimental outcomes (Lowe and Alway, 2002). The genetic homogeneity of animals used in research also means that conclusions from animal studies may have limited application to humans as they have large variability in their responses to exercise (McPhee et al., 2011; Erskine et al., 2010) as it is not representative of the diversity found in human populations.

Another benefit of using animals for muscle research is that animals can be sacrificed and whole muscles can be removed after an intervention. This is in contrast to human muscle biopsies which contain a small amount of tissue which may not represent adaptations throughout the entire muscle. Additionally, use of laboratory animals grants the investigator tighter experimental control than could be achieved in human studies without great cost. The environment, activity levels and nutritional intake can be precisely controlled and made almost identical for each subject.

Interventions in animals may be more appropriate for the research question than those possible in humans. The overload methods used in studies within this thesis

have been shown to induce hypertrophy much greater than that found with resistance training (Lowe and Alway, 2002) and would therefore be more appropriate to reveal the limits of any trade-off between fibre size and oxidative capacity.

The outline of the thesis

This thesis describes a series of studies on the role of microcirculation in skeletal muscle performance and the hypertrophic response. The aims of the thesis were to 1) gain a better understanding of the importance of skeletal muscle capillarisation in fatigue resistance, therefore providing evidence for its use as a therapeutic target to improve exercise tolerance in diseases affecting the microcirculation and 2) determine the importance of capillarisation in the hypertrophic response in ageing and in mice with different baseline muscle mass. Rodent models were used in chapters 2, 3, 5 and 6.

Chapter 2 used unbiased occlusion of capillaries through microsphere injection in a rat model to test the hypotheses that 1) fatigue resistance is reduced in proportion to the reduction in functional capillaries and 2) remodelling in response to overload induces angiogenesis that rescues the effects of capillary rarefaction.

Chapter 3 investigates the effects of angiogenic stimuli (voluntary running and overload) on the maintenance and restoration of muscle performance, respectively, in a rat model of CHF.

In **Chapter 4** the effects of superimposing endurance training in young and old highly resistance-trained men are described. It was predicted that, according to the size principle, muscle FCSA decreases with endurance exercise to accommodate for an increase in oxidative capacity and that this decrease in fibre size is greater in old participants due to an attenuated angiogenic response.

Chapter 5 describes the adaptation to concurrent overload and regular endurance exercise in young and old mice. It was hypothesised that inclusion of both stimuli leads to impaired hypertrophy in response to overload, smaller increases in fatigue resistance with endurance exercise and that responses to these stimuli are blunted in old mice due to lower capillary density and impaired angiogenesis.

Chapter 6 compares the response of three mouse strains with different baseline muscle mass to concurrent overload and endurance stimuli. According to the size principle, fibres with small FCSA are expected to demonstrate greater hypertrophy

than those with larger FCSA due to smaller diffusion distances between the periphery of the fibre to the interior of the fibre. Additionally, the combination of both overload and endurance exercise was expected to lead to blunted adaptations to both stimuli, as in chapter 5.

Prelude to Chapter 2

While greater functional capillary density is associated with improved muscle performance and impaired capillary density due to disease or inactivity is associated with reductions in muscle fatigue resistance, the role of the microcirculation has not been studied without concomitant changes in skeletal muscle. Here the effects of impaired skeletal muscle microcirculation on fatigue resistance are studied independently of other factors in otherwise healthy animals through both acute and chronic microsphere injection. The use of an angiogenic stimulus is also used to determine if fatigue resistance can be restored.

Chapter 2

IMPAIRED SKELETAL MUSCLE PERFORMANCE AS A CONSEQUENCE OF RANDOM FUNCTIONAL CAPILLARY RAREFACTION CAN BE RESTORED WITH OVERLOAD-DEPENDENT ANGIOGENESIS

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ABSTRACT

To what extent microvascular rarefaction contributes to impaired skeletal muscle function remains unknown. Our understanding of whether pathological changes in the microcirculation can be reversed remains limited by a lack of basic physiological data in otherwise healthy tissue. The principal objectives here were to: (1) quantify the effect of unbiased microvascular rarefaction on limb perfusion and muscle performance, and (2) determine if these changes could be reversed. We developed a novel protocol in rats whereby microspheres injected into the femoral artery allowed a unilateral reduction in functional capillary density in the extensor digitorum longus (EDL), and assessed acute and chronic effects on muscle function. Simultaneous bilateral EDL force and hindlimb blood flow measurements were made during electrical stimulation. Following functional capillary rarefaction there was an acute microsphere dose-dependent reduction in muscle fatigue resistance (r^2=0.0570, $p < 0.001$), despite preserved femoral artery perfusion. Histological analysis of EDL samples taken from injected animals confirmed a positive correlation between the proportion of functional capillaries and fatigue resistance (r^2= 0.720, p=0.002). Such impaired performance persisted for at least 2 weeks (p=0.016). Concomitant mechanical overload improved both anatomical capillary density (556 mm^2 ± 63 vs. 813 mm^2 ± 114, $p<0.05$) and fatigue resistance (0.36 ± 0.09 vs. 0.65 ± 0.11, $p<0.05$), confirming that the capacity for muscle remodelling was retained following chronic distributed ischaemia, and that the impact of capillary rarefaction could be alleviated. These results demonstrate that loss of functional capillaries is detrimental to muscle function, even in otherwise healthy tissue, independent of arterial perfusion. Restoration of muscle performance following a mechanical overload stimulus indicates that angiogenic treatments to alleviate microvascular rarefaction may be key to restoring exercise tolerance.

INTRODUCTION

Critical functions of the microcirculation that optimise muscle performance during exercise include supporting adequate gas exchange, delivery of nutrients and removal of metabolites. The extent of microvascular supply in skeletal muscle is associated with the integrative effects of activity (Andersen and Henriksson, 1977, Jensen et al., 2004, Hoier et al., 2012, Gavin et al., 2015) in a feedback manner, such that muscles with experimentally increased capillary density exhibit increased fatigue resistance (Hudlicka et al., 1977). In contrast, reduced capillarity occurs in skeletal muscle after experimentally induced (Kindig et al., 1999, Nusz et al., 2003) and clinical (Schaufelberger et al., 1995, Duscha et al., 1999, Wadowski et al., 2018) chronic heart failure (CHF), from which we can infer that poor fatigue resistance in disease may be accounted for, at least in part, by deleterious changes in the microcirculation.

Not only the number but also the distribution of capillaries plays an important role in tissue oxygenation (Degens et al., 1994a, Egginton and Gaffney, 2010). If capillary loss is also accompanied by an increased heterogeneity of capillary spacing in the muscle tissue, then this may cause tissue oxygenation to be reduced more than expected from reduced capillary density alone (Piiper and Scheid, 1991, Degens et al., 2006b), and hence contribute to a further decline in functional capacity of the muscle.

Impaired oxygen supply in disease may also be exacerbated by a reduction in the proportion of capillaries supporting continuous red blood cell flow (Kindig et al., 1999, Richardson et al., 2003). In support of this, an ~4-fold reduction in mechanical output of the heart resulted from a stochastic occlusion of 25% of terminal arterioles in the coronary microcirculation, suggesting that distributive (microvascular) ischaemia may be more detrimental than that with a focal (arterial) origin (Hauton et al., 2015). Thus, microvascular rarefaction per se may represent a mechanism for exercise intolerance associated with diseases adversely affecting the microcirculation (e.g. CHF, diabetes). A lack of experimental data on the physiological effects of microvascular rarefaction, in the absence of influence from concomitant changes due to disease, presents a constraint to our understanding of the interaction between muscle performance and capillary supply. Consequently, it is imperative that the basic physiological effects that arise from a compromised microcirculation are established. The primary objective of this study was therefore

to assess the effect on muscle performance of an isolated reduction in functional microvascular density, in otherwise healthy tissue.

Future benefits of deciphering this relationship may include modifying existing treatments (Zamani et al., 2017) to recover impaired exercise tolerance in microvascular disease. We therefore studied how muscle function and blood flow were affected by acute and chronic reductions in the number of functional (perfused) capillaries, and how the angiogenic response to mechanical overload (Degens et al., 1992, Zhou et al., 1998, Deveci and Egginton, 2002, Egginton et al., 2011, Ballak et al., 2016) was affected by an underlying constrained microcirculation in otherwise healthy tissue. Compensatory overload after synergist extirpation occurs due to the additional functional demands imposed on remaining muscles. Pronounced hypertrophy (Zhou et al., 1998, Deveci and Egginton, 2002) coupled with increased fatigue resistance (Frischknecht and Vrbova, 1991, Ballak et al., 2016) due to angiogenic remodelling (Egginton et al., 1998, Egginton et al., 2011, Williams et al., 2006b, Ballak et al., 2016) proceeds shortly after overload surgery.

Specifically, we tested the hypotheses that fatigue resistance will be reduced in proportion to the decreased number of functional capillaries, but that the capacity for remodelling is retained such that the angiogenic mechanical stimulus of muscle overload can rescue the effects of capillary rarefaction. Using microsphere injections to induce unbiased arteriolar blockade, we quantified the influence of a reduced number of perfused capillaries on muscle performance and hindlimb blood flow. Our findings suggest that capillary rarefaction is a major physiological contributor to skeletal muscle fatigability, and hence limits exercise tolerance, but that mechanical stimuli can successfully reverse pathological changes and restore muscle function to normal levels.

METHODS

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 (70/08674). All experiments conformed to the principals and regulations described by guidelines published in the Journal of

Physiology (Grundy, 2015). Ethical approval was also provided by Manchester Metropolitan University (SE171810).

Animal surgery: acute effects of microsphere injection

Anaesthesia in male Wistar rats [body mass (M b): 258 ± 16 g, range: 224–296 g, n=25) was induced with isoflurane (4% in 100% O2) and subsequently maintained by constant alfaxalone (Alfaxan: Jurox, Crawley, UK) infusion (30–35 mg kg^{-1} h^{-1}) delivered via an external jugular vein catheter. A tracheotomy tube allowed spontaneous breathing. A catheter (PE20) was implanted into the carotid artery to record heart rate and measure blood pressure with a pressure transducer (AD Instruments, Oxford, UK).

Spatially unbiased occlusion of microvessels in the extensor digitorum longus (EDL) muscle was achieved through injection of polystyrene microspheres (Triton Technology Inc., San Diego, CA, USA) via a catheter (PE10 heat-pulled to reduce tip size) implanted in a branch of the femoral artery (superficial epigastric artery). Microspheres (mean diameter 10 μm) stochastically blocked terminal arterioles, preventing blood flow in narrower downstream capillaries (Eriksson and Myrhage, 1972, Egginton and Hudlicka, 1999) and thereby inducing acute functional microvascular rarefaction. No evidence was found in any sample of trapped microspheres in the capillary bed during histological analysis of capillary perfusion. Initial experiments indicated that injecting 10:1 dilutions of the microsphere suspension (as per the manufacturer's instructions and accepted practice in the field; Deveci & Egginton, 1999) induced a significant and persistent increase in local femoral blood flow. To reduce this bias, microspheres were centrifuged (number of microspheres=100,000–1,000,000; this range was found to be appropriate for inducing progressively more severe arteriolar occlusion), the supernatant was removed and washed spheres were re-suspended in saline. This eliminated the confounding effect of the carrying solution on femoral artery vascular conductance (FVC) during the 10 min following injection.

Animal surgery: chronic effects of arteriolar blockade

Muscle overload has been shown to precipitate an angiogenic response via upregulation of vascular endothelial growth factor (VEGF) and its main signalling receptor Flk-1 in what is considered to be a mechanotransduction-mediated

response (Egginton et al., 1998, Egginton et al., 2011, Williams et al., 2006b). Therefore, unilateral overload to induce adaptive remodelling of the EDL was induced by surgical extirpation of the synergist, tibialis anterior (TA) (Zhou et al., 1998, Deveci and Egginton, 2002), in two new groups of animals. One group (OV: M b at terminal experiment: 281 ± 20 g, range: 248–310 g, n=8) underwent TA removal only, while in addition to TA removal, the other group (OV+MS: terminal M b: 283 ± 17 g, range: 261–305 g, n=8) also received an injection of a moderate dose of microspheres (350,000; ascertained above) after removal of the TA, following the protocol used in acute experiments. A further group (chronic MS: terminal M b: 302 ± 9 g range: 290–315 g, n=7) underwent only an injection of microspheres, which was of greater magnitude (700,000) to accommodate the probable reduced microsphere delivery to the EDL via blood flow to the TA. When transformed to a mass-specific dose, this remained within the range[2] at which resting blood flow remained unperturbed (~4,762,000 microspheres per gram EDL, Fig. 2). Surgical procedures were undertaken in animals with body mass approximating that used in acute experiments. Data from intact control animals (terminal M b: 257 ± 15 g, range: 224–288 g, n=15) was used for comparison. Surgical anaesthesia was induced and maintained by isoflurane inhalation. Post-operative analgesia [buprenorphine (Vetergesic, Ceva, Amersham, UK) 0.05 mg kg^{-1}] and antibiotic [enrofloxacin (Baytril, Bayer, Reading, UK) 2.5 mg kg^{-1}] were given in all cases. Following a 14-day recovery period, EDL fatigue resistance and hindlimb blood flow were quantified. Figure 1 shows outlines the study design.

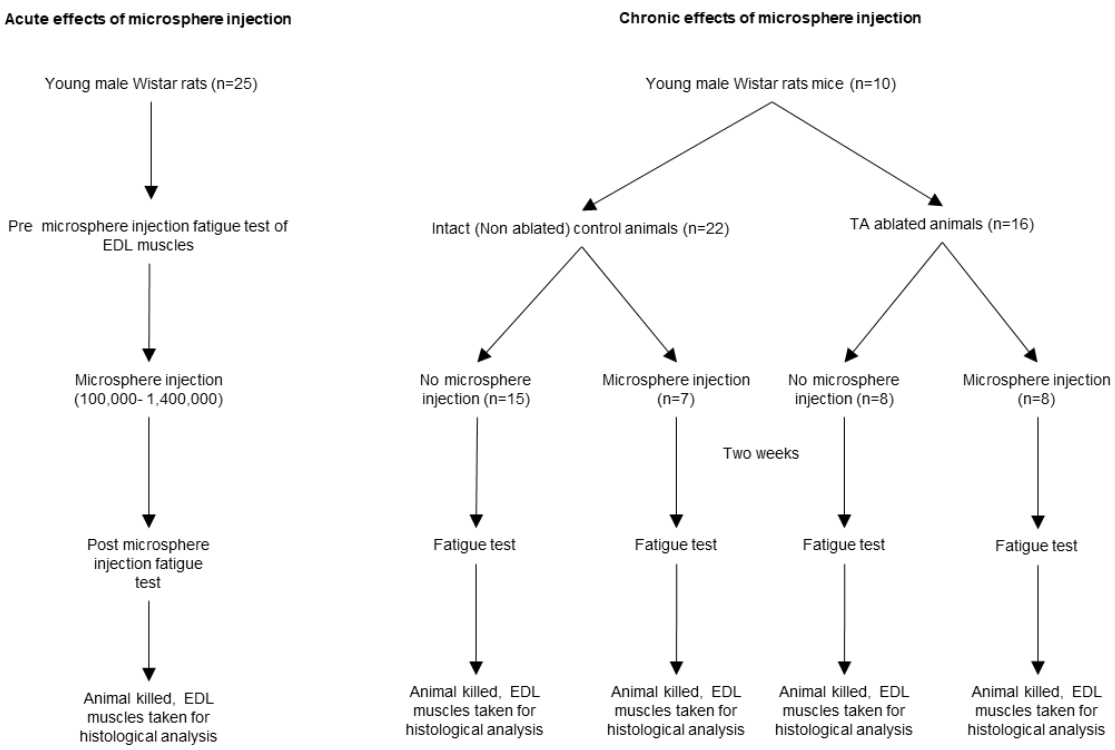


Figure 1 Study design for acute and chronic effects of microsphere injection on fatigue resistance.

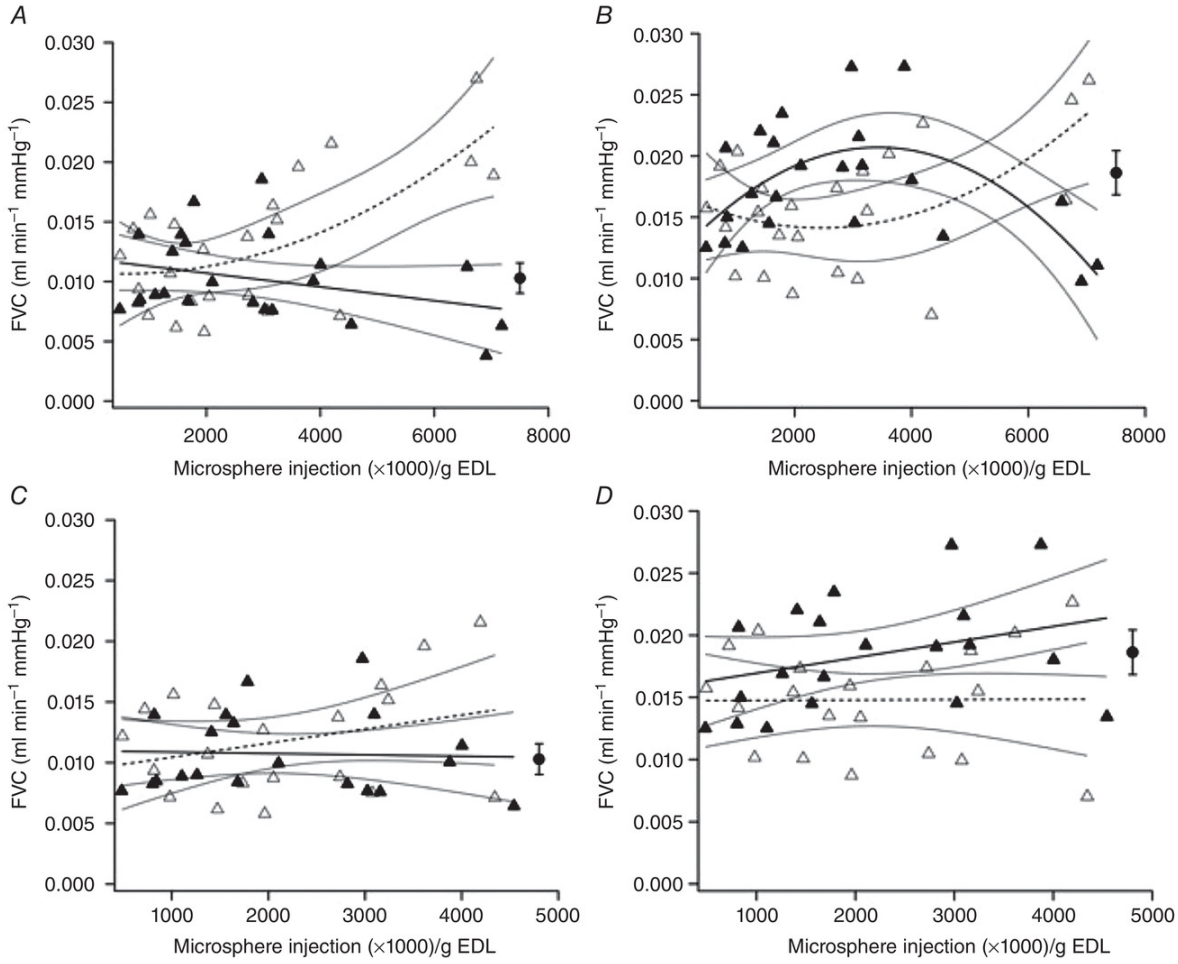


Figure 2: Ipsilateral (open triangles) and contralateral (filled triangles) data are shown in each panel. 95% confidence intervals are displayed around regression lines. There was a dose-dependent effect on ipsilateral resting (A) (r^2=0.444, p=0.003) and end-stimulation (B) (r^2=0.304, p=0.027) FVC. In the resting contralateral limb (A) there was a non-significant relationship (r^2=0.103, p=0.135) but decreasing end-stimulation flow (r^2=0.393, p=0.007) was detected with higher dose (B). Given the outliers observed at the highest doses (A, B), these data were omitted and the analyses re-run. Omitting these confounding data (C, D) we found that there was no effect on resting (C) (ipsilateral: r^2=0.089, p=0.200; contralateral: r^2=0.002, p=0.864) or end-stimulation (D) (ipsilateral: r^2=0.000, p=0.974; contralateral: r^2=0.110, p=0.153) FVC for mild to moderate arteriolar occlusion (p>0.05). The effect of femoral ligation on contralateral FVC is also displayed (filled circle); no equivalent ipsilateral value was calculated due to cessation of hindlimb blood flow.

Quantifying muscle performance and hindlimb perfusion

Bilateral EDL twitch force was measured simultaneously by connecting the muscle of each limb to a lever arm force transducer system (305B-LR: Aurora Scientific, Aurora, ON, Canada), following extirpation of the synergist TA to allow unimpeded access to the EDL. The EDL was indirectly stimulated with electrodes adjacent to the popliteal nerve (Hudlicka et al., 1977). To measure femoral artery blood flow throughout the experiment, perivascular flow probes (0.7PSB; Transonic, Ithaca, NY, USA) were placed at the proximal aspect of the profunda femoris artery bifurcation. Before fatigue measurements, muscle length and current delivery were set to ensure maximal isometric twitch force production. Resistance to fatigue was quantified in the EDL whereby a 30 s period of 1 Hz twitches to activate the metabolic machinery was followed by a fatigue test (Egginton and Hudlicka, 1999) during which the muscle was stimulated for 180 s with 10 Hz twitches (0.3 ms pulse width, supramaximal voltage).

Following this initial bout of stimulation to establish reference fatigue curves and blood flow responses, a saline solution (0.6 ml) containing microspheres was injected to occlude arterioles in the EDL with an unbiased method. To ensure that the bulk of flow from the femoral artery passed through the EDL, minimising blockage of arterioles in other limb muscles from systemic application (verified by histology in pilot studies, data not shown), functional hyperaemia was induced in the EDL before and after injection by 30 s of 4 Hz stimulation (0.3 ms pulse width, supramaximal voltage). The number of injected microspheres was within the range 100,000–1,400,000, and is expressed as microspheres per gram of EDL. After a post-injection period of at least 10 min, during which time all variables (blood pressure, heart rate, femoral blood flow) returned to stable baseline levels, the protocol for inducing fatigue was repeated to establish the acute effects of reduced microvascular perfusion on muscle performance. Pilot studies demonstrated that a second stimulation did not significantly affect blood flow or fatigue resistance in muscles with an intact microcirculation. To assess the effects of major microvascular blockade in another group of animals the proximal femoral artery was ligated. Muscle force, blood flow and pressure data were acquired using PowerLab 8/35 and analysed in LabChart 8 (both from AD Instruments).

A fatigue index (FI) was calculated as muscle tension at the end of stimulation/peak muscle tension at the start of the test, using the average of five consecutive twitches.

Relative FI was calculated for each muscle as the ratio of pre- to post-microsphere injection or ligation values. FVC was calculated as femoral blood flow/mean arterial pressure. Reported FVC was normalised for EDL mass to account for muscle hypertrophy in chronically treated animals. Hyperaemic scope was calculated as end-stimulation FVC/resting FVC.

Following the final fatigue stimulation, capillary plasma perfusion was visualised by an arterial injection of a fluorescein isothiocyanate (FITC)-labelled dextran solution (50 mg kg^{-1}, MW = 500,000; Sigma, Poole, UK) while the muscle was stimulated supramaximally at 4 Hz to ensure the FITC dextran had full vascular access to the EDL. Whether these 'functional' (i.e. perfused) capillaries were able to support red blood cell flux was not explicitly quantified. Muscles were left in situ for at least 1 min after stimulation to ensure appearance of dye in capillaries (Snyder et al., 1992) and animals were then killed by a Schedule 1 method.

Histology

Immediately following death of the animal, the EDL muscle was excised, blotted dry and weighed. The muscle was then mounted in OCT embedding medium (Thermo Scientific, Loughborough, UK), frozen in liquid nitrogen-cooled isopentane and stored at −80°C. In a subset of animals, 10 µm cross sections were cut with a cryostat to determine FCSA and capillary indices such as C:F and CD. Care was taken to avoid oblique cross sections which would lead to unrepresentatively high FCSA. Tortuous capillaries may undulate in and out of the muscle cross section, therefore it is possible to count multiple points of the same vessel, artificially raising the C:F and CD (Olfert et al., 2016). Capillary boundaries were labelled with a carbohydrate-binding protein known to identify rodent endothelial cells (Griffonia simplicifolia lectin I, 5 µl ml^{-1} FL-1101 GSL I; Vector Labs, Peterborough, UK). To identify muscle fibre types, serial sections were blocked with 10% goat serum (Vector Labs) in PBS for 60 min, then incubated for 120 min with monoclonal antibodies BAD-5 (1:600), SC-71 (1:600), 6H1 (1:50) and BF-F3 (1:100) in blocking solution for types I, IIa, IIx and IIb, respectively (Developmental Studies Hybridoma Bank, Iowa City, IA, USA). After washing in PBS three times for 5 min, sections were incubated in secondary antibodies Alexa Fluor 350 IgG2b for type I (1:500), Alexa Fluor 488 IgG1 for type IIa (1:500) and Alexa Fluor 555 IgM for types IIb and IIx (1:500) (Thermo Fisher Scientific Inc., Waltham, MA, USA) in blocking solution in the dark. After being washed once more, the slides were mounted using ProLong

Diamond Antifade mountant (Thermo Fisher Scientific). Digital images of whole-muscle cross-sections were acquired at ×20 magnification using a confocal microscope (Leica TCS SP5). Subsequent image analysis with BTablet and AnaTis (BaLoH Software, Ooij, The Netherlands) enabled calculation of a capillary perfusion index (perfused/unperfused vessels). Three regions of interest (475 × 475 μm^2) on the muscle image were used to establish an unbiased counting frame with which to quantify muscle fibre type composition, capillarisation and capillary supply (domain) area, taking into account the regional heterogeneity of capillary distribution and regional differences in fibre type composition (Kissane et al., 2018). Regions of interest were spaced equidistantly across the medial-lateral axis of the muscle in a systematic random manner, namely without a priori determination of placement. Capillary localisation depended upon staining intensity and anatomical landmarks; to be identified as a capillary, staining had to be at a location where one can expect a capillary and be elevated above the surrounding stain intensity. Image processing using the thresholding function in ImageJ (Schneider et al., 2012) allowed removal of non-specific staining that was below the high-intensity staining of identified capillaries. Pilot experiments indicated that FITC migration from capillaries was a minor problem. A small degree of FITC extravasation occurred in stored samples but observations show this to be greater if sections rather than tissue blocks are stored. We therefore adopted a protocol whereby block storage was the preferred approach until batch analysis was possible, at which time sections were cut and quickly imaged. Estimation of the oxygen partial pressure distribution within representative EDL histological sections was performed in a custom MATLAB program (‘oxygen transport modeler’) (Al-Shammari et al., 2019). Model assumptions include estimates of capillary radius (1.8–2.5 × 10^{-4} cm), muscle oxygen demand (15.7 × 10−5 ml O_2 ml−1 s^{-1}), myoglobin concentration (10.2 × 10−3 ml O_2 ml^{-1}), O_2 solubility (3.89 × 10−5 ml O_2 ml^{-1} $mmHg^{-1}$) and diffusivity (1.73 × 10^{-7} cm^2 s^{-1}) (Al-Shammari et al., 2019). No direct measurement of these parameters was possible in this study, so these values were applied across all groups. Although we have adopted a modelling approach to facilitate group comparison, physiological data to suggest P O_2 buffering by myoglobin in skeletal muscle (Gayeski et al., 1985, Gayeski and Honig, 1986) supports an alternative perspective on muscle–capillary oxygen diffusivity, whereby tissue oxygenation is not directly influenced by diffusion distance (Hepple et al., 2000), contrary to classical theory (Krogh, 1919). Whilst the considerable technical challenge of

accurately measuring dynamic quantities (including PO_2) may confound the interpretation of these empirical data (Groebe, 1996), it is clear that this remains a controversial topic; nevertheless, while recognising its inherent methodological limitations, we consider it timely to apply a model that assumes PO_2 gradients to be a cardinal factor in determining muscle oxygenation, as it allows for theoretical exploration of oxygen flux during simulated perturbations in metabolic demand and altered capillary supply.

Statistical analyses

To test for an effect on FVC of injections of saline, microsphere dose and microsphere solution, an ANOVA with Tukey post hoc tests was performed. Associations between microsphere dose, muscle performance, FVC and histological parameters were assessed using ordinary least-squares regression. Mean arterial pressure before and after microsphere administration was assessed using paired-samples t tests. ANOVA with Tukey post hoc tests were used to determine whether chronic differences occurred after surgical intervention. Data are presented as mean ± SD. Statistical analyses were performed in SPSS (v. 25) and differences and correlations were considered significant at $p<0.05$.

RESULTS

Immediate effect of microsphere injections on FVC

No difference in resting FVC was observed before injection of saline, washed microspheres or microsphere solution (p=0.544). FVC was increased in animals injected with microspheres in the normal solution after 2 min (p=0.001) and 5 min (p=0.006), returning to parity after 10 min (p=0.208), compared to animals injected with saline or washed microspheres. There was no difference between FVC following injections of saline and washed microspheres (2 min: p=0.371; 5 min: p=0.952). Contralateral FVC was unaffected throughout (rest: p=0.752; 2 min: p=0.987; 5 min: p=0.965; 10 min: p=0.208).

Acute effects of arteriolar blockade on FVC

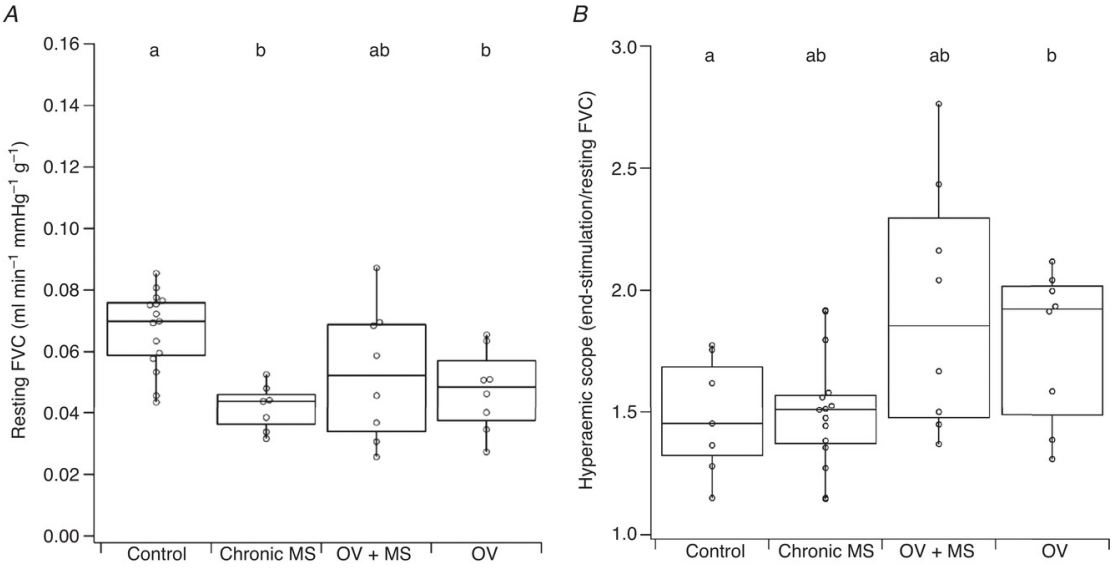
There were no significant bilateral differences in mean femoral blood flow at rest (ipsilateral 1.19 ± 0.26, contralateral 1.22 ± 0.27 ml min^{-1}, p=0.784) or at the end of stimulation (ipsilateral 1.71 ± 0.39, contralateral 1.86 ± 0.35 ml min^{-1}, p=0.380)

before injection of microspheres. Peak systolic femoral flow during rest (p=0.019) and flow amplitude (the difference between maximum and minimum flow recorded at systole and diastole) increased with microsphere dose (p=0.045); in contrast, at end-stimulation there was no effect of dose (p>0.05). There was no significant effect of dose or microsphere administration itself on resting (control: 123 ± 10, after injection: 122 ± 12 mmHg, p=0.282) or end-stimulation mean arterial pressure (control: 119 ± 15, after injection: 118 ± 15 mmHg, p=0.478). Resting (control: 12.2 ± 8.2, after injection: 13.8 ± 8.4 mmHg, p=0.664) and end-stimulation (control: 13.0 ± 8.4; after injection: 13.9 ± 8.8 mmHg, p=0.742) pulse pressures were similarly unaffected by dosage.

At higher doses than 100,00–700,000 microspheres there was a positive relationship between dose and FVC that was best described by a polynomial function both at rest (Fig. 2A) and end-stimulation (Fig. 2B). Contralateral FVC (Fig. 2A) was unaffected at rest, but the ipsilateral trend was paralleled at end-stimulation (Fig. 2B). Interestingly, the highest dose of microspheres elicited a persistent increase in resting FVC; omitting these data (n=3) indicated that FVC was unaffected at lower doses (Fig. 2C, D). Therefore, it appears that resting and active FVC is enhanced by the magnitude of microsphere dose above a threshold value.

Chronic effects of arteriolar blockade on FVC

Muscle overload (OV) induced a reduced resting FVC normalised to muscle mass (Fig. 3A; p=0.015). Two weeks after microsphere (MS) injection, resting FVC was lower than control (p=0.011), but in OV+MS no decrease was observed (Fig. 3A; p=0.147). The fractional increase in FVC (i.e. magnitude of functional hyperaemia) during stimulation was enhanced in OV (p=0.029; Fig. 3B) while chronic application



of microspheres did not have a significant effect on the hyperaemic response (chronic MS: p=0.999; OV+MS: p=0.217; Fig. 3B, Table 1).

Figure 3. Reduced resting FVC (normalised to muscle mass) was observed after chronic arteriolar blockade (chronic MS) and overload (OV). The magnitude of functional hyperaemia was equivalent to the control group in chronic MS and overload with microspheres (OV+MS), but enhanced after overload (OV). Unmatched lower-case letters denote statistical significance (p<0.05).

Table 1. *EDL fatigue resistance and blood flow (normalised to muscle mass) during rest and stimulation*

		Control	**Chronic MS**	**OV+MS**	**OV**
Fatigue index		0.47 ± 0.09^{a}	0.36 ± 0.03^{b}	0.65 ± 0.11^{c}	0.64 ± 0.08^{c}
Maximum twitch tension (N)		0.35 ± 0.10^{a}	0.35 ± 0.07^{a}	0.52 ± 0.10^{b}	0.48 ± 0.08^{b}
Maximum twitch tension per g (N g^{-1})		2.41 ± 0.64^{ab}	1.90 ± 0.44^{a}	2.72 ± 0.60^{b}	2.54 ± 0.40^{ab}
Mean femoral flow (ml min $^{-1}$g^{-1})	Rest	8.13 ± 1.62^{a}	5.37 ± 1.12^{b}	6.38 ± 2.77^{ab}	5.58 ± 1.47^{b}
	End-stimulation	11.70 ± 2.49^{a}	7.81 ± 1.90^{b}	10.56 ± 3.46^{a}	10.44 ± 1.09^{a}
Peak (systolic) femoral flow (ml min^{-1}g^{-1})	Rest	27.00 ± 6.79^{a}	14.82 ± 2.63^{b}	20.52 ± 5.24^{b}	17.64 ± 4.17^{b}
	End-stimulation	34.46 ± 8.24^{a}	19.26 ± 5.39^{b}	29.48 ± 7.83^{a}	28.20 ± 6.51^{ab}
Flow amplitude (ml min $^{-1}$g^{-1})	Rest	25.22 ± 7.00^{a}	13.39 ± 2.92^{b}	18.95 ± 4.80^{ab}	16.50 ± 4.25^{b}
	End-stimulation	31.40 ± 8.45^{a}	16.46 ± 5.26^{b}	25.60 ± 8.05^{ab}	24.92 ± 7.13^{ab}
FVC (ml min^{-1} mmHg^{-1}g^{-1})	Rest	0.067 ± 0.013 ^{a}	0.042 ± 0.008 ^{b}	0.053 ± 0.022^{ab}	0.047 ± 0.013^{b}
	End-stimulation	0.100 ± 0.020 ^{a}	0.062 ± 0.016 ^{b}	0.089 ± 0.024^{a}	0.086 ± 0.015^{ab}
	Hyperaemic scope	1.50 ± 0.24^{a}	1.48 ± 0.24^{ab}	1.79 ± 0.31^{ab}	1.92 ± 0.51^{b}

Unshared superscript letters denote statistical significance (p<0.05) as assessed by Tukey post hoc tests.

Muscle fatigue

In the acute model, before injection there was no bilateral difference in the fatigue index (FI) of EDL (ipsilateral: 46.9 ± 8.5%, n=25; contralateral: 48.5 ± 8.4%; n=24, F=0.477, p=0.493). A significant, dose-dependent, inverse relationship between the number of microspheres injected and relative FI was detected in the ipsilateral (p<0.001), but not in the contralateral control EDL (p=0.182). The FI in rats with ligated femoral artery was significantly impaired compared to simultaneously measured contralateral FI (p<0.001) (Fig. 4A).

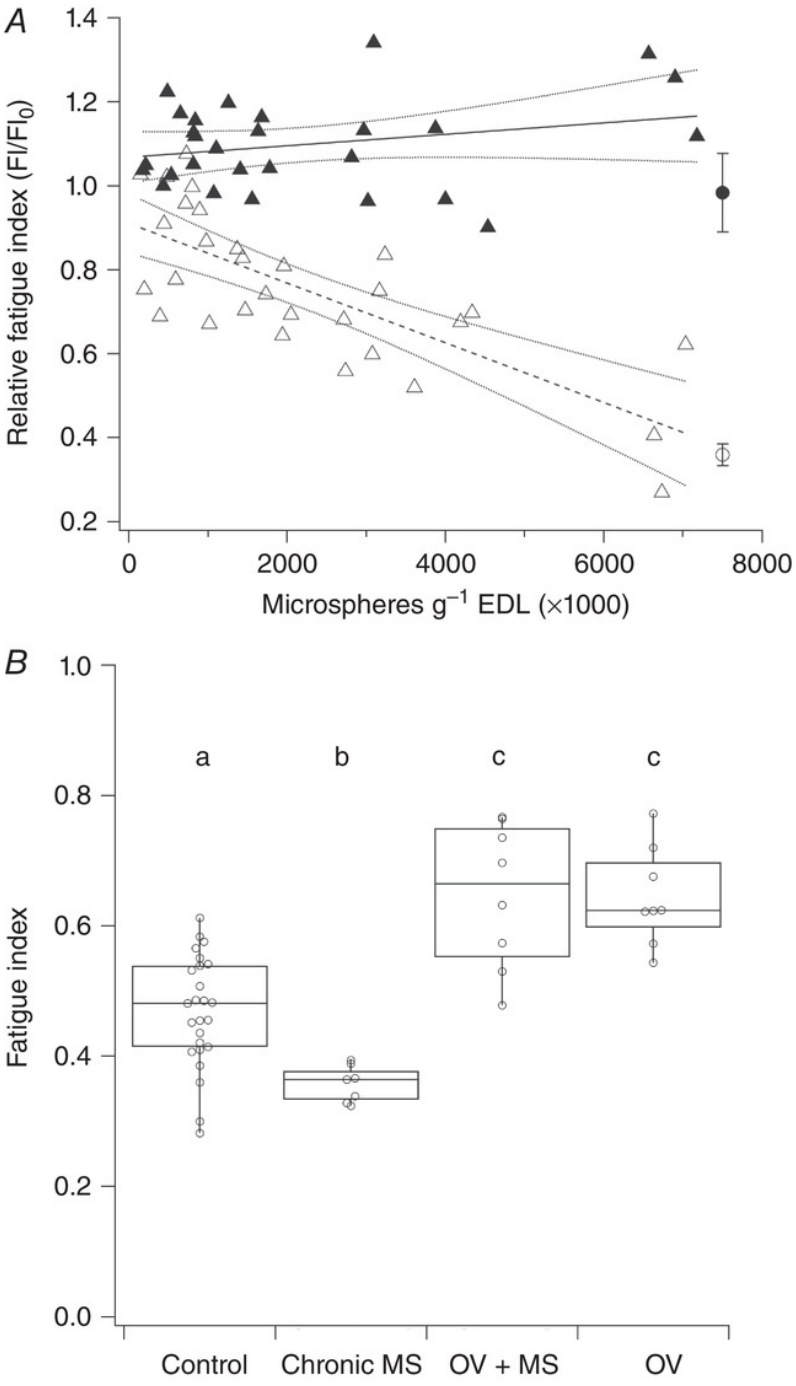


Figure 4. A, *there was a significant decrease in ipsilateral relative FI (i.e. pre-injection/post-injection FI) with increasing dose of microspheres injected (open triangles, dashed line: r^2=0.570, p<0.001) while there was no effect on the contralateral FI (filled triangles, continuous line) (r^2=0.067, p=0.182). The effect of femoral artery ligation is displayed for ipsilateral (open circle) and contralateral (filled circle) extensor digitorum longus muscle. B, chronic occlusion of arterioles by microspheres (chronic MS) caused impaired FI (p=0.016) relative to control while both overload with microspheres (OV+MS) and overload (OV) improved FI (p<0.001). Unmatched lower-case letters denote statistical significance (p<0.05).*

FI was improved in OV (p<0.001) but was impaired compared to control values in chronic MS (p=0.016; Fig. 4B, Table 1). In contrast, OV+MS had enhanced FI (p<0.001). There was no difference between OV and OV+MS FI (p>0.05), but in chronic MS animals FI was significantly impaired compared to OV+MS (p<0.001; Table 1, Fig. 4B).

Histology

A significant positive relationship was found between capillary perfusion and FI (p=0.002), indicating that a reduction in perfusion after microsphere injection was associated with declining muscle performance (Fig. 5). No association existed between contralateral capillary perfusion and FI (r^2=0.038, p=0.591), indicating that potential microsphere spill-over to the contralateral limb was negligible. Acute microsphere injection resulted in significant dose-dependent increases in perfused capillary domain area (CDA) (p=0.006; Figure 5a) and heterogeneity of capillary spacing (log_RSD) (p=0.025). Compared to controls, overload caused ipsilateral muscle hypertrophy (ipsilateral/contralateral EDL mass) of 19% (p<0.001) while no change in muscle mass occurred in chronic MS (p=0.705; Table 2). Total capillary density (CD) was higher after overload than for control and chronic MS (p<0.01; Table 2). There was no difference in total (anatomical) CD between OV and OV+MS (p=0.969). An increased anatomical CDA was measured following chronic microsphere injections (p<0.01); in contrast, relatively large data variability resulted in no statistical difference for perfused CDA between groups (Table 2, Fig. 6). The heterogeneity of capillary spacing was unaffected by overload and chronic microsphere injection (p>0.05). No differences were found between groups in EDL fibre-type composition (Type I: p=0.935; Type IIa: p=0.821; Type IIb/IIx:

p=0.950) (Table 2). In contrast, overload caused an increased cross-sectional area of Type IIa and IIb/IIx fibres (Table 2).

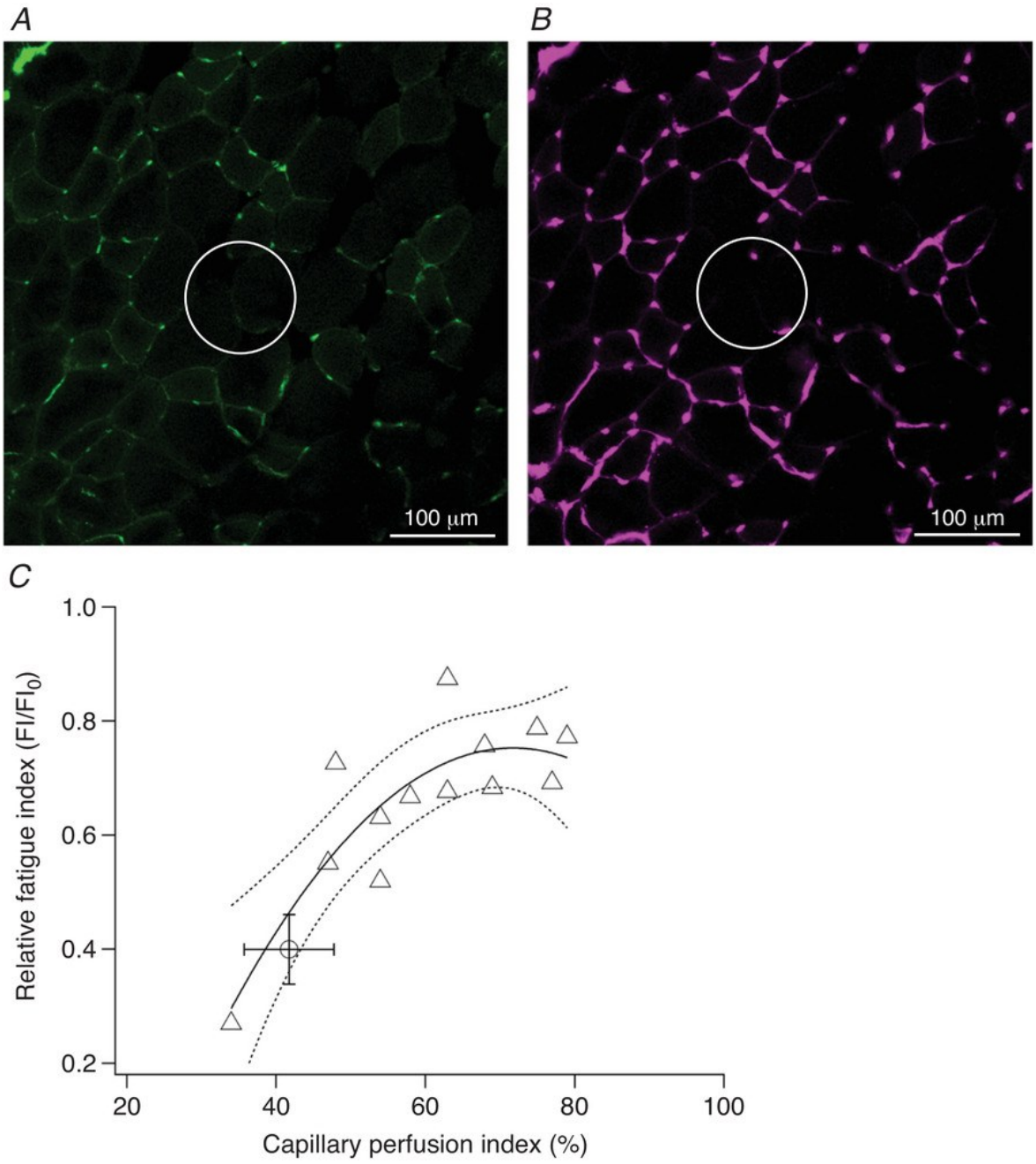


Figure 5. Dextran-FITC-labelled perfused (A) and total (B) capillary supply after moderate microsphere injection. Comparison of circled regions highlights functional microvascular rarefaction as capillaries remain unperfused after arteriolar blockade. There was a significant polynomial relationship (r^2=0.720, p=0.002) between ipsilateral capillary perfusion index (i.e. proportion of total capillaries that were perfused) and relative fatigue index (FI) in animals undergoing acute injection of microspheres (C). The effect of femoral ligation is also shown for comparison (open circle). Regression line and 95% confidence intervals are plotted.

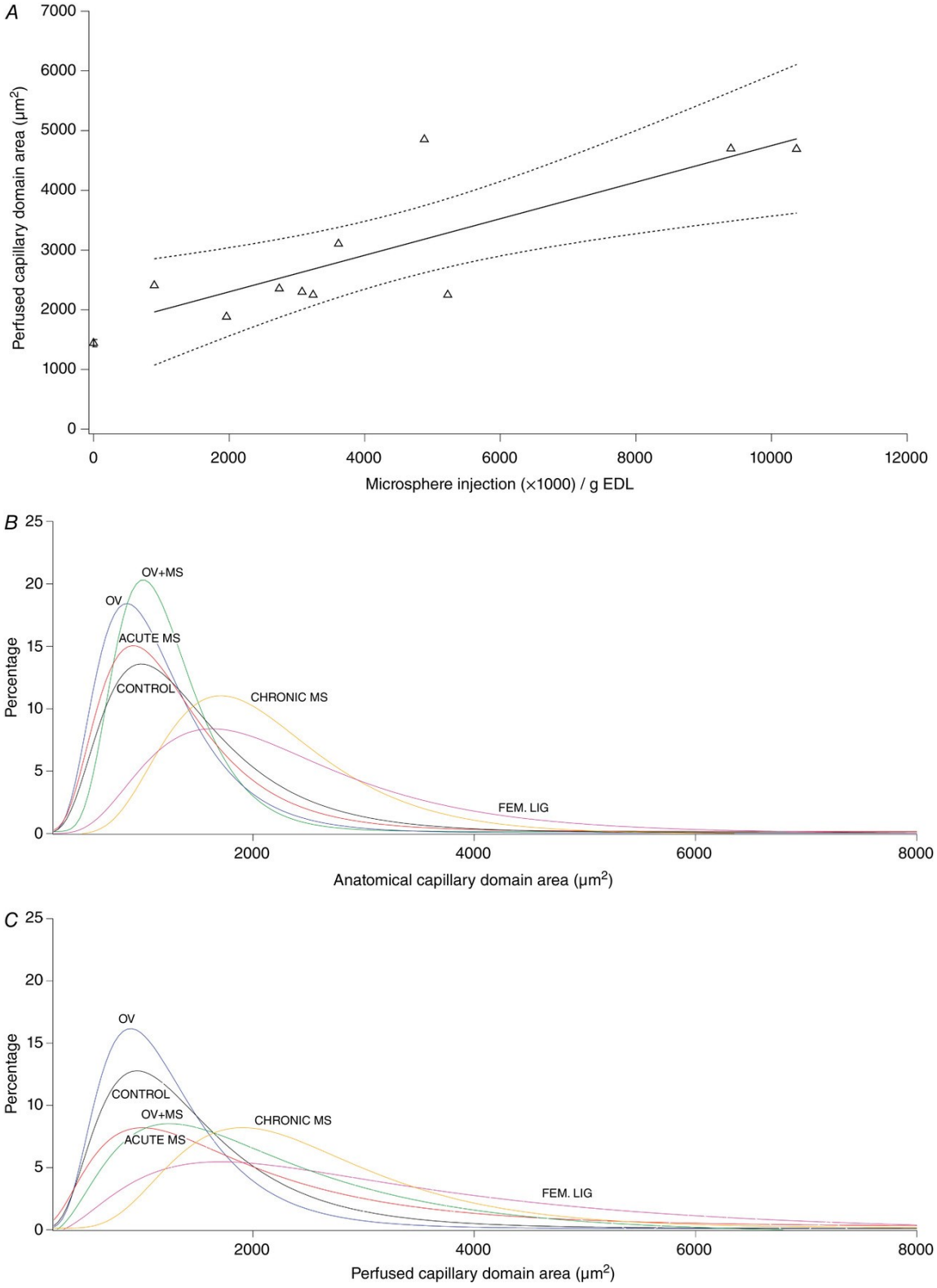


Figure 6. A , acute perturbation in capillary domain area (CDA) associated with microsphere injection. Mean domain area increases with arteriolar occlusion. Domain size increased (r^2=0.632, p=0.006) with increasing microsphere dosage and consequent arteriolar occlusion. Mean ± SEM control value is also shown (far left). B and C , lognormal frequency distribution of capillary domain area (exemplar data from each group). Capillary domain areas were calculated for each capillary in a

sample and each was binned into one of an incrementally increasing group, each with a range of 200 μm^2 (e.g. 201–400 μm^2, 401–600 μm^2 etc). The representation of each domain area size in the muscle was calculated as a percentage of total domains analysed. The effects of acute (medium dose of microspheres, equivalent to the chronic injections) and chronic distributed ischaemia and overload are displayed. Overload precipitates a narrowing of the total CDA curve indicating that domain sizes occur across a smaller range, which is consistent with an angiogenic response. Acute and chronic application of microspheres causes a downwards shift in the curve, indicating that there is a greater distribution of domain sizes as larger capillary domain areas are precipitated by arteriolar blockade. There was a rightwards shift in the curve for total and perfused CDA in chronic MS and femoral ligation animals, corresponding to an undesirable increased mean domain area and consequently a longer average diffusion distance to and from the capillary.

Table 2. *Morphometric characteristics of EDL*

	Control	**Chronic MS**	**OV+MS**	**OV**
EDL mass (mg per g M_b)	0.57 ± 0.05^{a}	0.59 ± 0.06^{a}	0.68 ± 0.06^{b}	0.67 ± 0.03^{b}
Bilateral EDL mass hypertrophy	1.02 ± 0.09^{a}	1.06 ± 0.010^{a}	1.19 ± 0.07^{b}	1.19 ± 0.06^{b}
Anatomical capillary density (mm^{2})	617 ± 61^{a}	556 ± 63^{a}	813 ± 114^{b}	836 ± 131^{b}
Perfused capillary density (mm^{2})	564 ± 107^{a}	420 ± 95^{b}	512 ± 48^{ab}	728 ± 165^{c}
Anatomical capillary: muscle fibre	1.47 ± 0.30^{ab}	1.29 ± 0.13^{a}	1.66 ± 0.19^{b}	1.63 ±0.22^{b}
Perfused capillary: muscle fibre	1.25 ± 0.29^{ab}	0.96 ± 0.19^{a}	0.92 ± 0.18^{a}	1.42 ± 0.31^{b}
Anatomical CDA (μm^{2})	1399 ± 156^{a}	1841 ± 204^{b}	1414 ± 158^{a}	1337 ± 155^{a}
Perfused CDA (μm^{2})	1854 ± 397^{a}	2506 ± 765^{a}	2274 ± 519^{a}	1759 ± 521^{a}
Anatomical $\log_{RSD}$	0.107 ± 0.008^{a}	0.096 ± 0.010^{a}	0.100 ± 0.012^{a}	0.104 ± 0.008^{a}
Perfused $\log_{RSD}$	0.127 ±0.016^{a}	0.109 ± 0.008^{a}	0.112 ± 0.019^{a}	0.112 ± 0.014^{a}
Type I (%)	3.8 ± 2.1^{a}	3.9 ± 2.2^{a}	3.3 ± 1.8^{a}	3.5 ± 2.4^{a}
Type 1 FCSA (μm^{2})	922 ± 217^{a}	1008 ± 138^{a}	1042 ± 180^{a}	1010 ± 173^{a}
Type IIa (%)	21.4 ± 5.0^{a}	19.9 ± 5.4^{a}	21.5 ± 4.2^{a}	23.2 ± 6.2^{a}
Type IIa FCSA (μm^{2})	933 ± 99^{a}	1012 ± 133^{ab}	1169 ± 120^{b}	1113 ± 120^{b}
Type IIb/IIx (%)	74.8 ± 6.9^{a}	76.2 ± 7.3^{a}	75.1 ± 4.2^{a}	73.3 ± 8.5^{a}
Type IIb/IIx FCSA (μm^{2})	1644 ± 189^{a}	2085 ± 310^{b}	2269 ± 182^{b}	2121 ± 223^{b}

Abbreviations: CDA, capillary domain area; FCSA, fibre cross-sectional area. Superscript letters denote statistical significance (p≤0.05) as assessed by Tukey post hoc tests.

DISCUSSION

The main observations of the present study are that (1) the extent of capillary rarefaction is correlated with muscle fatigue resistance, independent of changes to arterial blood flow, (2) capillary rarefaction did not attenuate the hypertrophic response to an overload stimulus, and (3) overload-induced angiogenesis alleviated the decline in muscle performance.

Effect of capillary rarefaction on muscle performance

Our novel approach provides insight into the effects of up to 70% capillary occlusion on muscle performance. This range encompasses the 32% reduction in the number of capillaries per muscle fibre reported in muscles of CHF patients (Duscha et al., 1999). The strong relationship between muscle fatigue resistance and functional (perfused) capillaries (Fig. 5C) is indicative of the critical function of the microcirculation, as also observed in cardiac muscle (Hauton et al., 2015). Our findings may be particularly relevant to the development of heart failure, where the magnitude of capillary rarefaction in skeletal muscle progressively increases with time after experimental induction of the condition (Nusz et al., 2003), and where the decline in fatigue resistance depends upon clinical severity (Buller et al., 1991). Considered together with the angiogenic-driven improvement in muscle fatigue resistance that follows long-term stimulation (Hudlicka et al., 1977) and mechanical overload (Tables 1 and 2, Fig. 4B) (Deveci and Egginton, 2002), there is strong evidence to indicate that maintaining adequate capillarisation is a component that determines optimal muscle function, and that rarefaction is a significant contributory factor to reductions in exercise tolerance in disease (Duscha et al., 1999, Nusz et al., 2003).

Our current understanding of the effects of peripheral disturbances in blood flow distribution on skeletal muscle performance, independent of confounding variables seen with co-morbidities, is largely based on the iliac/femoral artery ligation model (Lotfi et al., 2013), which induces a comprehensive restriction on hindlimb perfusion (Couffinhal et al., 1998, Hudlická et al., 2008). In this model, fatigue resistance of skeletal muscle is initially compromised due to the constraint on arterial flow and corresponding limitation on the supply of substrates to, and removal of heat and waste products from, active muscle tissue, with subsequent improvements coincident with development of collateral circulation (Couffinhal et al., 1998, Hudlická et al., 2008, Hellingman et al., 2010). However, this approach does not consider the more subtle effects of capillary rarefaction, which result in localised pockets of ischaemia (as indicated by increased capillary supply area while capillary distribution is unaffected), within muscle tissue, on skeletal muscle performance independent of blood flow, as may occur in heart failure patients (Nusz et al., 2003). Unbiased blockage of arterioles in the current study (Fig. 5) demonstrates that at all

but the lowest microsphere doses, acute reductions in functional capillary supply, which would be impossible to imitate using established ligation models (Lotfi et al., 2013), have a negative impact on skeletal muscle performance (Fig. 5A). We can thus simulate the early stages of microvascular disease in skeletal muscle, where limited changes in the microcirculation occur without affecting arterial perfusion, which may also become impaired with disease progression (LeJemtel et al., 1986). It is important to be cautious in extrapolating from our findings in the context of therapeutic intervention because the animals used in these experiments were young adults; diseases that affect the microcirculation including CHF and diabetes mellitus typically occur in more elderly humans. However, the basic relationships established would probably be enhanced by such co-morbidities. For example, in addition to microvascular rarefaction (Nusz et al., 2003), impaired capillary red blood cell flow (Kindig et al., 1999, Richardson et al., 2003), muscle wasting (Fülster et al., 2013), impaired functional hyperaemia (Sullivan et al., 1989) and reduced oxidative capacity (Bowen et al., 2015, Southern et al., 2015) all contribute to suboptimal muscle performance in disease. The data presented in this paper present the effects of microvascular rarefaction without such concomitant changes in healthy tissue and therefore provide insight into the role of the microcirculation for muscle function.

Interestingly, the relationship between FI and functional capillary density (Fig. 5) indicates that skeletal muscle is sensitive to small perturbations in the microcirculation, although to a lesser extent than cardiac muscle (Hauton et al., 2015), which has a correspondingly highly oxidative metabolic phenotype (De Sousa et al., 2002). This suggests that there is little functional reserve in the capillary bed, i.e. a small degree of rarefaction (c. 10%; Fig. 5C) can be observed without deleterious effects on muscle performance but there is a steep decline thereafter. Indeed, theoretical (Al-Shammari et al., 2014) models of oxygen transport within muscle and experimental studies of muscle structure (Degens et al., 2006b) have demonstrated that an optimal distribution of capillaries exists, and any disturbance to this pattern only decreases intramuscular PO_2 (Fig. 7). Increased capillary domain areas with distributed ischaemia may therefore impose a restriction on muscle performance as oxygen delivery becomes spatially limited (Figs 5 and 6). Consequently, compensatory mechanisms to ameliorate local ischaemia, such as microvascular dilatation to locally maximise flow (Klitzman et al., 1982), appear inadequate in maintaining muscle performance in otherwise healthy tissue.

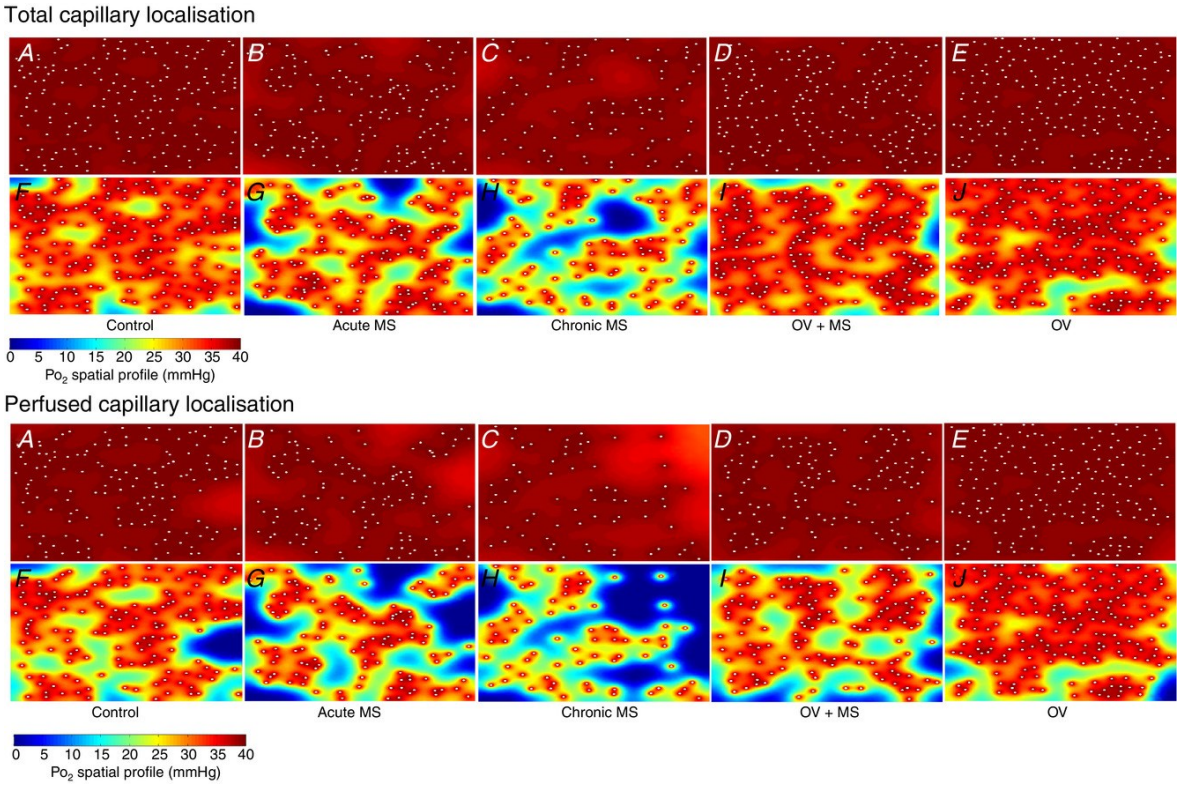


Figure 7. Simulation of muscle P O2 at rest (A–E) and maximal rate of oxygen consumption (F–J) in representative images. Acute effects (B and G) are seen after injection of 200,000 microspheres. Model assumptions are provided in Methods. Areas of muscle hypoxia (highlighted in blue, where PO_2 < 0.5 mmHg) during exertion are increased after microvascular blockade (G and H) and this profile corresponds to an attenuated fatigue resistance.

The EDL contains oxidative and glycolytic fibres (Table 2) (Pullen, 1977, Kissane et al., 2018) and localised ischaemia may particularly constrain oxidative metabolism in Type I and IIa fibres, contributing to increased fatigability. Unbiased blockade of arterioles is predicted to disrupt the spatial distribution (Fig. 6, Table 2) of perfused capillaries, as was indeed observed in the acute model, potentially contributing to declining muscle performance due to reduced tissue oxygenation (Greene et al., 1989, Degens et al., 2006b, Goldman et al., 2006, Al-Shammari et al., 2014). However, no statistical difference in heterogeneity of capillary spacing was found between chronic models, indicating that the unbiased arteriolar blockade resulted in a stochastic loss of functional capillaries. Increased total CDA after chronic microsphere injection is indicative of a greater diffusion distance across respiring muscle tissue and while the equivalent perfused CDA (Table 2) is not statistically different from other groups, the trend to an increased mean value suggests a developing biological significance with a limitation on oxygen delivery that is likely

to contribute to reduced fatigue resistance (Al-Shammari et al., 2014). Interestingly, no shift in fibre-type composition was detected after chronic arteriolar blockade or overload (Table 2), indicating that the observed reductions in fatigue resistance after microsphere injection were principally driven by microvascular disruption. In CHF, other factors such as a slow-to-fast shift in fibre type composition (Drexler et al., 1992) may compound the effects of capillary rarefaction, and together with fibre atrophy aggravate the capillary rarefaction-induced reductions in muscle fatigue resistance. Effective therapeutic interventions may thus lie in the development of exercise training protocols that prevent or reverse capillary rarefaction, as seen in animal models of hypertension (Amaral et al., 2000, Amaral et al., 2001, Melo et al., 2003, Fernandes et al., 2012) and in heart failure patients (Gustafsson et al., 2001, Eleuteri et al., 2013).

Based on our acute model (Fig. 4A), the moderate volume of microspheres injected in chronic experiments was expected to impose a deterioration in FI of ~30%. This impairment persists even 2 weeks after injection in chronic MS, indicating that little or no angiogenesis occurred to rectify the loss of functional capillaries and consequent loss of function (Tables 1 and 2, Fig. 4B). This contrasts with the upregulation of capillary growth during chronic systemic hypoxia (Deveci et al., 2001, Deveci and Egginton, 2002), suggesting that compensatory mechanisms to overcome microvascular constraint, even in otherwise healthy animals, are insufficient to recover function in the longer term. Note that contrasting results for exposure to chronic hypoxia have been reported (Sillau and Banchero, 1977, Snyder et al., 1985, Bigard et al., 1991), although methodological differences preclude direct comparison (see Deveci et al . 2001). However, persistently reduced FI in chronic MS is surprising as partial recovery occurs after extensive reduction in femoral flow following ligation (Hudlická et al., 2008). Arterial occlusion may therefore be a more potent stimulus for capillary proliferation than finer-scale distributed ischaemia, suggesting wide-scale tissue hypoxia stimulates a greater angiogenic response than local pockets of tissue hypoxia. Importantly, hypertrophic angiogenic remodelling of muscle was not diminished and was accompanied, as seen in other studies (Degens et al., 1993a, Egginton et al., 1998, Egginton et al., 2011, Zhou et al., 1998, Deveci and Egginton, 2002, Ballak et al., 2016), with enhanced resistance to fatigue after overload (Fig. 4B, Table 1), regardless of microvascular impediment. This indicates that an underlying capacity for microvascular regeneration can be harnessed given a mechanical stimulus of

sufficient intensity. A similar increase in capillarity occurs with overload after arterial ligation (Deveci and Egginton, 2002), highlighting the potency of blood flow-independent, mechanical stimuli for recovery of muscle capillarity and performance that could be harnessed in rehabilitation programmes.

Hindlimb perfusion and capillary rarefaction

In the acute microsphere model, resting and end-stimulation hindlimb blood flow were not adversely influenced by increasing arteriolar blockade (Fig. 3). In fact, resting blood flow was slightly elevated following administration of the highest dose, which may reflect a reactive hyperaemic response (Williams and Segal, 1993) to overcome local muscle ischaemia. Whatever the cause, the overall resting and contraction-induced muscle blood flow was not limited by arteriolar blockade in the short term, and the decline in muscle fatigue resistance was thus probably related to diffusion limitations, as indicated by increased capillary domain size (Table 2, Fig. 6), rather than insufficient perfusion via the feed artery blood flow. The similarity in FI after both the highest dose of microspheres and femoral artery ligation is therefore informative because, despite comparatively high blood flow in the microsphere model, muscle performance is impaired due to reduced functional capillary density (Fig. 5C). In contrast, reduced arterial blood flow delivery rather than microvascular deficit determines the reduced performance of muscle in the ligation model. Interestingly, the femoral artery blood flow waveform was affected by rarefaction, with increased resting flow amplitude and peak flow associated with cardiac systole in more severe microvascular rarefaction. These observations in part agree with a theoretical model that predicted elevated blood flows but a smoother waveform with vessel rarefaction (Olufsen et al., 2012). We speculate that as arteriolar blockade increases, a femoral blood flow response is stimulated in an attempt to alleviate the increased ischaemic areas within the muscle.

In the chronically ischaemic hindlimb, skeletal muscle fatigability is considerably increased despite the number of capillaries per muscle fibre remaining undisturbed (Hudlická et al., 2008), highlighting the damaging influence of long-term restricted blood flow. Thus, depressed exercise-induced limb blood flow in disease may exacerbate the effect of capillary rarefaction on fatigue resistance by imposing a further convective constraint of oxygen delivery and waste product removal (Wilson et al., 1984). Dysfunction of the microcirculation by development of non- and slow-flowing capillaries (Kindig et al., 1999, Richardson et al., 2003, Padilla et al., 2006)

that is apparent in heart failure and diabetes is expected to compound this rarefaction-derived constraint on performance by further reducing the functional capillary density.

The absence of a chronic effect on hyperaemia after microsphere administration (Table 1, Fig. 4B) indicates that no constraint was imposed upon the upregulation of femoral artery perfusion during stimulation, although a significantly lower end-stimulation flow rate was measured (Table 1), which parallels the attenuated blood flow measured during gradually induced peripheral ischaemia (Tang et al., 2005). The amplitude of blood flow did not differ between chronic groups (Table 1), mirroring the similar mean arterial pressures and indicating the minimal effect of capillary rarefaction on upstream haemodynamics. No effect on resting blood flow (normalised to muscle mass) was reported in earlier overload experiments (Egginton et al., 1998) so it is unclear why this parameter was reduced in our chronic models (Table 1), although factors such as ambient temperature (He et al., 2002) may have exerted an influence. Nevertheless, this serves to highlight that improvement in FI after overload is independent of increased blood flow, and is instead driven by mechanical stimuli acting on muscle fibres and their vascular supply (Egginton et al., 1998, Egginton et al., 2011, Deveci and Egginton, 2002). Enhanced functional hyperaemia relative to control occurred in OV (Table 1), while peak flow did not differ from control values (Table 1), in agreement with previous data (Egginton et al., 1998). OV+MS had a similar hyperaemic response and peak flow values to OV, and peak flow was increased relative to chronic MS (Table 1). Note that the functional hyperaemia reported herein is relatively low when compared to that observed in the EDL (hyperaemic scope: 5.7) during unanaesthetised maximal exercise (Armstrong and Laughlin, 1985), indicating that there remained a potential for further increases in flow. Nevertheless, improvements in the capacity for upregulating arterial flow, as well as microvascular density, were elicited by overload and overcame any potential limitations on mean flow associated with chronic microvascular rarefaction.

Computational models predict that the magnitude of vessel rarefaction determines the scale of increased vascular resistance (Greene et al., 1989) and reduced muscle blood flow (Heuslein et al., 2015). Placement of femoral artery flow probes distal to the profunda femoris enabled gross hindlimb blood flow to be quantified, but the proportion of flow drawn by EDL was not explicitly measured. Furthermore, up to 30% of capillaries remained perfused after acute ligation, indicating that collateral

artery blood flow prevented total ('no-flow') muscle ischaemia (thus avoiding profound hypoxia). Quantification of individual muscle blood flow (Degens et al., 1998, Deveci and Egginton, 1999) arteriolar blockade may improve our interpretation of haemodynamic effects by accounting for any change in EDL-specific perfusion, but no differential effects have previously been reported.

A cautionary note on peripheral microsphere injections

Bolus injections of saline and washed microspheres did not change FVC, i.e. there was no haemodynamic effect attributable to the administration of microspheres with our protocol. In contrast, administration of suspension medium alone produced a persistent increase in FVC, perhaps due to inclusion of thimerosal (0.01%), an organomercury preservative agent, and the surfactant Tween 80 (0.05%) in the manufacturer's microsphere preparation. Thimerosal induces vascular smooth muscle relaxation and vessel dilation (Förstermann et al., 1986, Rosenblum et al., 1992), while Tween 80 is associated with a biphasic response whereby arterial blood flow is reduced immediately after injection followed by a hyperaemia (Grund et al., 1995). That the microsphere suspension solution causes significant changes in localised blood flow in the rat is of interest due to the widespread use of microspheres to quantify organ- and tissue-specific blood flow (Prinzen and Bassingthwaighte, 2000). Spuriously high rates of blood flow will probably occur near the injection site after administration of diluted microsphere suspensions, confounding haemodynamic outcomes not representative of normal physiological conditions.

Future research

To determine if functional capillary density is closely coupled with fatigue resistance in human skeletal muscle, an acute study could use injections of increasing concentrations of dissolvable microspheres with a known half-life into the microcirculation of a muscle and a fatigue protocol used to measure muscle performance. Chronic studies of capillary rarefaction through microsphere injection in humans may not be possible due to the implications of prolonged ischemia. While the present study demonstrates the importance of functional capillarisation in muscle performance in a rat model, these findings may not be applicable without performing a similar acute study in humans.

Conclusions

Acute reductions in the number of functional (perfused) capillaries reduced skeletal muscle fatigue resistance, in the absence of changes in blood flow and perfusion pressure. This suggests that peripheral capillary rarefaction per se caused a decline in skeletal muscle endurance. Chronic mechanical overload of muscle with a reduced number of functional capillaries successfully recovered fatigue resistance, indicating that the underlying remodelling capacity of muscle can be harnessed to overcome microvascular constraint. A parallel exists between the observations reported here and the increased muscle fatigability observed in patients with CHF, chronic obstructive pulmonary disease and diabetes, supporting the hypothesis that impaired performance of skeletal muscle is in part due to capillary rarefaction, and that the magnitude of rarefaction is a significant aspect of overall muscle performance. These data suggest that significant therapeutic benefits may be accorded to such patients by restoring skeletal muscle microvasculature, by means of flow-independent angiotherapy to enhance muscle fatigue resistance, ameliorate disease prognosis and improve quality of life.

Prelude to Chapter 3

In chapter 2 it was determined that reductions in functional capillary density with microsphere injection led to impaired fatigue resistance, both acutely and chronically. It was also shown that reductions in fatigue resistance due to chronic loss of functional capillaries can be restored through the use of an angiogenic stimulus. As stated in the introduction, one condition characterised by skeletal muscle capillary rarefaction is chronic heart failure. Having determined that fatigue resistance is closely linked to functional capillary density I aimed to see if angiogenic stimuli could prevent or rescue the reductions in fatigue resistance associated found with chronic heart failure.

Chapter 3

IMPAIRED SKELETAL MUSCLE FATIGUE RESISTANCE FOLLOWING COMPENSATORY CARDIAC HYPERTROPHY IS RECOVERED BY FUNCTIONAL OVERLOAD- OR AEROBIC EXERCISE-INDUCED ANGIOGENESIS

ABSTRACT

Microvascular rarefaction may contribute to declining skeletal muscle performance in cardiac and vascular diseases. It remains uncertain to what extent microvascular rarefaction occurs in the earliest stages of these conditions, if impaired blood flow is an aggravating factor and whether angiogenesis restores muscle performance. To investigate this the effects of aerobic exercise and functional muscle overload on the performance, femoral blood flow (FBF) and microvascular perfusion of the extensor digitorum longus (EDL) were determined in a chronic rat model of compensatory cardiac hypertrophy (CCH). CCH was induced with surgically-imposed abdominal-aortic coarctation. CCH was associated with hypertension (157 mmHg ± 25 vs. 122 ± 3 in control, $p<0.05$) and increased relative heart mass (0.38 ± 0.04 vs. 0.27 ± 0.04 in control, $p<0.05$). Reduced FBF at end-stimulation (0.96 mL min^{-1} ± 0.19 vs. 1.74 mL min^{-1} ± 0.23 in control $p<0.05$) coincided with attenuated fatigue resistance (0.27 ± 0.10 vs. 0.49 ± 0.06 in control, $p=0.039$) immediately after aortic banding, indicating an arterial perfusion constraint on muscle performance. Reduced functional capillary density (259 mm^2 ± 24 vs. 570 mm^2 ± 94 in control, <0.05) and fatigue resistance (0.37 ± 0.05 vs. 0.49 ± 0.06, $p<0.05$) occurred 4 weeks after aortic banding, indicating a microvascular limitation to muscle performance. Although overload-induced EDL hypertrophy was attenuated after aortic banding (12% vs. 23% in unbanded animals, $p=0.027$), the fatigue resistance was similar to controls after exercise and overload (both $p>0.999$) and associated with the prevention of a reduction in functional capillarity in the EDL of rats with CCH ($p<0.05$). These data show that reductions in skeletal muscle performance during cardiac hypertrophy can be countered by pro-angiogenic interventions, providing a future therapeutic target to improve the function of skeletal muscle in clinical disease.

INTRODUCTION

Persistent exercise intolerance is a hallmark of chronic heart failure (CHF), compromising quality of life and contributing to a poor clinical prognosis. Skeletal muscle performance is, however, poorly correlated with left ventricular ejection fraction in CHF (Rogers, 2001, Franciosa et al., 1981), suggesting that peripheral factors determine the severity of exercise intolerance (Pandey et al., 2015). Further pathological changes in CHF contributing to impaired exercise performance include sarcopenia and a slow-to-fast shift in muscle fibre-type composition (Drexler et al., 1992), but there is growing evidence for constraints imposed by structural and functional changes in the microcirculation in the muscle (Tickle et al., 2020, Duscha et al., 1999, Richardson et al., 2003). Reduced skeletal muscle microvascular density occurs in both experimentally induced (Bowen et al., 2017, Nusz et al., 2003, Richardson et al., 2003, Kindig et al., 1999) and clinical (Duscha et al., 1999, Schaufelberger et al., 1995, Wadowski et al., 2018) CHF, and this rarefaction imposes a limit on the transfer of oxygen to respiring muscle tissue thereby contributing to the rapid onset of fatigue (Hauton et al., 2015, Tickle et al., 2020). Peripheral muscle capillary rarefaction after imposition of cardiac dysfunction indicates that systemic effects may occur rapidly (< 21 days) after development of perturbed heart function (Nusz et al., 2003). Coupled to the deleterious effects of rarefaction, a disturbed anatomical distribution of capillaries may also compromise muscle function because increased heterogeneity of capillary spacing can reduce tissue oxygenation (Degens et al., 2006b, Piiper and Scheid, 1991). Furthermore, animal models of CHF demonstrate reduced red blood cell velocity and flux (Richardson et al., 2003) in addition to an increased proportion of capillaries with intermittent RBC flux at rest and during contractile activity (Kindig et al., 1999). The consequent increase in transit time potentially explains the higher oxygen extraction observed in CHF (Katz et al., 2000), but also suggests that muscle oxygenation is impaired leading to suboptimal mitochondrial function (Behnke et al., 2007). Improving muscle and exercise performance in CHF may therefore depend upon restoration of optimal topology of the microcirculation. However, it remains unknown whether capillary distribution and/or capillary perfusion is changed during CHF and whether these putative changes affect skeletal muscle oxygenation, performance and capacity for adaptative remodelling.

Capillary proliferation from pre-existing vessels (angiogenesis) in skeletal muscle is an essential adaptation to match tissue performance to changing functional demands (Brodal et al., 1977, Zumstein et al., 1983) that is triggered by both physical and metabolic stimuli (Hudlicka, 1991). For example, the increased longitudinal strain on the abluminal capillary surface (Egginton et al., 2001) during overload not only results in hypertrophy (Deveci and Egginton, 2002, Tickle et al., 2020, Zhou et al., 1998) and enhanced fatigue resistance, but also induces angiogenesis (Ballak et al., 2016, Egginton et al., 1998, Tickle et al., 2020, Frischknecht and Vrbova, 1991, Egginton et al., 2011). The significance of angiogenesis for function is illustrated by the observation that even after experimentally-induced capillary rarefaction, overload-mediated angiogenesis can recover function and capillarisation in otherwise healthy muscle (Tickle et al., 2020).

In addition to the pro-angiogenic effects of longitudinal strain, capillary proliferation can be induced by enhanced vascular shear stress *via* application of a hyperaemic stimulus such as occurs during chronic electrical stimulation of muscle (Hudlicka et al., 1977, Egginton and Hudlicka, 1999), or vasodilator administration (Egginton et al., 2016, Mandel et al., 2016). While the hyperaemic stimulus undoubtedly contributes to the expansion of the microvascular bed (Waters et al., 2004, Olesen et al., 2010) with prolonged aerobic exercise training (Andersen and Henriksson, 1977), the exercise-induced angiogenesis is also mediated by metabolic (e.g. hypoxia, glucose metabolism) factors (Olfert et al., 2016).

In clinical (Esposito et al., 2018, Gustafsson et al., 2001, Esposito et al., 2010a) and experimental (Ranjbar et al., 2017) CHF, enhanced capillarity and angiogenic signalling (e.g. *via* vascular endothelial growth factor, VEGF) are found after exercise therapy indicating that the capacity for remodelling is still present 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 hypothesised to drive repair of endothelial damage rather than expanding microvascular density *per se* (Chong et al., 2004). Reduced VEGF expression has also been reported , indicating regulatory dysfunction and the potential of multiple factors (e.g. severity and specific type of CHF), to negatively affect capillary proliferation in muscle tissue (Arakawa et al., 2003). 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 principal objective of this paper was therefore to quantify the functional and structural effects of angiogenic stimuli on skeletal muscle during compensatory cardiac hypertrophy (CCH), a model of incipient cardiac dysfunction. We tested in this rat model the following specific hypotheses: 1) CCH has a deleterious effect on skeletal muscle microcirculation and fatigue resistance. 2) Exercise has a preventative effect on the adverse effects of CCH on skeletal muscle. 3) Overload has a restorative angiogenic effect on impaired skeletal muscle capillarisation and fatigue resistance in rats with CCH. By quantifying the effects of cardiac dysfunction on skeletal muscle function and microcirculation, we provide new data on the microvascular bed as a potential therapeutic target for skeletal muscle dysfunction in chronic diseases such as CHF.

METHODS

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. Ethical approval was also provided by Manchester Metropolitan University (SE171810).

Aortic constriction and EDL overload

Compensatory cardiac hypertrophy was induced *via* abdominal aortic constriction. Groups of aortic banded animals were allocated as follows: i) banded control (Aob: 304 ± 6g, n=7); ii) unilateral overload of the EDL (Aob+OV: 376 ± 6g, n=7); iii) voluntary wheel running (Aob+EX: 305 ± 12g, n=7); iv) acute banded, in which the immediate effects of banding were quantified (Acute-Aob: 245 ± 40, n=6). Corresponding non-banded groups were also used: i) intact (Control: 257 ± 8g; n=7); ii) overload (OV: 343 ± 9g, n=7); iii) wheel running (EX: 316 ± 41g, n=8). Only EX and Aob+EX had access to a cage wheel, all other groups were sedentary. Figure 1 outlines the study design.

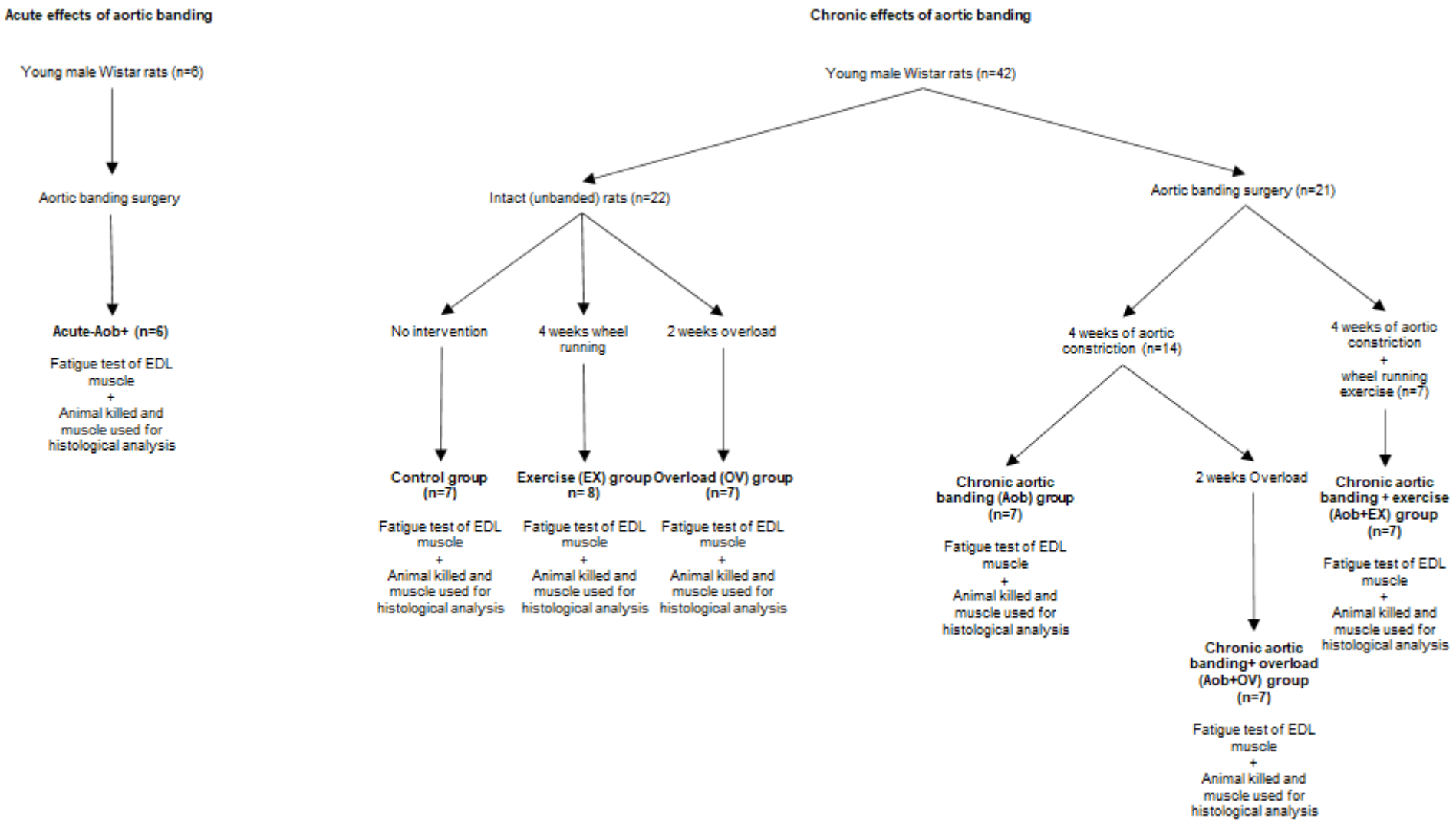


Figure 1 study design

Under isoflurane anaesthesia (induction 4%, maintenance 2.5%) male Wistar rats underwent surgery to constrict the abdominal aorta immediately cranial to the renal artery bifurcations using a titanium clip (post-mortem measured diameter: 0.37 ± 0.07mm Ligaclip®; Ethicon Endo-Surgery Inc., Cincinnati, OH, USA) (Cornelussen et al., 1994, Degens et al., 2006a, Levy et al., 1996). Aob and Aob+EX were otherwise intact. Unilateral mechanical overload of the extensor digitorum longus (EDL) (Zhou et al., 1998)(Zhou et al 1998, Deveci and Egginton 2002; Tickle et al. 2020) was performed in Aob+OV 4 weeks after clip placement. In this procedure, extirpation of the tibialis anterior induces stretch and functional overload of the EDL. Post-operative analgesia (buprenorphine (Vetergesic®, Ceva, Amersham, UK) 0.05 mg kg^{-1}) and antibiotic (Enrofloxacin (Baytril®, Bayer, Reading, UK) 2.5 mg kg^{-1}) were provided after all surgical procedures. Muscle fatigue resistance and hindlimb perfusion in Aob and Aob+EX was quantified 4 weeks after aortic constriction, and in Aob+OV after an additional 2 weeks.

Wheel running

EX and Aob+EX animals were housed in a cage with *ad libitum* access to an instrumented running wheel that enabled real-time monitoring of night-time exercise performance (distance moved, exercise duration and running speed) *via* bespoke LabView software ('Rodent voluntary exercise analysis system' developed in conjunction with University of Manchester, UK). To ensure access to the running wheel was the same for each animal and exclude erroneous data due to disturbance, all cage wheels were locked during weekly cage cleaning and welfare checks.

Fatigue resistance and hindlimb perfusion

Protocols for measuring muscle fatigue resistance and arterial blood flow are 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 (30-35 $mg \cdot kg \cdot hr^{-1}$) delivered *via* a catheter in the external jugular vein. 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 at optimal length was measured by linking each muscle to a lever arm force transducer (305B-LR: Aurora Scientific, Aurora, ON, Canada) and providing supramaximal electrical stimulation *via* the popliteal nerve

(Hudlicka et al., 1977). Unimpeded access to the EDL was facilitated by extirpation of the overlying and synergist tibialis anterior (TA). To determine EDL fatigue resistance, a 30-s period of 1-Hz twitches, to activate the metabolic machinery, was followed by 10-Hz impulses (0.3 ms pulse width, supramaximal voltage) for 180 s (Egginton and Hudlicka, 1999, Tickle et al., 2020). A fatigue index (FI) was calculated as muscle force at the end of the test divided by peak muscle force at the beginning of the test. A mean of five consecutive twitches was selected to represent end stimulation and peak force data.

The femoral artery blood flow (FBF) was measured bilaterally throughout each experiment with perivascular flow probes (0.7PSB; Transonic, Ithaca, NY, USA) at the proximal aspect of the *profunda femoris* arterial bifurcation (Tickle et al., 2020). The muscle-mass-specific increase in flow, i.e. hyperaemic increment above resting, was calculated to account for potential differences in EDL mass and normalised to femoral vascular conductance using arterial pressure measured in the tail.

Following the conclusion of each experiment, capillary perfusion was visualised by an injection of fluorescein isothiocyanate (FITC) – labelled dextran solution (50 mg kg^{-1}), MW = 500 000; Sigma, Poole, UK) *via* the carotid artery (Tickle et al., 2020). Before and after injection, the EDL in both legs were stimulated at 4 Hz for 30 s to induce functional hyperaemia and capillary recruitment. EDL were left *in situ* for a minimum of 1 min after injection to maximise dye localisation in perfused capillaries (Snyder et al., 1985) after which animals were killed by a Schedule 1 method.

Histology

Immediately after animals were euthanised, the EDL muscles were removed, blotted dry and weighed. The muscle was then frozen on cork with OCT embedding medium (Thermo Scientific, Loughborough, UK) using liquid-nitrogen-cooled isopentane and stored at -80°C. Cryostat sections (10 µm) were prepared and labelled with biotinylated *Griffonia simplicifolia* lectin I (5 $\mu L \cdot mL^{-1}$ FL-1101 GSL I; Vector Labs, Peterborough, UK) and streptavidin Alexa Fluor 350 conjugate. For fibre typing, serial sections were blocked in 10% goat serum in PBS for 60 minutes (Vector Laboratories, USA) and then incubated for 120 minutes in monoclonal antibodies BAD-5 (1:600), SC-71 (1:600), 6H1 (1:50) on one slide and BF-F3 (1:100) on another in blocking solution for fibre types I, IIa, IIx and IIb, respectively (Developmental Studies Hybridoma Bank, USA). After three 5-minute washes in

phosphate-buffered saline (PBS) the sections were incubated in the dark with secondary antibodies Alexa Fluor IgG2b for type I (1:500), Alexa Fluor IgG1 for type IIa (1:500) and Alexa Fluor 555 IgM for types IIb and IIx (1:500) (Thermofisher Scientific, USA) in blocking solution. After three further 5 min washes the slides were mounted with ProLong Diamond Antifade mountant (Thermofisher Scientific, USA). Whole-muscle cross-sectional images were acquired with a confocal microscope (Leica TCS SP5) at x20 magnification. Subsequent image analysis with BTablet and Anatis (BaLoH Software, Ooij, The Netherlands) enabled determination of a capillary perfusion index (perfused/unperfused capillaries). To account for the regional heterogeneity of capillary distribution and fibre type composition (Kissane et al., 2018) three regions of interest (475 x 475 μm^2) of the whole muscle image were used to establish an unbiased counting frame with which to quantify muscle fibre type composition (numerical proportion of fibre types) and the fibre cross-sectional area, FCSA), capillarisation (expressed as capillary to fibre ratio (C:F) and capillary density (CD)) and capillary domain area (CDA). The CDA is defined as the area surrounding a capillary delineated by equidistant boundaries from adjacent capillaries and is an index of the area of tissue to which a capillary may supply oxygen to working muscle (Al-Shammari et al., 2014, Hoofd et al., 1985). The heterogeneity of capillary distribution is provided by Log_RSD. Sample sizes for histological parameters were lower than for the functional measurements in EX (n=5) and OV (n=5) due to tissue damage.

Statistical Analyses

Differences in FI, FBF and histological parameters between groups shown in figure 1 were quantified 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 and Bengele, 1973). Statistical testing was conducted in SPSS (v.25). Data are presented throughout as mean ± SD and differences or effects were considered significant where $p<0.05$.

RESULTS

Cardiovascular effects of aortic banding

Acute-Aob animals had an intermediate carotid artery blood pressure that did not significantly differ from any group. Tail blood pressure was lower in Acute-Aob

animals ($p=0.001$) and was higher in EX ($p=0.039$) and Aob+EX ($p=0.016$) compared to Control (Table 1). Aortic banding acutely resulted in a greater carotid:tail blood pressure ratio ($p<0.001$). Carotid blood pressure was higher after chronic banding compared to all unbanded groups (Table 1; $p<0.05$). There were no significant differences in heart rate between groups.

Aortic banding induced significant cardiac hypertrophy ($p<0.001$), irrespective of being combined with exercise or overload. The heart to body mass ratio was higher in EX than Control and OV animals ($p<0.05$). Exercise resulted in cardiac hypertrophy in the EX animals only (compared to Control: $p<0.001$) and aortic banding resulted in cardiac enlargement ($p=0.005$) irrespective of overload and exercise. The exercise induced hypertrophy was less than the aortic banded hypertrophy ($p=0.005$).

Table 1. *Cardiovascular response to aortic banding. Unshared letters denote statistical significance (p<0.05) as determined by ANOVA with post-hoc tests.*

	Control	**EX**	**OV**	**Acute-Aob**	**Aob**	**Aob + EX**	**Aob + OV**
M_b (g)	254 ± 44 ^{ab}	316 ± 41 ^{c}	343 ± 23 ^{cd}	245 ± 40 ^{a}	304 ± 17 ^{bc}	305 ± 33 ^{bc}	375 ± 17 ^{d}
Heart mass (% M_b)	0.27 ± 0.04 ^{a}	0.32 ± 0.04 ^{b}	0.26 ± 0.04 ^{a}	-	0.38 ± 0.04 ^{c}	0.35 ± 0.04 ^{bc}	0.35 ± 0.04 ^{bc}
BP (carotid) (mmHg)	122 ± 3 ^{a}	124 ± 13 ^{a}	123 ± 8 ^{a}	139 ± 12 ^{ab}	157 ± 25 ^{b}	148 ± 14 ^{b}	147 ± 11 ^{b}
BP (tail) (mmHg)	92 ± 7 ^{ab}	115 ± 11 ^{c}	92± 13 ^{a}	58 ± 16 ^{d}	115 ± 21 ^{bc}	119 ± 12 ^{c}	96 ± 15 ^{abc}
carotid:tail BP	1.33 ± 0.13 ^{a}	1.08 ± 0.11 ^{a}	1.36 ± 0.22 ^{a}	2.54 ± 0.73 ^{b}	1.39 ± 0.22 ^{a}	1.25 ± 0.14 ^{a}	1.55 ± 0.23 ^{a}
HR (bpm)	394 ± 43 ^{a}	383 ± 53 ^{a}	347 ± 48 ^{a}	356 ± 68 ^{a}	359 ± 59 ^{a}	400 ± 43 ^{a}	397 ± 31 ^{a}

M_b: Body mass

Wheel running

The velocity and duration of each exercise bout, and therefore total distance travelled were lower in Aob+EX than EX rats ($p<0.05$), but motivation to exercise (number of bouts) was otherwise undiminished after band application, indicating a constraint on exercise stamina imposed by CCH (Fig. 2).

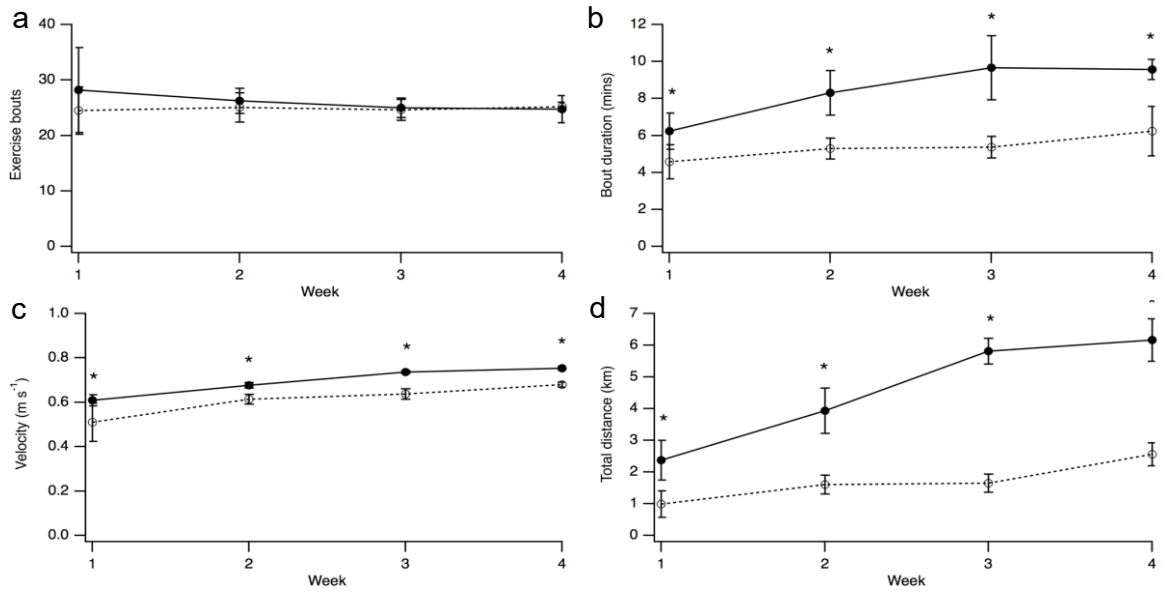


*Figure 2. Impaired voluntary wheel running exercise performance after aortic banding. While inclination to exercise was undiminished (a), bout duration (b), velocity (c) and total distance travelled (d) were reduced in banded animals (dashed line) compared to exercise controls (solid line). *: statistical significance ($p<0.05$).*

Muscle performance

Fatigue index (FI) immediately after band application (Acute-Aob) was less compared to Control ($p<0.001$) and remained lower 4 weeks after aortic banding ($p=0.039$ Fig. 3). FI was higher in Aob+EX and Aob+OV than Aob ($p<0.05$) and comparable to Control in Aob+EX ($p>0.05$). In unbanded animals however, only overload ($p=0.036$) but not exercise ($p=0.954$) improved FI.

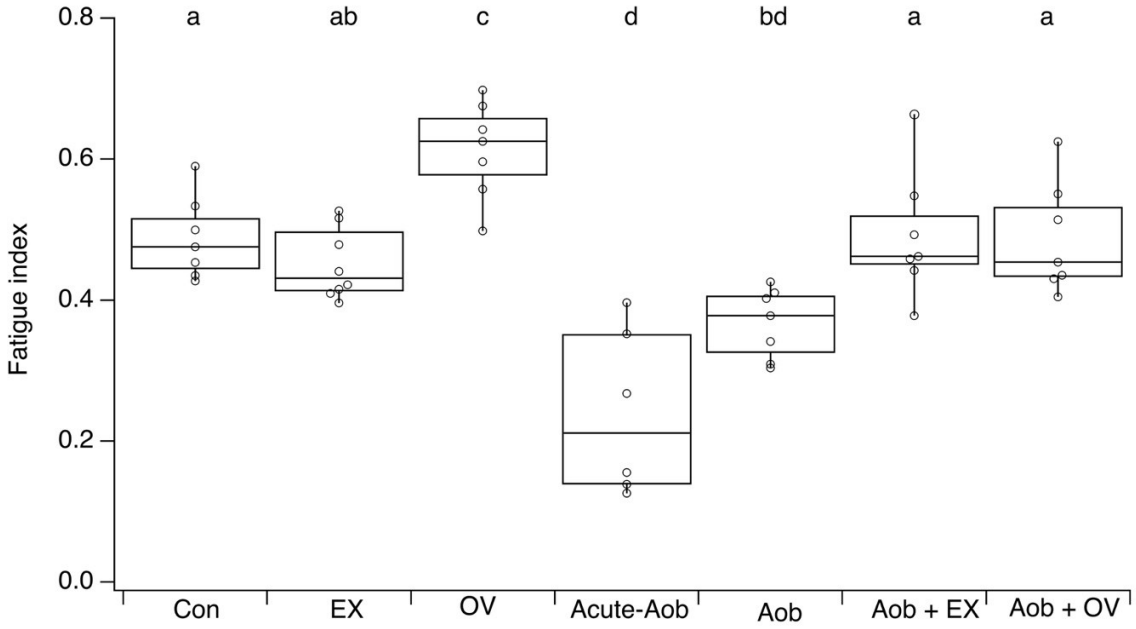


Figure 3. Impaired muscle fatigue resistance during compensatory cardiac hypertrophy was recovered after exercise or overload. Fatigue index (FI) was reduced immediately after band application and remained lower for 4 weeks. Exercise (Aob+EX: P = 0.029) and overload (Aob+OV: p=0.039) recovered fatigue resistance to control levels (p>0.05) in rats with aortic banding. Superscript letters denote significance(p<0.05).

Femoral artery perfusion

Resting femoral artery flow (mL min^{-1}) was lower in Acute-Aob compared to all other groups ($p<0.05$, Fig 4a). End-stimulation FBF was lower in Acute-Aob than EX, Aob+EX and Aob+OV groups ($p<0.05$), but not significantly different from Control, OV and Aob (Fig. 4b; Table 3). Hyperaemic scope was similar in all groups. The magnitude of hyperaemic flow per gram of EDL did not differ significantly between groups, even when accounting for femoral vascular conductance per g muscle tissue (FVC) (Table 3).

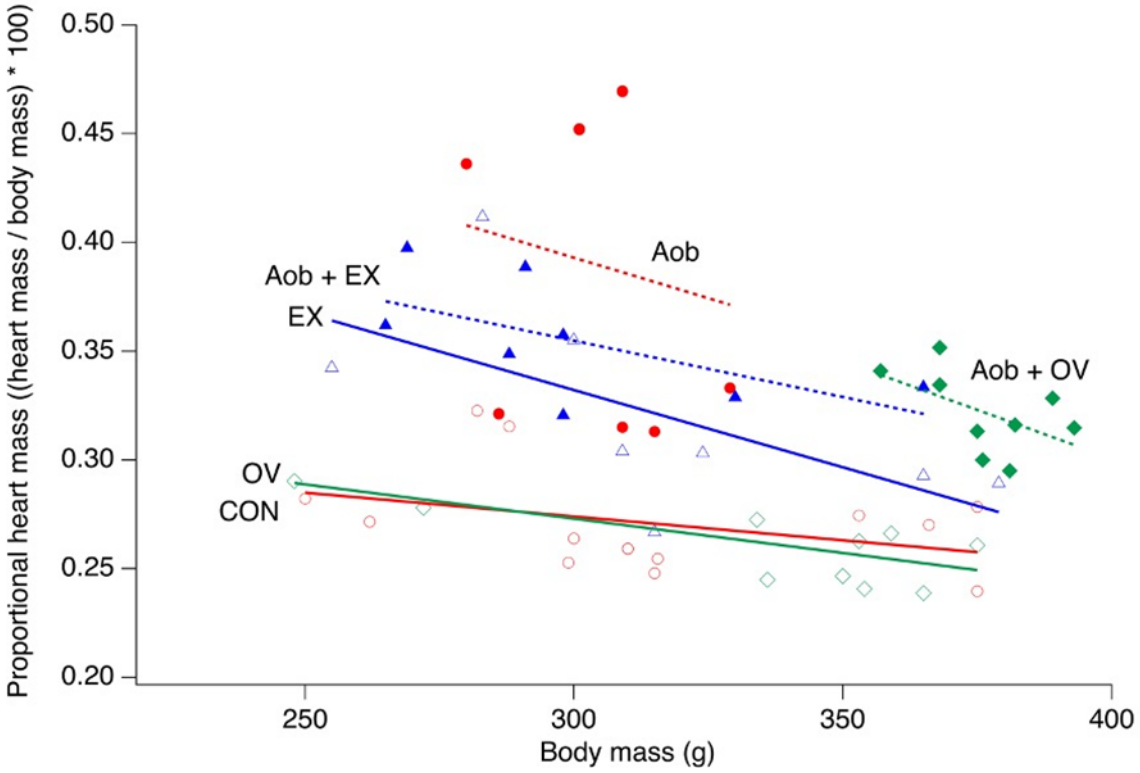


Figure 4: Exercise and aortic banding precipitate cardiac mass gain.

After controlling for the effect of body mass on heart mass using ANCOVA, there remained a significant effect of treatment: Control and OV heart mass was lower than in all other groups (P<0.05); cardiac enlargement occurred in EX (compared to Con: P = 0.045), that did not differ in magnitude from the compensatory hypertrophy in Aob + EX (P=0.774) and Aob + OV (P>0.999), although the magnitude of enlargement was smaller than in Aob (P=0.005); there was no difference between banded groups' heart mass (P>0.05). There was no difference in the regression slopes between groups (P=0.885), indicating that a common scaling relationship existed with body mass regardless of treatment. Control (red open circles, n=14); EX (blue open triangles, n=8); OV (green open diamonds, n=11); Aob (red filled circles, n=11); Aob + EX (blue filled triangles, n=8); Aob + OV (green filled diamonds, n=9). Group sample sizes are larger than for measurements of muscle performance and blood flow because not all in situ experiments were successful.

Table 3. *EDL isometric twitch performance and femoral artery blood flow. Unshared letters denote statistical significance ($p<0.05$) as determined by ANOVA with Tukey post-hoc tests.*

		Control	EX	OV	Acute-Aob	Aob	Aob + EX	Aob + OV
Fatigue index		0.49 ± 0.06 [a]	0.45 ± 0.05 [ab]	0.61 ± 0.07 [c]	0.27 ± 0.10 [d]	0.37 ± 0.05 [bd]	0.49 ± 0.09 [a]	0.49 ± 0.08 [a]
Maximum twitch tension (N)		0.32 ± 0.09 [a]	0.35 ± 0.17 [a]	0.40 ± 0.18 [ab]	0.26 ± 0.09 [a]	0.31 ± 0.03 [a]	0.29 ± 0.06 [a]	0.54 ± 0.11- [b]
Maximum twitch tension per g EDL (N g^{-1})		2.25 ± 0.58 [a]	1.80 ± 0.82 [a]	1.85 ± 0.86 [a]	2.17 ± 0.89 [a]	1.83 ± 0.28 [a]	1.71 ± 0.21 [a]	2.32 ± 0.59 [a]
Rise time 25-75% (ms)		7.2 ± 0.5 [a]	6.7 ± 0.8 [a]	7.1 ± 0.8 [a]	6.8 ± 0.9 [a]	6.5 ± 1.0 [a]	6.7 ± 1.1 [a]	5.8 ± 0.9 [a]
Fall time 75-25% (ms)		19.4 ± 2.9[ab]	16.0 ± 5.0 [a]	23.7 ± 6.9 [b]	21.2 ± 3.1 [ab]	14.8 ± 4.5 [a]	17.3 ± 2.9 [ab]	14.8 ± 3.0 [a]
Femoral flow ($mL\ min^{-1}$)	rest	1.09 ± 0.22 [a]	1.32 ± 0.39 [a]	0.97 ± 0.19 [a]	0.48 ± 0.21 [b]	1.23 ± 0.19 [a]	1.39 ± 0.32 [a]	1.27 ± 0.29 [a]
	end-stimulation	1.74 ± 0.23 [ab]	2.07 ± 0.81 [a]	1.92 ± 0.95 [a]	0.96 ± 0.48 [b]	1.80 ± 0.46 [ab]	2.13 ± 0.42 [a]	2.04 ± 0.50 [a]
Hyperaemic scope (end-stimulation / rest)		1.62 ± 0.25 [a]	1.61 ± 0.61 [a]	1.96 ± 0.76 [a]	1.95 + 0.59 [a]	1.48 + 0.34 [a]	1.60 ± 0.49 [a]	1.62 ± 0.31 [a]
Hyperaemic increase per g EDL ($mL\ min^{-1}\ g^{-1}$)		4.28 ± 1.47 [a]	3.75 ± 3.62 [a]	4.59 ± 4.09 [a]	4.06 ± 2.65 [a]	3.28 ± 2.42 [a]	4.31 ± 1.82 [a]	3.34 ± 1.76 [a]
Hyperaemic increase FVC ($mL\ min^{-1}\ mmHg^{-1}\ g^{-1}$)		0.050 ± 0.018 [a]	0.039 ± 0.035 [a]	0.047 ± 0.045 [a]	0.063 ± 0.04 [a]	0.035 ± 0.033 [a]	0.048 ± 0.030 [a]	0.048 ± 0.015 [a]

Muscle mass and histology

EDL mass as a proportion of body mass was increased in OV compared to Control, Aob and Aob+EX ($p<0.05$), but was similar in EX and Aob+OV ($p>0.05$) (Table 4). The magnitude of muscle hypertrophy after overload was, however, greater in OV compared to Aob+OV ($p=0.027$; Table 4).

Fibre type composition did not differ from Control with any intervention. Type I, IIa and IIb/IIx FCSA were, however, larger in Aob+OV when compared to Control *(*$p<0.05$), while IIb/x fibres were larger than Control in EX, Aob, and Aob+OV (Table 4; $p<0.05$). Overload did not increase FCSA in unbanded animals relative to Control and did not increase in Aob+OV relative to Aob.

While anatomical C:F was not different to Control in any group, it was greater in Aob+OV compared to Aob ($p=0.028$) and Aob+EX ($p=0.002$). Perfused C:F was reduced in Aob ($p=0.009$), while Aob+EX and Aob+OV were not significantly different from the Control (Table 4; $p<0.05$).

Anatomical CD did not differ between Control and any aortic banded animals ($p>0.05$). OV had an increased anatomical CD compared with Con ($p=0.029$), whereas EX demonstrated a similar anatomical CD to Control. Development of CCH in Aob, Aob+EX and Aob+OV precipitated a significant reduction in perfused CD ($p<0.05$) compared to non-banded groups (Table 4). Perfused CD in OV and EX did not differ significantly from Control.

The perfused CDA was greater in Aob of compared to all other groups ($p<0.001$), but in the Aob+EX and Aob+OV the perfused CDA was indistinguishable from unbanded groups (Table 4; $p>0.05$).

Neither anatomical Log_{RSD} nor perfused Log_{RSD} were significantly different from Control in any group.

Table 4. *Microcirculatory and muscle fibre morphometric characteristics. Unshared letters denote statistical significance (P<0.05) as determined by ANOVA with Tukey post-hoc tests.*

	Control	EX	OV	Aob	Aob + EX	Aob + OV
EDL mass (mg per g M_b)	0.56 ± 0.06 ^{a}	0.61 ± 0.05 ^{ab}	0.66 ± 0.07 ^{b}	0.56 ± 0.06 ^{a}	0.56 ± 0.06 ^{a}	0.63 ± 0.05 ^{ab}
Bilateral EDL mass hypertrophy	-	-	1.23 ± 0.11 ^{a}	-	-	1.12 ± 0.04 ^{b}
Anatomical capillary density (mm^2)	616 ± 51 ^{a}	696 ± 123 ^{ab}	824 ± 142 ^{b}	606 ± 123 ^{a}	670 ± 78 ^{ab}	722 ± 124 ^{ab}
Perfused capillary density (mm^2)	570 ± 94 ^{a}	668 ± 116 ^{a}	728 ± 165 ^{a}	259 ± 24 ^{b}	397 ± 95 ^{b}	366 ± 111 ^{b}
Anatomical capillary: muscle fibre	1.47 ± 0.30 ^{ab}	1.41 ± 0.12 ^{ab}	1.61 ± 0.23 ^{ab}	1.39 ± 0.39 ^{a}	1.25 ± 0.11 ^{a}	1.84 ± 0.22 ^{b}
Perfused capillary: muscle fibre	1.25 ± 0.29 ^{ab}	1.36 ± 0.13 ^{a}	1.42 ± 0.31 ^{a}	0.63 ± 0.24 ^{c}	0.76 ± 0.19 ^{bc}	0.90 ± 0.51 ^{abc}
Anatomical capillary domain area (μm^2)	1392 ± 119 ^{a}	1526 ± 268 ^{ab}	1339 ± 184 ^{ab}	1888 ± 407 ^{b}	1547 ± 191 ^{ab}	1417 ± 201 ^{a}
Perfused capillary domain area (μm^2)	1823 ± 368 ^{ab}	1575 ± 287 ^{a}	1507 ± 225 ^{a}	4525 ± 586 ^{c}	2499 ± 641 ^{ab}	2668 ± 923 ^{b}
Anatomical log_{rSD}	0.111 ± 0.010 ^{a}	0.103 ± 0.010 ^{a}	0.101 ± 0.009 ^{a}	0.104 ± 0.008 ^{a}	0.099 ± 0.011 ^{a}	0.096 ± 0.011 ^{a}
Perfused log_{rSD}	0.127 ± 0.016 ^{ab}	0.105 ± 0.010 ^{a}	0.109 ± 0.010 ^{ab}	0.131 ± 0.012 ^{b}	0.124 ± 0.012 ^{ab}	0.116 ± 0.015 ^{ab}
EDL fibre composition: Type I (%)	3.9 ± 2.3 ^{a}	2.2 ± 1.3 ^{a}	3.5 ± 2.7 ^{a}	4.4 ± 2.4 ^{a}	4.8 ± 1.7 ^{a}	4.1 ± 1.5 ^{a}
EDL fibre composition: Type IIa (%)	21.9 ± 5.2 ^{a}	25.3 ± 3.9 ^{a}	23.2 ± 6.9 ^{a}	23.5 ± 5.4 ^{a}	26.3 ± 3.3 ^{a}	23.3 ± 3.6 ^{a}
EDL fibre composition: Type IIb/IIx (%)	74.2 ± 7.2 ^{a}	72.5 ± 4.5 ^{a}	73.2 ± 9.5 ^{a}	72.1 ± 7.4 ^{a}	68.9 ± 3.6 ^{a}	72.9 ± 3.9 ^{a}
Type I FCSA (μm^2)	922 ± 217 ^{a}	922 ± 322 ^{a}	986 ± 182 ^{ab}	1151 ± 176 ^{ab}	969 ± 164 ^{a}	1340 ± 189 ^{b}
Type IIa FCSA (μm^2)	939 ± 105 ^{a}	1102 ± 212 ^{ab}	1109 ± 133 ^{ab}	1178 ± 226 ^{ab}	1054 ± 158 ^{a}	1379 ± 103 ^{b}
Type IIb/x FCSA (μm^2)	1754 ± 296 ^{a}	2520 ± 520 ^{bc}	2061 ± 187 ^{abc}	2435 ± 308 ^{bc}	2079 ± 446 ^{ab}	2730 ± 447 ^{c}

DISCUSSION

In this paper we have demonstrated that compensatory cardiac hypertrophy coincides with capillary rarefaction and a reduction in muscle fatigue resistance. Chronic mechanical stretch *via* overload and voluntary aerobic exercise respectively recovered or prevented loss of capillaries in muscle tissue, thereby preventing the reduction in fatigue resistance in rats with compensatory cardiac hypertrophy. These data indicate that the skeletal muscle microcirculation may be a new therapeutic target to restore muscle function in patients with chronic diseases such as CHF.

Effects of acute aortic banding

There was a striking effect of banding and exercise or overload on EDL fatigue resistance, highlighting the sensitivity of skeletal muscle to both local and systemic influences. Aortic banding precipitated an immediate decline in muscle performance that remained reduced after long-term aortic banding. In the acute situation, the reduced muscle fatigue resistance may be related to an attenuated arterial blood supply, consistent with impaired ischaemic muscle performance in conditions of constrained feed artery perfusion (Murthy et al., 2001, Fulgenzi et al., 1998).

Effects of chronic aortic banding

The acute drop in femoral blood flow after aortic banding had disappeared 4 weeks after aortic banding, and was not significantly enhanced by exercise or overload. Yet, muscle fatigue resistance was still reduced 4 weeks after banding. We infer that limitations on muscle performance after long-term aortic banding were due to changes within the muscle itself rather than on arterial blood supply.

We recently demonstrated that fatigue resistance is determined by perfused capillary density (Tickle et al., 2020), emphasising that microvascular impairment can contribute to attenuated muscle performance. Capillary rarefaction is associated with impaired exercise tolerance in clinical (Gerovasili et al., 2009) and animal models of heart disease (Duscha et al., 1999, Nusz et al., 2003) and given the absence of changes in arterial flow with CCH, the reduction in the perfused muscle microcirculation was a potential constraint on muscle function. Indeed, functional indices of EDL capillarisation were negatively affected after development of CCH. Furthermore, a larger capillary domain area (CDA) in Aob potentially reduced fatigue resistance due to diffusion limitations on oxygen delivery. These data are in line with other reports of reduced anatomical CD in CHF (Nusz et al.,

2003), hypertension (Serne et al., 2001) and hindlimb ischaemia (Dai et al., 2002). The situation may well be progressive as it has been observed that prolonged hypertension leads to capillary apoptosis (Prewitt et al., 1982). If so, this is significant, as our data show that already after 4 weeks of aortic banding there is microvascular deficit as reflected by the reduced muscle fatigue resistance.

Adverse changes in functional capillarity and fatigue resistance in Aob manifested during otherwise normal femoral blood flow. Maintenance of femoral flow despite chronic hypertension has been reported in other studies of abdominally coarcted rats with impaired peripheral circulatory structure or function (Boegehold et al., 1991, Levy et al., 1996, Overbeck, 1980). For example, Boegehold et al (1991) reported peripheral arteriolar rarefaction in the hindlimb of rats subjected to abdominal aortic constriction despite normal femoral arterial pressure. Similarly, peripheral vascular resistance and impaired hindlimb vasodilation develop after surgically imposed abdominal aortic stenosis, despite no increase in local femoral blood pressure (Ungvari et al., 2004, Overbeck, 1980, Bell and Overbeck, 1979). Together, these findings suggest that the mechanism(s) that determine functional rarefaction is pressure-independent and therefore derived from systemic factors related to the development of cardiac dysfunction. The established anti-angiogenic effects of reactive oxygen species and inflammatory cytokines , which are both upregulated during development of cardiac dysfunction (Ungvari et al., 2004, Agnoletti et al., 1999, Sun et al., 2007), may underlie the progression of microvascular rarefaction. This may be further compounded by disuse, as inactivity has been found to lead to decrements in microvascular supply (Kissane and Egginton, 2019) and Aob+EX travelled shorter distances than control EX rats.

Despite impairments in the number of functional capillaries, Log_{RSD} was maintained in Aob, suggesting that at least in this early phase of CCH and hypertension, heterogeneity of functional and anatomical capillary spacing is unaffected, preventing further declines in muscle oxygenation and performance (Al-Shammari et al., 2014, Degens et al., 2006b). A slow-to-fast shift in skeletal muscle fibre type composition and muscle atrophy may develop with CHF and are also thought to contribute to the exercise intolerance during CHF (De Sousa et al., 2002, Mancini et al., 1992, Carvalho et al., 2003), but such changes did not occur in our banded groups. We can therefore exclude these factors as underlying the reduced fatigue resistance in our aortic banded rats. However, such changes in fibre type composition are reported to occur 12 weeks and fibre atrophy by 24 weeks after

stenosis of the ascending aorta (Carvalho et al., 2003), and it is possible that after longer periods of abdominal aortic constrictions these factors may also become contributing factors to a reduced muscle fatigue resistance.

The surgically-induced abdominal coarctation led to chronic hypertension above the stenosis that over time resulted in CCH (Cornelussen et al., 1994, Degens et al., 2006a, Levy et al., 1996, De Sousa et al., 2002). While tail blood pressure was reduced immediately upon band application (this study; (Ungvari et al., 2004) it subsequently recovered which was probably realised by the increased blood pressure.

Prevention of reduced fatigue resistance during aortic banding by exercise

Wheel-running exercise did not enhance FI in intact animals but maintained muscle performance in rats with CCH. This suggests that the level of exercise completed by animals was not sufficient to register an improvement in the fatigue resistance of the EDL muscle in EX, but was of sufficient intensity to offset the pathological effects of CCH on skeletal muscle in banded rats. While inclination to exercise was undiminished in rats with CCH, the duration and intensity of each running bout was lower than that found in control animals, leading to a lower overall distance travelled, mirroring previous work in banded rats (De Sousa et al., 2002). In addition to a shorter duration, the running velocity of rats with CCH was also less than that of the controls, and it is possible that an earlier onset of fatigue may contribute to declining locomotor muscle power output. Nevertheless, even attenuated exercise performance was sufficient to restore EDL function to normal levels, thereby highlighting the beneficial effect of limited aerobic activity to reverse pathological changes that occur in skeletal muscle with CCH. It has been reported that reductions in oxidative capacity are also reversed following voluntary exercise in clinical CHF (Adamopoulos et al., 1993, Hambrecht et al., 1995, Stratton et al., 1994) and an aortic coarctation model (De Sousa et al., 2002, Gomes et al., 2016). Together these data indicate that aerobic exercise provides a potent restorative effect on skeletal muscle structure and function in various stages of disease.

Voluntary wheel running after banding (Aob+EX) preserved functional capillary domain area and perfused C:F, thereby maintaining tissue oxygenation (Al-Shammari et al., 2014) and FI. Also the $\log_{RSD}$ was maintained, preventing the development of local hypoxic regions within the muscle. Many of the reported

benefits of exercise therapy in CHF (Belardinelli et al., 1999) may thus be related to the maintenance of an adequate capillary supply in CHF.

Recovery of fatigue resistance during Aob by overload

An earlier study using aortic coarctation-induced CCH reported a muscle-specific effect of CCH on FI, with functional impairments occurring in the soleus and tibialis anterior, but not in the EDL (Levy et al., 1996). Although we have no explanation for the discrepancy in the absence of reduced fatigue resistance in the EDL in the study by Levy et al (1996) and the reduction we observed in aortic banded rats, it could be related to differences in the way fatigue resistance was determined ((Allman and Rice, 2002).

Improved functional capillarity with overload was accompanied by enhanced fatigue resistance, as reported previously (Ballak et al., 2016, Deveci and Egginton, 2002, Egginton et al., 2011, Tickle et al., 2020, Zhou et al., 1998, Degens et al., 1992), and parallels the upregulation of angiogenic growth factor expression and capillarity observed after resistance exercise in healthy subjects (Gavin et al., 2007, Holloway et al., 2018, Ferguson et al., 2018) and CHF patients (Williams et al., 2007). Alleviation of symptoms associated with heart disease may be hampered by coincident pathologies such as reduced peripheral blood flow. Interestingly, angiogenesis following overload may occur in conditions of experimentally reduced blood flow (Deveci and Egginton, 2002, Egginton et al., 1998), highlighting the potential of flow-independent mechanical stimuli to improve muscle capillarity and fatigue resistance even in advanced disease. While mechanical overload through synergist ablation is not directly analogous to resistance training (Murach et al. 2020), the successful application of muscle overload to rats with CCH suggests that therapeutic resistance exercise may be beneficial in patients with CHF. Resistance training has the benefit of imparting additional cardiovascular strain for short bouts in contrast to the long durations required with aerobic exercise (Duncker et al., 2014) while providing an effective method for improving muscle function (Giuliano et al., 2017). Increases in blood pressure can be mitigated further by not performing the Valsalva manoeuvre during resistance training (Soucek et al., 2009).

While EDL hypertrophy occurred after OV the relative mass gain was lower after development of CCH, although there were no significant differences in maximal twitch force or fibre cross-sectional area (FCSA) between OV and Aob+OV. An impaired muscle hypertrophy has also been reported in a rat model of metabolic

syndrome, where limited overload-induced muscle growth was attributed to dysfunctional mTOR signalling, a central regulator of growth and proliferation (Katta et al., 2010, Paturi et al., 2010). Raised levels of inflammatory cytokines, particularly TNFα, suppress mTOR activity in heart failure (Schiaffino et al., 2013) and may in part explain the reduced overload-induced gain in muscle mass during abdominal aortic constriction.

Greater capillarisation is associated with enhanced skeletal muscle fibre hypertrophy after resistance exercise training (Snijders et al., 2017a), highlighting the critical importance of the microcirculation to muscle growth. Reduced functional capillarisation with CCH may therefore contribute to the blunted hypertrophic response. Given the potential for regrowth of the functional microcirculation in Aob+OV, a longer period of overload may accommodate a full restoration of functional capillary density.

Critically, establishment of a functional impairment was prevented by aerobic exercise (Aob+EX) and reversed by mechanical overload (Aob+OV), where FI was identical to Control. OV enhanced FI, as found in previous experimental studies (Frischknecht and Vrbova, 1991, Tickle et al., 2020, Degens et al., 1993b). This benefit extended to animals with CCH, where an FI improvement of c.32% was recorded in Aob+OV compared to a 25% enhancement in OV. Therefore, a targeted mechanical stimulus without (obvious) systemic reciprocal effects leads to a rapid restoration of muscle performance. This parallels the enhanced muscle performance in CHF patients after resistance exercise therapy (Braith and Beck, 2008, Braith et al., 2005, Selig et al., 2004, Giuliano et al., 2017).

More advanced cardiac disease is associated with endothelial dysfunction that may limit the capacity to increase blood flow under conditions of increased tissue oxygen demand (Bank et al., 2000, Kubo et al., 1991), while vascular disease may also compound the effects of CHF on exercise performance by leading to reduced gross blood supply to the periphery *via* claudication and impaired functional hyperaemia (Sanada et al., 2005). Chronic experimental groups did not exhibit reduced hyperaemic flow, so endothelial function was not yet significantly affected in CCH. These interpretations bear a caveat: we have determined the effects on skeletal muscle function of incipient cardiac dysfunction rather than advanced disease, when co-morbidities may contribute confounding effects that make the identification of potential causal factors difficult.

Future research

While these findings are encouraging in supporting the use of angiogenic stimuli to improve muscle performance in CHF, approximate replication in humans is warranted to recommend the use of resistance training or running to improve muscle performance in CHF through capillary growth. Also, use of high load resistance training or running may not be feasible given the severity of symptoms in many CHF patients.

One way to use a similar approach in humans would be to use blood flow restriction training, this allows for increases in muscle size and strength with low intensity resistance loads and may have the added benefit of improving capillarisation (Nielsen et al. 2020).

Conclusions

In summary, we have demonstrated that functional microvascular rarefaction with CCH underlies the impaired skeletal muscle fatigue resistance in CCH. Loss of functional capillarisation can, however, be prevented through aerobic exercise and restored by functional overload (analogous to resistance exercise), contributing to the successful recovery of muscle performance. Therefore, improved fatigue resistance in Aob+EX and Aob+OV as a consequence of improved functional capillarity supports the hypothesis that skeletal muscle performance in CHF is primarily determined by peripheral factors, specifically a loss of functional capillaries in muscle. The key determining role of muscle microcirculation in development of exercise impairments during cardiac dysfunction underscore how angiogenic restorative growth should be a key therapeutic target.

Prelude to Chapter 4

Previous chapters focussed on the role that functional skeletal muscle microcirculation plays in fatigue resistance in otherwise healthy animals and in a rat model of chronic heart failure. Subsequent chapters focus on the role of the microcirculation in skeletal muscle plasticity.

The size principle of striated muscle suggests that fibre size is inversely related to oxidative capacity and therefore combining resistance and endurance exercise would blunt the adaptive responses to each training modality. In order to maintain or increase oxidative capacity while fibre size is increased, oxygen transport to the fibre can be improved by increasing capillary density. In the next chapter an endurance exercise programme is superimposed on to the regular resistance training of young and older resistance trained men. In accordance with the size principle, reductions in fibre size are predicted to occur alongside an increase in oxidative capacity. Given that the angiogenic response has been found to be blunted in both elderly rodents and people and studies of older people have found that greater capillarisation is linked to an improved hypertrophic response, even larger reductions in fibre size are expected in old resistance-trained men.

Chapter 4

ENDURANCE TRAINING-INDUCED INCREASE IN MUSCLE OXIDATIVE CAPACITY WITHOUT LOSS OF MUSCLE MASS IN YOUNG AND OLD RESISTANCE-TRAINED MEN

ABSTRACT

The concept of interference suggests that endurance training in resistance-trained individuals may induce some loss of resistance training-induced gains in muscle mass. This effect is perhaps more pronounced in older people. Here we investigated the impact of superimposed endurance training in young (28.5 ± 4.8 years; n=8) and old (67.5 ± 5.5 years; n=7) highly resistance-trained men. Alongside their usual resistance training programme participants underwent a 10-week endurance cycling training programme consisting of five 6-min intervals at 75% max heart rate (HRmax) separated by 4-min intervals at 90% HRmax. Resistance training was always performed prior to endurance training if performed on the same day but not all participants performed resistance and endurance training on the same day. The anatomical cross-sectional area (ACSA) of the thigh muscles, as determined with MRI, was 24% smaller in old compared to young participants ($p<0.001$). Although the maximal oxygen consumption (VO_2max) was also lower in the old group (2.69 $L \cdot min^{-1}$ vs. 3.24 $L \cdot min^{-}1$ in the young group, $p<0.001$), the VO_2max per kg body mass did not differ significantly between the young and old participants. Histological analyses of biopsies of the *m. vastus lateralis* showed that endurance training induced an increase in the succinate dehydrogenase activity in both young and old participants ($p \leq 0.043$), and an increase in the number of capillaries around type I fibres (5.02 ± 0.85 vs 5.95 ± 0.51 in young, 4.40 ± 0.92 vs. 4.76 ± 0.82 in old, $p=0.017$). The superimposed endurance training did not induce a significant decrease in thigh ACSA, fibre cross-sectional area, or knee extensor maximum voluntary isometric force. These observations indicate 1) that adding endurance training to resistance training can lead to positive endurance-related adaptations without negative consequences for muscle size and strength and 2) that the concurrent training or interference effect may only apply to extreme situations that are rarely encountered, even in highly-trained people.

INTRODUCTION

Success in weightlifting or long-distance running typically require muscle strength or endurance, respectively. Therefore, power athletes typically do resistance exercise that promotes muscle hypertrophy and a concomitant increase in strength, with little to no endurance exercise. Endurance exercise on the other hand induces, among other adaptations, increases in stroke volume, muscle oxidative capacity and angiogenesis with a reduction in type II fibre cross-sectional area (FCSA), resulting in an increase in whole body maximal oxygen uptake (VO_2max) (Baar, 2006). While many sports do require both explosive power and endurance, it is thought that adding endurance training to resistance exercise blunts the adaptation to resistance exercise and *vice versa*. In 1980, Hickson coined the term “interference effect”, now known as the concurrent training effect (CTE), to describe the blunted adaptation to resistance training in concurrent trained subjects when compared to those who only performed resistance training (Hickson, 1980).

It has been observed that there is an inverse relationship between fibre size and oxidative capacity, and it is thought that oxygen, ADP and ATP diffusion limitations put a constraint on fibre size (van Wessel et al., 2010, Degens, 2012, Kinsey et al., 2007, van der Laarse et al., 1998). There thus seems to be a trade-off (van der Laarse et al., 1998) between endurance (and high oxidative capacity) and power or force generating capacity (large fibre size) of a muscle fibre that provides and explanation for the CTE (van Wessel et al., 2010).

Yet, in contrast to the CTE concept, strength training in master endurance athletes has been found to have a positive effect on performance (Piacentini et al., 2013) and we recently showed that team athletes had adaptations similar to both endurance and power athletes (Degens et al., 2019). The effect of superimposed endurance training in resistance-trained individuals is yet to be determined. It has been shown, however, that endurance training combined with resistance exercise may not diminish the strength gain of resistance training (Petre et al., 2018) and animal models have shown that the fibre size constraint can be broken. For instance, oestrogen-related receptor gamma (Errγ) overexpression in myostatin null mice exhibit as large muscle fibres as the myostatin null mice despite having a higher oxidative capacity (Omairi et al., 2016), and functional overload resulted in both hypertrophy and an increased oxidative capacity even in old mice (Ballak et al., 2016). In both cases this was associated with a denser capillary bed, suggesting

that angiogenesis is instrumental in breaking the size constraint. However, no systematic study has yet investigated whether also in humans with larger muscle fibres than rodents (Wust et al., 2009) such an adaptation is possible, and in particular whether superimposing endurance exercise will negate the gains in muscle fibre size in long-term resistance-trained people. The potential detrimental effect of endurance training on the muscle (fibre) size of highly resistance trained men may be even more pronounced in old highly resistance-trained men, if, like in overloaded mouse muscle (Ballak et al., 2016), the angiogenic response is attenuated in old age.

The aim of the present study was to assess the impact of superimposing endurance training onto the usual resistance training programmes of both young and old highly resistance trained men on (1) the oxidative capacity and (2) size of the muscle fibres, and (3) the number of capillaries around a fibre (CAF). Given that CTE suggests that the hypertrophic response is attenuated in concurrent compared to resistance training alone, and based on the size principle we hypothesised that superimposing endurance training in both old and young highly resistance-trained men will lead to an increase in fibre oxidative capacity and a decrease in fibre size. This decrease in fibre size will be more pronounced in older highly resistance-trained men due to an attenuated angiogenic response.

METHODS

The Kaunas Regional Biomedical Research Ethics Committee (Authorisation number BE-10-4) provided ethical approval for the study. All subjects provided informed consent prior to participation. Ethical approval was also provided by Manchester Metropolitan University (SE171810).

Subjects

Fifteen highly resistance-trained men engaged in regular resistance training were recruited with the help of the Lithuanian Bodybuilding & Fitness Federation and divided into a young (n=8) (28.5 ± 4.8 years) and old (n=7) (67.5 ± 5.5 years) group. Young participants had performed regular resistance training for a minimum of 5 years and old participants for a minimum of 20 years.

I was included as one of the participants in the study. While this was important to increase the number of participants there are ethical implications in researchers

enrolling in their own research as this could potentially influence the data in favour of their desired outcome. Removal of my data from the population would not change the findings of the study.

Experimental design

The volunteers participated in a 10-week endurance-training programme that was superimposed on their usual training programme. Before and after the training programme, body fat percentage was determined, an MRI scan of the upper leg was performed and a *vastus lateralis* muscle biopsy was taken. In addition, the maximal oxygen consumption (VO_2max) and the maximal voluntary isometric contraction torque (MVC) of the knee extensor muscles were measured.

Endurance training programme

The endurance-training programme was home based and not conducted in a laboratory under researcher control but was preceded by a supervised familiarisation session in the laboratory. The participants had to provide written comments on each training session. These reports indicated at least a 90% completion of the set number of endurance training sessions. To monitor exercise intensity, participants were given a heart rate monitor. Compliance was based on self-reporting and not data from heart rate monitors.

The participants were instructed to perform cycle ergometry 3 times per week. To ensure adherence, they were encouraged to carry out additional endurance training at a convenient time, either in combination with their resistance training during the same gym visit, as a separate session during the same day, or at different day from resistance training. When both modalities were performed on the same day or within the same session, resistance training was always completed first. The intensity of the endurance-training was increased during the first 6 weeks from 45 min cycling at 75% of the maximal heart rate (HRmax) in the first week to 5 intervals of 6 min cycling at 75% HRmax interspersed with 4 min cycling at 90% HRmax in the final 4 weeks.

Anthropometric and VO_{2max} measurements

Body fat percentage was measured using bioelectrical impedance (Tanita, Tokyo, Japan). Fasting and hydration status were not controlled for. To assess VO_2max, participants cycled at 40 W for 3 min, after which the load was increased by 5 W every 10 s while maintaining a pedalling rate of around 70 rpm until volitional fatigue.

Participants were required to continue until their heart rate was at least the 90 percent of the predicted HRmax and the respiratory exchange ratio of > 1.1. A portable breath-by-breath analyser (Oxygen Mobile; Jaeger/VIASYS Healthcare, Hoechberg, Germany) was used and heart rate was monitored during the test (S-625X; Polar Electro, Kempele, Finland).

The MVC was measured using an isokinetic dynamometer (System 3; Biodex Medical Systems, Shiley, New York) at a knee angle of 50°, 70° and 90° (0° = anatomical zero/full leg extension) for 2 seconds with 60 seconds rest in between in a random sequence. Subjects were seated upright in the dynamometer chair with double shoulder seat belts stabilizing the upper body and were encouraged to perform each contraction as hard as possible.

MRI imaging and analyses

Images of the thigh were taken using a 1.5 T MRI scanner (Signa Explorer, GE Health Care, China) 7 days after the last training session to allow for muscle swelling to subside. With the participant in a supine position, a Cor FSE protocol was used and multiple 4-mm thick serial transverse sections were taken along the length of the thigh with no inter-slice gap. Images were analysed using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.net/Downloads). At 60% femur length (from distal), the total cross-sectional area of were measured. In addition, the size of the femur and the bone marrow cavity were measured to determine differences between young and old participants, as well as the proportion of the thigh occupied by subcutaneous fat. The optical density of the muscle was measured as an indication of fat content. Figure 1a shows an example of an MRI image of the thigh.

Histological analyses

Biopsies of the *m. vastus lateralis* were taken after MRI imaging at approximately 60% of femur length (from distal) under aseptic conditions and local anaesthesia with 2 % lidocaine employing a conchotome. The biopsies were frozen in isopentane cooled with liquid nitrogen and stored at -80°C until use.

Cross-sections (10 µm) were cut at -20 °C using a cryostat (Leica CM3050 S, Leica Microsystems, Nussloch, Germany). Sections were incubated in blocking solution (10% goat serum in PBS (phosphate buffered saline)) for 60 minutes, then incubated for 120 minutes with BAD5 (3:100) and SC-71 (1:50) for type I and type

IIa myosin heavy chain, respectively (Developmental Studies Hybridoma Bank, USA). After washing in PBS three times for 5 minutes sections were incubated for 60 minutes in secondary antibodies Alexa Fluor 350 IgG2b for type I (1:500), Alexa Fluor 488 IgG1 for type IIa (Thermofisher Scientific, USA) and Rhodamine labelled lectin *Ulex Europaeus* Agglutinin I (1:200) (Vector Laboratories, California, USA) capillaries. After three 5-minute washes in PBS, the slides were mounted using ProLong Diamond Antifade mountant (Thermofisher) and imaged at 10x maginification (Fig. 1b). Serial sections were stained for succinate dehydrogenase (SDH) as described previously (Wust et al., 2009), with the optical density (OD) of the stain at 660 nm used as a quantitative indication of oxidative capacity (Fig. 1c). For each section, a calibration curve was created using a series of filters with a known OD to adjust for variation in background staining and lighting. The OD of the SDH stain and fibre cross-sectional area (FCSA) of each fibre was determined using ImageJ. Also the number of capillaries around a fibre (CAF) was determined for each fibre type. The roundness of the fibre was calculated as perimeter^2/(4π·FCSA). Larger roundness values indicate a greater deviation from circularity (Barnouin et al., 2017).

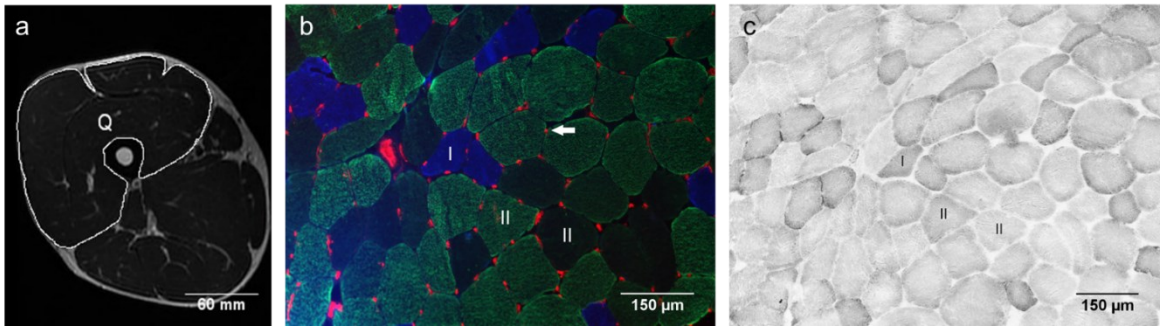


Figure 1: a shows an MRI scan of a left thigh at approximately 60% of femur length (from distal end) with labelled quadriceps muscles (Q). b shows a muscle cross-section immunofluorescently stained for type I (blue fibres) and type II (green and non-stained fibres) fibres, and capillaries (white arrow) stained with Rhodamine-labelled Ulex Europaeus Agglutinin I. c is a serial section of b stained for succinate dehydrogenase.

Statistics

All statistical analyses were completed with SPSS software. A Shapiro-Wilk test showed that all data were normally distributed. Repeated-measures analysis of

variance (ANOVA) was used with pre and post-exercise, fibre type (I vs. II) and knee angle (50°, 70° and 90°) as within factors, and age as a between factor. Three-way interactions were excluded. If interactions were found, Bonferroni post-hoc tests were done to locate differences. Differences were considered significant at $p<0.05$.

RESULTS

Participant characteristics

Table 1 shows that younger participants were taller ($p=0.042$), had a higher body mass ($p=0.001$) and BMI ($p=0.002$) compared to older participants. There were no significant differences between young and old in body fat and subcutaneous fat percentage in the thigh area. While younger individuals had higher VO_2max ($L \cdot min^{-1}$), HRmax ($p<0.001$) and max power (W) ($p \leq 0.023$) than older individuals, there was no significant difference between young and old in VO_2max ($mL \cdot min^{-1} \cdot kg^{-1}$) and max power ($W \cdot kg^{-1}$) per kg body mass.

Body mass, BMI, VO_2max and power were not significantly changed with endurance training.

Table 1: *Participant characteristics, maximal voluntary isometric knee extension contraction torque (MVC) and maximal oxygen uptake (VO_2max). Data are mean ± standard deviation, and range in parenthesis.*

Variables	Young (n = 8)		Old (n = 7)	
	PRE	POST	PRE	POST
Age (y)	28.5 ± 4.8 (23 – 35)		67.5 ± 5.5 (61 – 77)	
Training experience (y)	10.6 ± 5.1 (5 – 20)		36.7 ± 12.1 (20 – 50)	
Height (cm)	182 ± 5 (176 – 188)		175[y] ± 7 (162 – 180)	
Body mass (kg)	98.9 ± 7.6 (89.4 – 109.4)	101.0 ± 8.7 (85.9 – 110.7)	83.0[y] ± 8.1 (68.5 – 93.2)	82.8[y] ± 8.0 (69.0 – 93.0)
BMI ($kg \cdot m^{-2}$)	30.4 ± 2.1 (27.3 – 34.5)	30.5 ± 2.4 (26.8 – 34.1)	27.2[y] ± 1.1 (26.1 – 28.8)	27.2[y] ± 0.9 (26.0 – 28.7)
Fat mass (%)	18.0 ± 6.3 (9.7 – 31.7)	18.2 ± 6.0 (8.5 – 29)	22.3 ± 3.4 (19.0 – 27.9)	22.1 ± 3.3 (19.0 – 28.0)

Thigh subcutaneous fat (%)	20.4 ± 5.0 12.7 – 31.5	19.8 ± 4.6 8.3 – 24.0	22.4 ± 3.6 17.6 – 30.0	21.7 ± 3.0 18.3 – 27.0
Thigh muscle ACSA (cm^2)	242 ± 18 (207–262)	244 ± 13 (220 – 267)	183^{y} ± 16 (161 – 210)	190^{y} ± 17 (163–211)
VO_2max ($L \cdot min^{-1}$)	3.24 ± 0.48 (2.56 – 3.60)	3.41 ± 0.27 (2.99 – 3.79)	2.68^{y} ± 0.39 (2.03 – 3.15)	2.59^{y} ± 0.27 (2.28 – 3.00)
VO_2max ($mL \cdot min^{-1} \cdot kg^{-1}$)	32.2 ± 5.3 (25.4 – 39.5)	33.9 ± 3.0 (29.8 – 38.1)	33.1 ± 7.2 (24.4 – 42.0)	31.8 ± 4.7 (28.1 – 39.5)
HRmax (bpm)	179 ± 11 (163 – 194)	176 ± 8 (168 – 191)	151^{y} ± 12 (136 – 165)	150^{y} ± 7 (140 – 157)
$Power_{max}$ (W)	356 ± 48 (270 – 430)	377 ± 20 (360 – 415)	292 ^{y} ± 42 (235 – 345)	301 ^{y} ± 32 (260 – 330)
$Power_{max}$/BM ($W \cdot kg^{-1}$)	3.54 ± 0.57 (2.74 – 4.39)	3.76 ± 0.41 (3.34 – 4.42)	3.58 ± 0.66 (2.67 – 4.47)	3.70 ± 0.50 (3.07 – 4.22)
Fibre roundness	1.32 ± 0.04 (1.24 – 1.37)	1.33 ± 0.04 (1.27 – 1.40)	1.27 ± 0.08 (1.13 – 1.38)	1.28 ± 0.08 (1.13 – 1.44)

BMI: body mass index; ACSA: anatomical cross-sectional area; HRmax: maximal heart rate; VO_2max: maximal oxygen consumption; $Power_{max}$: power at VO_2max; $Power_{max}$/BM: $Power_{max}$ per body mass; ^{y} indicates a significant difference from young participants ($p<0.05$).

Bone properties

There were no significant differences in femur area, marrow area and marrow OD between young and older individuals (Table 2). The bone OD was, however, lower in the young than older participants (p=0.030), which suggests that the bones of the older participants contained more fat than those of the young participants. There was no significant effect of the training on the bone properties.

Table 2: *Bone characteristics from participants, averages taken from both limbs.*

Variables	**Young (n = 8)**		**Old (n = 7)**	
	PRE	**POST**	**PRE**	**POST**
Femur bone area (mm^2)	691 ± 58 (611 – 770)	702 ± 56 (604 – 768)	708 ± 36 (668 – 791)	707 ± 38 (655 – 766)
Femur bone OD	373 ± 77 (207 – 492)	348 ± 87 (181 – 457)	455^{y} ± 76 (380 – 630)	423^{y} ± 122 (176 – 588)
Marrow area (mm^2)	194 ± 45.8 (132 – 296)	195 ± 46.8 (115 – 279)	210 ± 35.1 (173 – 281)	193 ± 52.1 (168 – 273)

Marrow OD	1126 ± 243 (640 – 1533)	960 ± 255 (570 – 1329)	1281 ± 259 (809 – 1707)	1255 ± 322 (668 – 1580)

OD: optical density; [y] indicates a significant difference from young participants (p=0.03).

Muscle properties

The anatomical cross-sectional area (ACSA) of all muscles in the thigh (Table 1; p<0.001) and quadriceps muscles (Fig. 2a; p<0.001) were larger in the young than old participants. The quadriceps ACSA/femur ratio is significantly lower (p<0.001) in old participants compared to young (Fig. 2b). In addition, the MVC of younger participants was higher than that of the old at all angles (p<0.001) (Fig. 2c). There was a significant angle*age interaction for MVC (p=0.009). In young participants the MVC was greater at 70° and 90° than at 50° (p<0.001 and p=0.004 respectively). In old participants the MVC at 70° was greater than at 50° (p=0.005) and at 90° (p=0.001). There were no significant changes in muscle cross-sectional area, or MVC at any angle, after endurance training in either age group (Fig. 2a, 2c). The specific torque of the extensor muscles (MVC/quadriceps ACSA, Fig. 2d) did not differ significantly between young and old participants at any knee angle. The specific torques at 70° and 90° were greater than at 50° (p<0.001 and p≤0.007 respectively), and the specific torque at 70° was greater than at 90° (p=0.002).

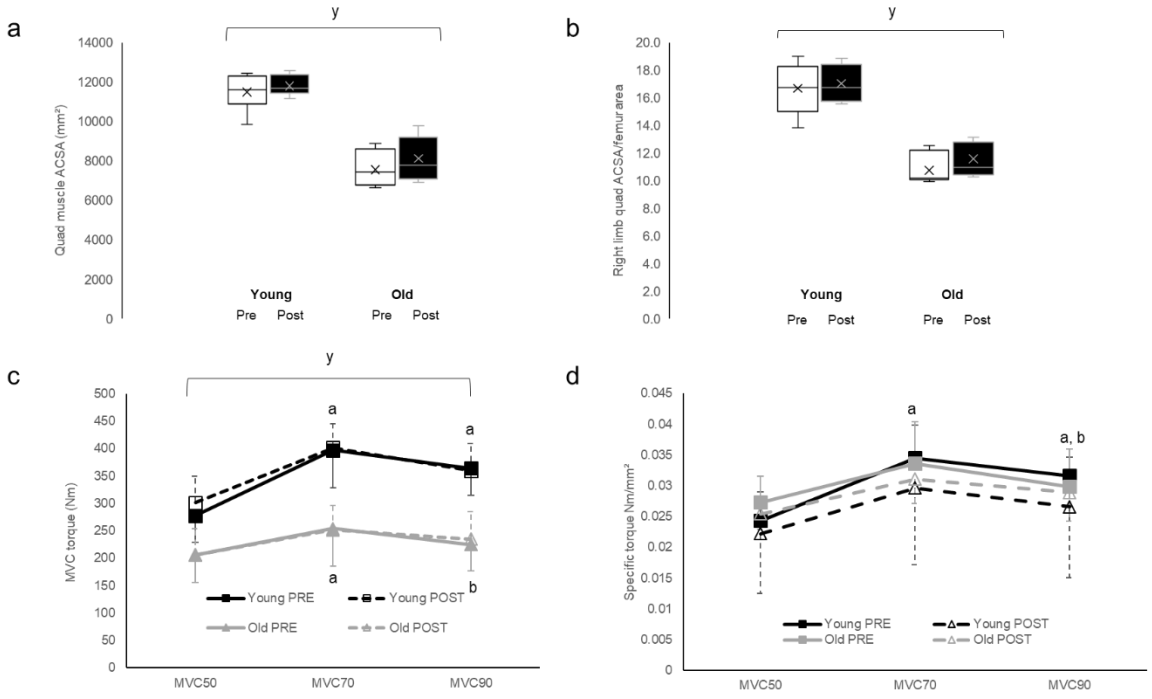


Figure 2: a total quadriceps muscle anatomical cross-sectional area (ACSA) pre and post endurance training in young and old highly resistance-trained men. b shows right quadriceps ACSA/femur area pre and post endurance exercise in young and old participants. c shows maximal voluntary isometric contraction of knee extension (MVC) at 50, 70 and 90° (MVC50, MVC70 and MVC90 respectively) of young and old participants pre and post endurance exercise. d shows the specific torque (MVC/quadriceps ACSA) of quadriceps muscles at MVC50, MVC70 and MVC90 for young and old participants pre and post endurance exercise. y indicates a significant difference to young participants at p<0.001; a indicates a significant difference to MVC50 at p≤0.007. b indicates a significant difference to MVC70 at p≤0.002.

The lower OD in all muscles of the young than the old participants (Fig. 3a) (p≤0.03) suggests that the intramuscular fat content is higher in the older than in younger participants. Figure 3b shows that the relationship between quadriceps muscle ACSA and VO_2max is similar pre and post endurance training (R^2=0.465 and 0.651, respectively).

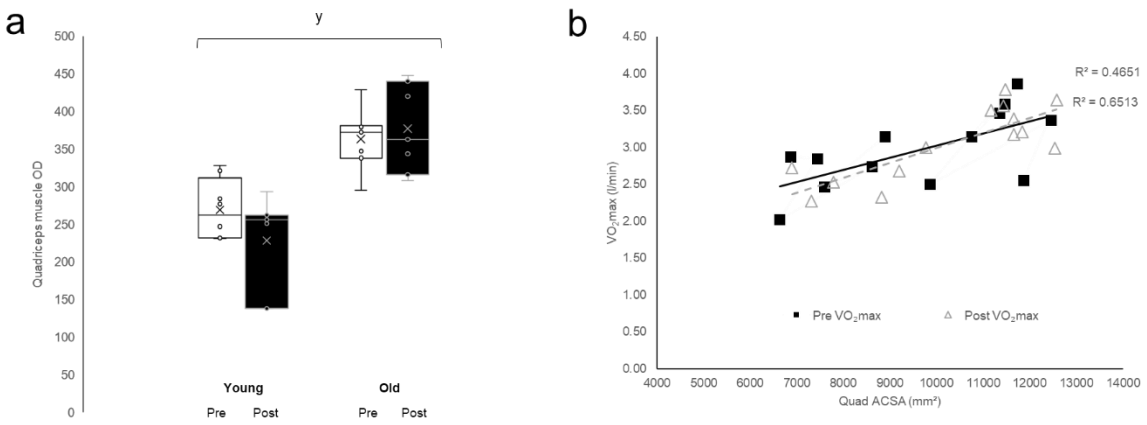


Figure 3: a shows the quadriceps muscle optical density (OD) in young and old highly resistance-trained men pre and post an endurance exercise programme. b shows the relationship between quadriceps anatomical cross-sectional area (ACSA) and maximal oxygen consumption (VO_2max) pre and post endurance exercise. y indicates a significant difference in OD compared to young subjects at p≤0.03.

Fibre type composition

Old participants had a larger proportion of type I fibres and a smaller proportion of type II fibres when compared to young participants (p=0.014) (Fig. 4a). Young participants had a greater proportion of type II fibres compared to type I (p=0.004)

whereas old participants had similar proportions of type I and II fibres. There was no significant effect of superimposed endurance training on fibre type composition.

Fibre cross-sectional area

There was a main effect of fibre type (p=0.017) without any significant type*age or type*training interactions, indicating that type II fibres were larger than type I, irrespective of age and endurance training (Fig. 4b). Neither training nor age had a significant effect on the FCSA.

Succinate dehydrogenase activity

The SDH activity was significantly higher in type I fibres compared to type II (Fig. 2c; p<0.001). Endurance training led to an increase in SDH activity in both type I and type II fibres (p=0.019 and p=0.043, respectively) in both age groups (Fig. 4c).

Capillarisation

Both type I and II fibres had lower CAF in old participants compared to the young (p=0.028). Type I fibres had a higher CAF than type II fibres (p=0.002) in both young and old participants. There was a training * fibre type interaction (p=0.034). Post-hoc analysis revealed that superimposing endurance training onto resistance training increased CAF for type I (p=0.007) but not for type II fibres (Fig. 4d), irrespective of age.

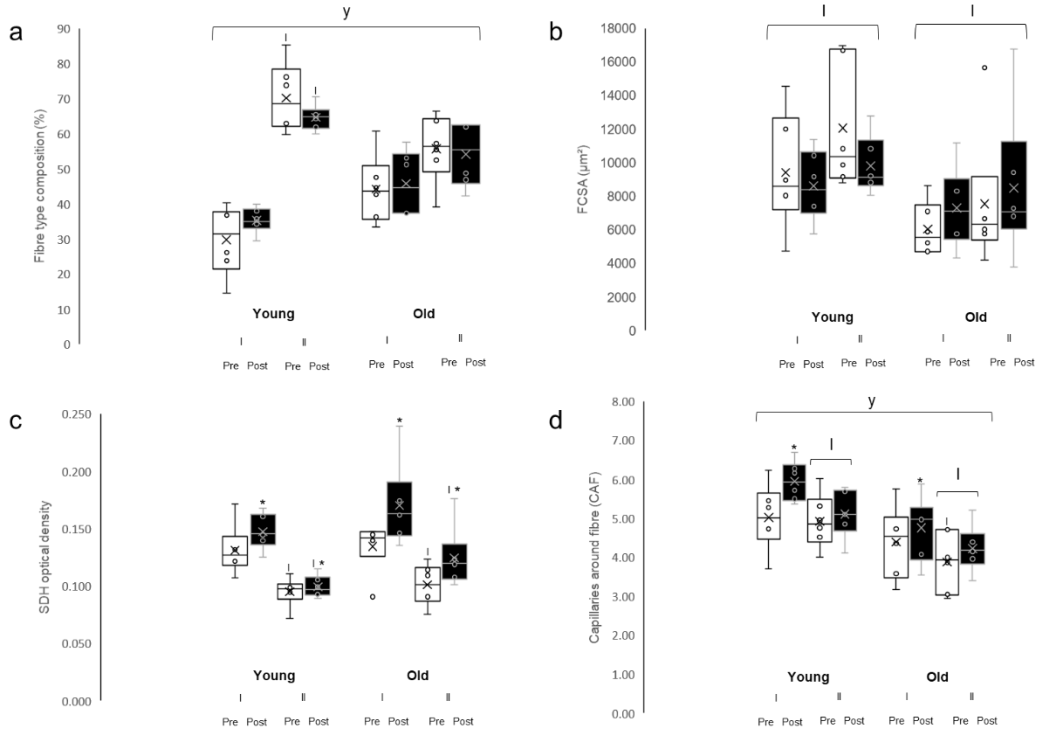


*Figure 4: a shows fibre type composition in the m. vastus lateralis from young and old highly resistance-trained men pre and post endurance exercise. b shows fibre cross-sectional area (FCSA) for type I and type II fibres pre and post endurance exercise in young and old highly resistance-trained men. c shows succinate dehydrogenase (SDH) staining optical density for type I and type II fibres pre and post endurance exercise in young and old participants. d shows the capillaries around fibres (CAF) for type I and II fibres pre and post endurance exercise in young and old participants. * indicates a training effect at p ≤ 0.043; y indicates a significant difference compared to young subjects at p ≤0.028; I indicates a significant difference compared to type I fibres at p ≤ 0.041.*

Fibre roundness

There were no significant changes in muscle fibre roundness after endurance training in either age group, nor was there a difference between fibre types or between young and old participants (Table 1).

DISCUSSION

The main finding of the present study is that superimposing endurance training on a regular resistance exercise programme of highly resistance-trained men induces an increase in oxidative capacity without a decrement in muscle (fibre) size in both young and old participants that was accompanied by angiogenesis. This suggests that even in people highly trained for strength, benefits of endurance exercise do not impair force production or lead to reductions in muscle size.

Age-related changes in muscles of highly resistance-trained men

We observed that even when maintaining high levels of resistance exercise there is still an age-related reduction in muscle mass, absolute maximal oxygen consumption ($L \cdot min^{-1}$) and muscle strength. This is also reported in elite master weightlifters where the relative rate of age-related decline in muscle power was similar to that of control subjects, but they still had a larger muscle power compared to age-matched control subjects (Pearson et al., 2002). This phenomenon is not limited to power athletes but the exercise performance in all athletic disciplines shows an age-related decline (Ganse et al., 2018) that in endurance athletes was

associated with an age-related decline in VO_2max at a similar relative rate to untrained subjects (Tanaka and Seals, 2008).

Here we showed that also in highly resistance-trained men the absolute VO_2max decreased with age, but the VO_2max per kg body mass was similar in young and old participants, suggesting that the decline in VO_2max with ageing is largely due to a loss of muscle mass (Fleg and Lakatta, 1988). This is further supported by our observation of a positive relationship between quadriceps ACSA and VO_2max.

The lower MVC torque in old compared to young participants seems to be mainly due to a loss of muscle mass. The higher optical density of muscle in the MRI images of old participants suggests that they have greater levels of intramuscular fat when compared to young subjects. The accumulation of intramuscular fat during ageing can reach levels of more than 10% (Schwenzer et al., 2009) and may result in a reduced specific tension that will further contribute to the lower muscle strength (McPhee et al., 2018). Yet, we observed that the specific torque (MVC torque per ACSA) was similar in our young and old participants. The similar specific force (per ACSA) may be explained by the reduction in pennation angle that accompanies the decrease in muscle size during ageing, where the fascicles are more in line of pull of the tendon (Degens et al., 2009a). Alternatively, the maintenance of specific tension in our resistance-trained participants suggests that although resistance training cannot prevent the age-related reductions in muscle size, it may help to maintain the ‘muscle quality’ or force per unit area of muscle (Reeves et al., 2004)

The smaller muscle ACSA was probably more related to a reduction in fibre number that has often been reported during ageing (McPhee et al., 2018) than a decrease in fibre size, as we did not find a significant difference in FCSA between young and old highly resistance-trained men. Such an absence of an age-related reduction in FCSA was also seen in master endurance cyclists (Pollock et al., 2018), but not in master sprinters (Korhonen et al., 2006). Whatever the cause of the discrepancy, these observations suggest that regular exercise may attenuate the age-related fibre atrophy, but not the age-related loss of muscle fibres, corresponding with the observation that motor unit loss is not attenuated in longstanding master athletes (Piasecki et al., 2019).

We found that the biopsies of our 61- to 77-year-old participants exhibited a greater proportion of type I fibres than the young, similar to the increased proportion of type I fibres found in muscles from older people (Larsson et al., 1978). This is, however,

an equivocal finding, as others have reported no significant age-related change in the fibre type composition of the *m. vastus lateralis* (Andersen, 2003, Barnouin et al., 2017).

Similar to what has been found in the recreationally active population (Barnouin et al., 2017), the capillarisation of the muscles from our old highly resistance-trained men was lower than that found in young participants. Since blood flow is, via shear stress, an important factor for the maintenance of the vascular bed (Hudlicka et al., 1992), the age-related capillary rarefaction may be due to a reduction in sheer stress resulting from impaired vasodilation and blood flow responses in ageing (Proctor and Parker, 2006).

We speculate that the higher bone optical density in the aged participants reflects a lower bone mineral density or higher fat content. Both are well documented in ageing (Riggs et al., 2004, Ambrosi et al., 2017) and are thought to contribute to impaired bone strength and increased fracture risk. Thus, although master power athletes benefit from improved bone mineral density compared to age-matched controls, decrements still occur with ageing (Piasecki et al., 2018).

Thus despite a resistance exercise and associated bone loading, the age-related decline in muscle mass, and bone and muscle quality suggest that there is an inherent ageing process that cannot be slowed, as suggested previously (Degens, 2012).

Is there a concurrent training effect?

Endurance exercise has long been associated with atrophy of type II fibres and an increase in muscle oxidative capacity (Baar, 2006, Staron et al., 1984) and therefore has been thought to diminish the resistance training-induced hypertrophy via the so-called concurrent training effect (Hickson, 1980). In addition, the inverse relationship between fibre size and oxidative capacity suggests that there is a trade-off between fibre size and oxidative capacity (van Wessel et al., 2010, van der Laarse et al., 1998), where due to this trade-off the endurance exercise-induced increase in oxidative capacity may cause muscle fibre atrophy. Yet, we have seen in rodent studies that this constraint on fibre size may be broken. For instance, hypermuscular myostatin null-mice overexpressing (Errγ) have a similar fibre size as the myostatin null mice, yet with an elevated oxidative capacity (Omairi et al., 2016), and hypertrophy of overloaded mouse plantaris muscles was accompanied by an increase in oxidative capacity (Ballak et al., 2016). However, the hypertrophied

fibres in the muscles of these mice are still smaller (1,500 μm^2 in (Ballak et al., 2016)) or the same size (up to 4,000 μm^2 in type IIb fibres; (Omairi et al., 2016)) as untrained human muscle fibres (4,000 μm^2; (Barnouin et al., 2017, Wust et al., 2009)). It could thus be that the size constraint is not yet reached, and that only in highly resistance-trained men with much larger fibres (8,000 μm^2 in our young group) any increase in oxidative capacity will constrain fibre size and induce atrophy. While we found that endurance training added to the regular resistance exercise of highly resistance-trained men induced an increase in oxidative capacity, this was not accompanied by a reduction in FCSA. These observations challenge the concept of a trade-off between fibre size and oxidative capacity.

There are several potential explanations to overcome the size constraint, such as a flattening of the fibres to reduce diffusion distances from the periphery to the core of the fibre, increased myoglobin levels to maintain oxygen availability to mitochondria even at low oxygen tension, movement of mitochondria to the periphery of the fibre and/or angiogenesis (Hendrickse and Degens, 2019). As in oxidative, more than in glycolytic, fibres mitochondria are more concentrated in the subsarcolemmal region (Wust et al., 2009), such a redistribution could also occur when endurance training is superimposed on resistance exercise. However, redistributing mitochondria to the subsarcolemmal region creates longer diffusion distances for ATP from the mitochondria to the ATP-consuming myofibrils in the core of the fibre (Kinsey et al., 2007) that could put in turn a diffusion limit on fibre size (Degens, 2012). It remains to be seen, however, whether such a redistribution occurred in our population. We did not see, however, any change in the roundness of the fibre (data not shown), indicating no significant change in the shape of the fibres, but we did see a significant increase in the number of capillaries around a fibre, in particular around type I fibres. A similar situation was seen in hypertrophied mouse plantaris where the increase in oxidative capacity and fibre size was accompanied by angiogenesis, and the attenuated hypertrophy in old mice was associated with impaired angiogenesis (Ballak et al., 2016). In the current study, we did not find evidence for an attenuated angiogenic response in the older highly resistance-trained men, similar to that seen in older women (Gavin et al., 2015). Thus, angiogenesis in our population may well have served to ensure an adequate oxygenation in the face of an increased oxidative capacity and helped to overcome the size constraint in both young and old highly resistance-trained men.

Muscle capillarisation may well be a determining factor in hypertrophy, as indicated by the attenuated hypertrophy in overloaded muscles from old mice that was associated with impaired angiogenesis (Ballak et al., 2016) and the lower hypertrophic response to resistance training in muscles with lower capillary density, particularly in elderly people (Snijders et al., 2017a, Moro et al., 2019). Based on these and the observations in the present study, we propose that endurance training prior to a dedicated resistance-training-only programme may augment increases in muscle mass (Hendrickse and Degens, 2019).

At first glance the increase in muscle oxidative capacity without a concomitant rise in VO_2max post training is difficult to understand, but VO_2max is limited by the cardiovascular system and not by the working muscle (McPhee et al., 2009). In addition, our data are in line with another study where the change in VO_2max did not correlated with the change in muscle SDH concentration after an endurance training programme (McPhee et al., 2011). Here we thus have muscular adaptations to the superimposed endurance-training programme, but apparently no, or only minimal cardiac adaptations. We have no explanation for this observation, but it could be that the weekly duration of the endurance training programme did not reach the threshold required for cardiovascular and cardiac adaptations to occur (Fagard, 2003). Another possibility to induce such adaptations is the use of high intensity endurance training, but then the risk of CTE may indeed develop (Sousa et al., 2019). Our study shows that moderate intensity endurance training of not too long duration elicits beneficial effects without inducing loss of muscle mass in resistance-trained people.

Limitations

Although participants did not follow a prescribed resistance-training regimen, all performed resistance exercise 4 times per week (2 sessions for upper body, 2 sessions for lower body). Another limitation is the homebased character of the endurance training, where therefore controls were minimal and HR monitor data were not available to researchers to check participants compliance. However, we did ask for a report of each session and analysis of these reports showed that each person completed at least 90% of the sessions. While resistance training was always performed first if participants trained both modalities in the same day, some participants undertook each modality on separate days, thus adding variability and making it more difficult to observe differences between groups.

Presenting muscle volume of the thigh musculature instead of ACSA would reduce the risk of measurement error. Additionally, ACSA underestimates the number of sarcomeres in parallel in the pennate quadriceps muscles, meaning that it is a less accurate indicator of hypertrophy than PCSA or muscle volume.

Electrical stimulation of the participants' muscles could have been performed and the interpolated twitch technique used to estimate true maximal torque. Electromyography (EMG) could also have been used to measure antagonist co-activation. Determining voluntary activation may have influenced the differences in strength as some studies have found reductions with ageing and coactivation of antagonist muscles has been shown to increase with age (Klass et al., 2009). These measures were not included to restrict the amount of time required for testing and electrical stimulation was not performed to limit the discomfort of participants.

Perspectives

Concurrent training in young and old resistance-trained men led to improvements in oxidative capacity without muscle fibre atrophy, thus providing evidence that the inverse relationship between fibre size and oxidative capacity can be overcome. Additionally, there were no reductions in MVC in both young and old subjects. Our observations add to evidence that challenges previous assumptions about the "jack of all trades, master of none" nature of concurrent training and support the concomitant use of resistance and endurance training in elderly people. A recent study of endurance, power and team athletes determined that team athletes, while not specialising in power or endurance specifically, had similar jump power to power athletes and similar VO_2max to endurance athletes (Degens et al., 2019) and incorporation of endurance training has been found to augment skeletal muscle hypertrophy under certain conditions (Murach and Bagley, 2016). As such, carefully considered incorporation of endurance training may provide endurance benefits to both old and young resistance-trained men without a reduction in muscle size or strength.

Prelude to Chapter 5

In chapter 4 it was shown that fibre cross sectional area and thigh anatomical cross-sectional area were not reduced after a superimposed endurance training programme while oxidative capacity and capillaries around type I fibres were increased. In the next chapter the effects of concomitant hypertrophic and endurance stimuli are studied in young and old mice. Similar to chapter 4 it is expected that inclusion of both training modalities will lead to impaired hypertrophy and blunted increases in fatigue resistance. Additionally, responses to these stimuli are predicted to be further blunted in old mice due to lower capillary density and diminished angiogenic response.

Chapter 5

REGULAR ENDURANCE EXERCISE OF OVERLOADED MUSCLE OF YOUNG AND OLD MALE MICE DOES NOT ATTENUATE HYPERTROPHY AND IMPROVES FATIGUE RESISTANCE

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*Please note this chapter is in American English in accordance with the guidelines of GeroScience

ABSTRACT

It has been observed that there is an inverse relationship between fiber size and oxidative capacity due to oxygen, ADP and ATP diffusion limitations. We aimed to see if regular endurance exercise alongside a hypertrophic stimulus would lead to compromised adaptations to both, particularly in older animals.

Here we investigated the effects of combining overload with regular endurance exercise in young (12 months) and old (26 months) male mice. The plantaris muscles of these mice were overloaded through denervation of synergists to induce hypertrophy and the mice ran on a treadmill for 30 min per day for 6 weeks.

The hypertrophic response to overload was not blunted by endurance exercise, and the increase in fatigue resistance with endurance exercise was not reduced by overload. Old mice demonstrated less hypertrophy than young mice (24.5% ± 8.3 vs 47.4 % ± 15.3, p=0.03), which was associated with impaired angiogenesis, evident in a smaller increase in capillary to fibre ratio (15.8% vs. 43.7% increase in young, p=0.010), and a 24% reduction in specific tension (p=0.045) with overload.

The data of this study suggest that combining endurance exercise and overload induces the benefits of both types of exercise without compromising adaptations to either. Additionally, the attenuated hypertrophic response to overload in old animals may be due to a diminished capacity for capillary growth.

INTRODUCTION

It has long been known that mechanical loading of a muscle leads to increases in strength, muscle fiber cross-sectional area and muscle mass, whereas regular endurance exercise induces, among other adaptations, a decrease in muscle mass, an increase in muscle oxidative capacity and angiogenesis (Baar, 2006). The combination of both hypertrophic and endurance stimuli is thought to result in a trade-off between the adaptations to each, resulting in a diminished response to the resistance or endurance exercise in comparison to these training modalities on their own (van Wessel et al., 2010). Part of such a diminished response may reflect an inverse relationship between fiber size and oxidative capacity that is thought to be due to diffusion limitations of oxygen (Degens, 2012, van Wessel et al., 2010, van der Laarse et al., 1998). These predictions have however never been formally tested. Therefore, here we seek to answer the fundamental question of whether there is a trade-off using a powerful hypertrophic stimulus (denervation-induced overload) alongside regular endurance exercise. Denervation-induced overload leads to a much greater hypertrophic response than resistance exercise, but elicits otherwise similar adaptations (Alway et al., 2005).

In the capillaries the exchange of oxygen, nutrients, heat and metabolites with the surrounding muscle fibers takes place. In line with the potential importance of oxygen diffusion limitations for fiber size is the observation that fiber size is the main determinant of capillary supply to a fiber (Ahmed et al., 1997, Degens et al., 1994b) and that hypertrophy is accompanied by angiogenesis (Degens et al., 1992, Egginton et al., 1998, Plyley et al., 1998). However, in mammalian muscle, capillaries can only be placed at the periphery of a fiber. Consequently, with ongoing growth the diffusion distance from the capillaries to the center of the fiber will increase and hence theoretically put a limit on fiber hypertrophy (Degens, 2012). This may be alleviated to some extent by reducing the fiber oxidative capacity, an adaptation opposite to endurance exercise-induced adaptations. It may therefore be predicted that the hypertrophic response to overload is reduced by regular endurance exercise, and the increase in oxidative capacity induced by regular endurance exercise is diminished when accompanied by a hypertrophic stimulus.

In addition to age-related reductions in muscle strength, muscle mass, capillarization, oxidative capacity and fatigue resistance, the adaptive responses to both hypertrophic and endurance stimuli have been shown to diminish with age in

humans and rodents (Ballak et al., 2016, Petrella et al., 2006, Larsson et al., 2019, Degens and Alway, 2003, Conley et al., 2000, Walters et al., 1991). The attenuated responses to overload and chronic electrical stimulation in old rodents were accompanied by a dampened angiogenic response (Ballak et al., 2016, Walters et al., 1991), and a lower capillarization in elderly people was associated with a poorer hypertrophic response (Snijders et al., 2017a, Moro et al., 2019). These observations suggest that a diminished angiogenic response and/or lower capillary network in old age may underlie the diminished muscle plasticity seen within this time period (Hendrickse and Degens, 2019). Given the importance of the capillary network for fatigue resistance (Tickle et al., 2020), a lower capillary density in old age (Hendrickse and Degens, 2019) may also result in reduced fatigue resistance, as has particularly been seen in old age during repetitive shortening, but not isometric, contractions (Callahan and Kent-Braun, 2011).

The first objective of our study was therefore to assess whether there is a trade-off between the functional and morphological adaptations to overload and regular endurance exercise. Thereto, the plantaris muscle from young and old mice was simultaneously subjected to overload by denervation of synergists and daily treadmill running. Denervation of synergists typically induces 30% hypertrophy in the overloaded muscle (Degens et al., 1992). The second objective was to assess whether muscle plasticity was 1) diminished in old age 2) and whether reduced muscle plasticity was related to a less dense microvascular network and attenuated angiogenesis. We hypothesized that 1) overload-induced hypertrophy is attenuated by endurance exercise and 2) an endurance exercise-induced increase in fatigue resistance is attenuated by overload. We also proposed that 3) both overload and endurance exercise induce angiogenesis and 4) that responses to both endurance exercise and overload are blunted in old mice that is associated with a less dense microvascular network and attenuated angiogenesis.

MATERIALS AND METHODS

Animals

Fourteen 12-month-old (young adult) and 10 26-month-old (old) male C57BL/6J mice were kept individually with *ad libitum* access to standard chow (LabDiet5001: protein 28.7%, carbohydrate 57.9%, fat 13.4%) and water at 22°C on a reversed 12 hour light/dark cycle at the Lithuanian Sports University. All animal procedures were approved by the Lithuanian State Food and Veterinary Service and Manchester

Metropolitan University (SE171810). Twelve-month-old mice were used in the young group as maturational muscle fibre growth continues until this age, meaning that comparisons between old animals and young animals below this age may mask any age-related loss of fibre size or changes in capillarisation (Larsson et al, 2019). Figure 1 outlines the study design.

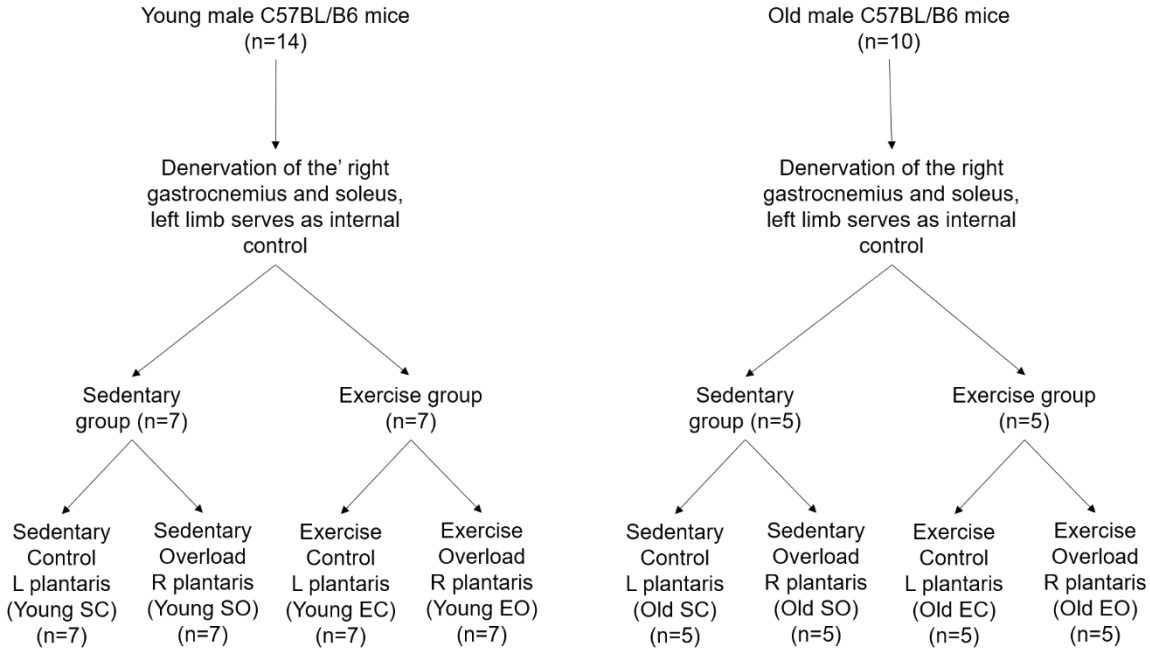

Figure 1. Study design. Fourteen young (12 months) and 10 old (26 months) male C57BL/B6 mice were subject to denervation surgery in which the plantaris muscles of the right hindlimb were overloaded through denervation of the gastrocnemius and soleus muscles. The left plantaris served as an internal control. Mice of each age group were then split into sedentary and exercise groups, the latter undergoing 30 minutes of treadmill running 5 days·week^{-1}. The sedentary mice had a sedentary control plantaris muscle (SC) and an overloaded plantaris muscle (SO) while the exercised mice had an exercised control plantaris muscle (EC) and an exercised overloaded plantaris muscle (EO).

Surgery to overload the plantaris muscle

To overload the plantaris muscle of the right leg, the gastrocnemius and soleus muscles were denervated by cutting the medial and lateral branches of the tibial nerve as described previously (Degens and Alway, 2003). A section of the nerve was removed to prevent reinnervation. The surgery was performed under aseptic conditions and animals were anesthetized with an intraperitoneal injection of a mix

of ketamine (100 mg·kg^{-1}) (Ketamidor, Richter Pharma AG, Wels, Austria) and xylazine (16 mg·kg^{-1}) (Sedaxylan, Eurovet, Bladel, Netherlands) in saline. After surgery, the wound was closed with suture and the animal was given ketoprofen (2.5 mg·kg^{-1}) (Rifen, Eurovet, Bladel, Netherlands) as an analgesic immediately after surgery and again 24 hours later. The contralateral plantaris muscle served as the internal control as it has previously been shown that unoperated limbs of overloaded animals show no differences in enzyme activity and muscle mass when compared to those of sham operated animals (Baldwin et al. 1977).

Endurance exercise

One week after recovery from surgery half of the animals were allocated to a control group and the other half to an endurance exercise group. The endurance exercise consisted of running at a speed of 12 m·min^{-1}, 30 min·day^{-1}, 5 days·week^{-1} at 0º for 6 weeks. This program has been shown to induce an increase in the oxidative capacity of at least the quadriceps muscle without causing muscle damage (Savage and McPherron, 2010). A shock-grid was used to encourage running and mice were shocked an average of 6 times per session. Mice that refused to run during the first 5 days were removed from the exercise group and replaced with an animal from the non-exercise group. This was to maintain the numbers of animals in each group but this meant that there were no dropouts in the study and introduced a selection bias into the study as only mice willing to run were included in the exercise group. Exercise was undertaken in the dark with a red light.

Terminal experiment

Seven weeks after the surgery to overload the plantaris muscle, the mice were anesthetized as described above. The age of the mice at the terminal experiments was 14 months and 28 months for the young-adult and old mice, respectively. The pedal withdrawal reflex was tested every 10 minutes and 20% of the original dose was administered to the animal when required. The gastrocnemius muscle of one leg was removed, exposing the sciatic nerve, and weighed. The animal was then moved to the *in-situ* force measurement apparatus and its body temperature kept at 36.5°C. The distal plantaris tendon was cut and attached to the force transducer (Dual-Mode Muscle Lever Systems 305C-LR Aurora Scientific, Ontario, Canada) using 3-0 silk suture.

The sciatic nerve was cut proximally and stimulated with incremental 0.2-ms pulses every 30 s until maximal twitch force was obtained. Then the current was increased

by 10% to supramaximally stimulate the muscle every 30 s to determine optimal length, defined as the length at which the muscle produced the maximal active twitch force.

The muscle was then stimulated twice, 5 min apart, at 200 Hz for 300 ms to determine maximal isometric tetanic force. Five minutes after the last tetanus, the muscle was subjected to a series of 330-ms 40-Hz trains once every second for 4 min to assess fatigue resistance. A fatigue index was calculated as the force in the last contraction divided by the force in the strongest contraction during the series.

The same process was then repeated on the contralateral limb. The overloaded and control muscles were stimulated in random order. After the contractile properties of the plantaris muscles were determined in both legs, the animal was euthanized with carbon dioxide. Both plantaris muscles were then removed, weighed and mounted in OCT embedding medium in liver (Thermo Scientific, Loughborough, UK), frozen in liquid nitrogen and stored at -80°C.

Muscle morphology

Serial 10-µm sections were cut from the mid-belly of each plantaris muscle at -20°C using a cryostat.

Two serial slides with three sections each were blocked with 10% goat serum (Vector Laboratories, USA) in phosphate-buffered saline (PBS) for 60 minutes. Then one slide was incubated for 2 hrs with monoclonal antibodies BA-D5 (1:600), SC-71 (1:600) and 6H1 (1:50) (Developmental Studies Hybridoma Bank, USA) in blocking solution to identify type I, IIa and IIx fibers, respectively. The serial slide was incubated for 2 hrs with BF-F3 (1:100) (Developmental Studies Hybridoma Bank, USA) and biotinylated *Griffonia simplicifolia* lectin I, (5 $\mu L \cdot mL^{-1}$) (Vector Laboratories, USA) to identify type IIb fibers and capillaries, respectively. After three 5-min PBS washes the first slide was incubated in secondary antibodies Alexa Fluor 350 IgG2b for type I (1:500), Alexa Fluor 488 IgG1 for type IIa (1:500) and Alexa Fluor 555 IgM for type IIx (1:500) (Thermofisher Scientific, USA). The second slide was incubated in Alexa Fluor 555 IgM for type IIb (1:500) and Streptavidin, Alexa Fluor 350 conjugate (1:200) (Thermofisher Scientific) for capillaries. After three more 5-min washes in PBS the slides were mounted with ProLong Diamond Antifade mountant (Thermofisher Scientific, USA).

Digital images of the deep and superficial regions of the plantaris muscle were taken using a fluorescent microscope (Zeiss Axio Imager Z1, Zeiss, Germany) at 20x magnification. For each image, fibers were traced and typed, and coordinates of capillaries were recorded manually using BTablet (BaLoH Software, Ooij, Netherlands). Using these data, fiber cross-sectional area (FCSA), fiber type composition, capillary density (CD), capillary to fiber ratio (C:F), capillary domain size (the area geometrically supplied by a single capillary, μm^2), local capillary to fiber ratio (LCFR) (the sum of the fractions of each domain which overlap the fiber), capillary fiber density (CFD) (LCFR divided by the cross sectional area of a fiber, mm^{-2}) and an index of the heterogeneity of capillary spacing (Log_RSD) were calculated using AnaTis (BaLoH software, Ooij, Netherlands) (Degens et al., 1992). An average of 158 fibers were analyzed per region. The percentage of non-contractile material was calculated by subtracting the total fiber area from the total domain area and dividing by the total domain area.

Succinate dehydrogenase

A third serial slide was stained for succinate dehydrogenase (SDH) activity as described previously (Ballak et al., 2016). The optical density (OD) of the stain at 660 nm was used as a quantitative indication of oxidative capacity (van der Laarse et al., 1989). For each sample a series of filters with a known OD were used to create a calibration curve to adjust for variation in background staining and lighting between sections. The OD of the SDH stain was determined using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.net/Downloads). Integrated SDH activity was calculated as the SDH OD multiplied by the FCSA.

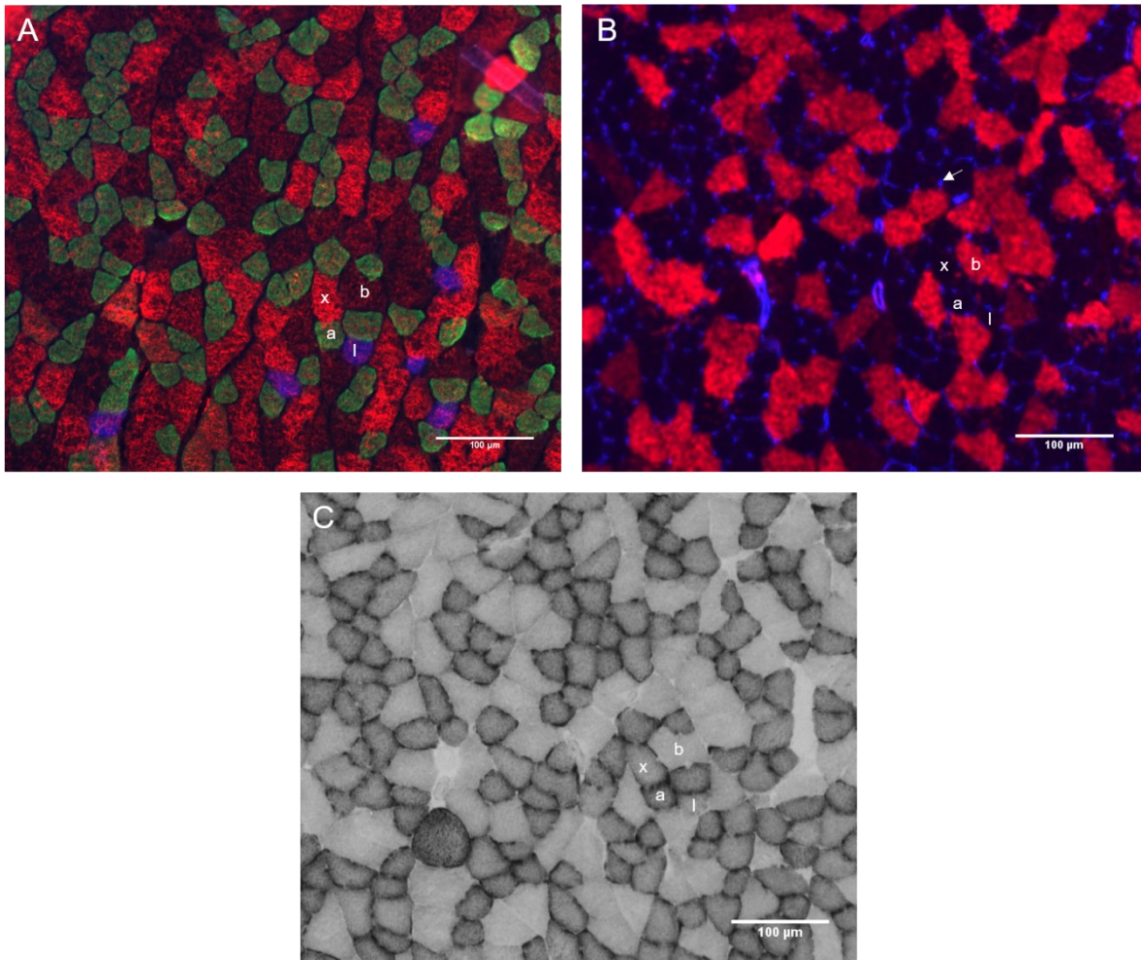

Figure 2. Serial sections of plantaris muscles from a young male sedentary control (SC). Sections were stained for (A) type I (blue, denoted by I), type IIa (green, by a) and type IIx fibers (red, denoted by x), (B) type IIb fibers (red, denoted by b) and capillaries (blue, denoted with an arrow) and (C) succinate dehydrogenase (SDH) activity.

Statistics

Data were analyzed with IBM SPSS version 25. Shapiro-Wilk tests were used to determine if data were normally distributed. In cases where data were not normally distributed, data were transformed logarithmically. A repeated-measures ANOVA with as within factors overload and muscle region, and between factors age, exercise and fiber type (5 levels: IIa, IIb, IIx, IIa/IIx, IIb/IIx) was performed. The effects of overload, age and exercise were also analyzed irrespective of fiber type on the pooled fibers. In addition to main effects, two-way interactions were considered between each factor pair. Effects and interactions were considered significant at $p<0.05$. A Bonferroni correction was applied to test differences between fiber types.

RESULTS

Body mass, muscle masses and connective tissue

The body mass of the mice was decreased 7 weeks after induction of overload ($p<0.001$) (Table 1). Old mice had a lower gastrocnemius muscle mass than young-adult mice ($p<0.001$), and the denervated gastrocnemius muscle mass was lower compared to control muscles ($p<0.001$; Table 1). The percentage of plantaris muscle cross-sectional area consisting of non-contractile material was similar in both age groups and unchanged by overload and exercise (Table 1). Control plantaris muscle mass was similar in both age groups. Overload led to an increase in plantaris muscle mass ($p<0.001$), but the 'age*overload' interaction ($p=0.03$) was reflected by a smaller increase in mass in the old than young-adult mice (Fig. 3A). There was no significant 'exercise*overload' interaction ($p=0.998$), indicating that the overload-induced increase in plantaris muscle mass was similar in trained and non-trained animals. Similarly, plantaris mass normalized to body mass was increased by overload ($p<0.001$) and this increase was smaller in old mice ($p=0.003$) (Table 1).

Table 1: *Body mass, gastrocnemius muscle mass, plantaris mass normalized to body mass and proportion of non-contractile material in young and old C57BL/6J mice after denervation to induce hypertrophy of the plantaris muscle and/or endurance exercise.*

	Young (12 months)				**Old (26 months)**				**Significant effects (p<0.05)**
	Sedentary pre	**Sedentary post**	**Exercise pre**	**Exercise post**	**Sedentary pre**	**Sedentary post**	**Exercise pre**	**Exercise post**	
Body mass (g)	38.1 ± 3.2	37.5 ± 4.8	41.5 ± 7.3	34.8 ± 5.8	41.8 ± 12.3	36.9 ± 9.4	30.6 ± 9.0	27.9 ± 8.7	T***
	SC	**SO**	**EC**	**EO**	**SC**	**SO**	**EC**	**EO**	
Gastrocnemius mass (mg)	129 ± 11	74 ± 11	133 ± 15	74 ± 10	122 ± 13	60 ± 7	90 ± 43	63 ± 15	O***, H***
Plantaris mass normalized to body mass (mg g $^{-1}$)	0.572 ± 0.114	0.849 ± 0.166	0.610 ± 0.097	0.882 0.105	0.548 ± 0.170	0.678 ± 0.258	0.646 ± 0.056	0.822 ± 0.112	O***, OH**
Non-contractile material (%)	8.34 ± 4.93	7.63 ± 8.22	7.33 ± 4.41	8.22 ± 2.70	10.03 ± 7.36	7.79 ± 4.93	6.98 ± 3.94	10.05 ± 9.76	

*SC = sedentary control, SO = sedentary overload, EC = exercise control, EO = exercise overload. Data are expressed as means ± standard deviation. T: time effect O: age effect, H: overload effect. ** denotes p<0.01 ***denotes p<0.001*

Contractile properties and fatigue resistance

The maximal isometric tetanic force of the plantaris muscles was lower in old than young-adult mice ($p<0.001$, Fig. 3B). It can be seen in figure 3B that the age*overload interaction ($p<0.001$) was reflected by a higher tetanic force of the overloaded than control muscles in young animals only ($p=0.002$). Specific tension was lower in old than young-adult animals ($p<0.001$, Fig. 3C), and while the specific tension was maintained with overload in young animals, it was lower in overloaded muscles of old animals (age*overload interaction $p=0.045$). Fatigue resistance was lower in old animals ($p=0.001$), and the plantaris muscle of exercised mice had a greater fatigue resistance than those from non-exercised mice, irrespective of age or overload ($p<0.001$, Fig. 3D).

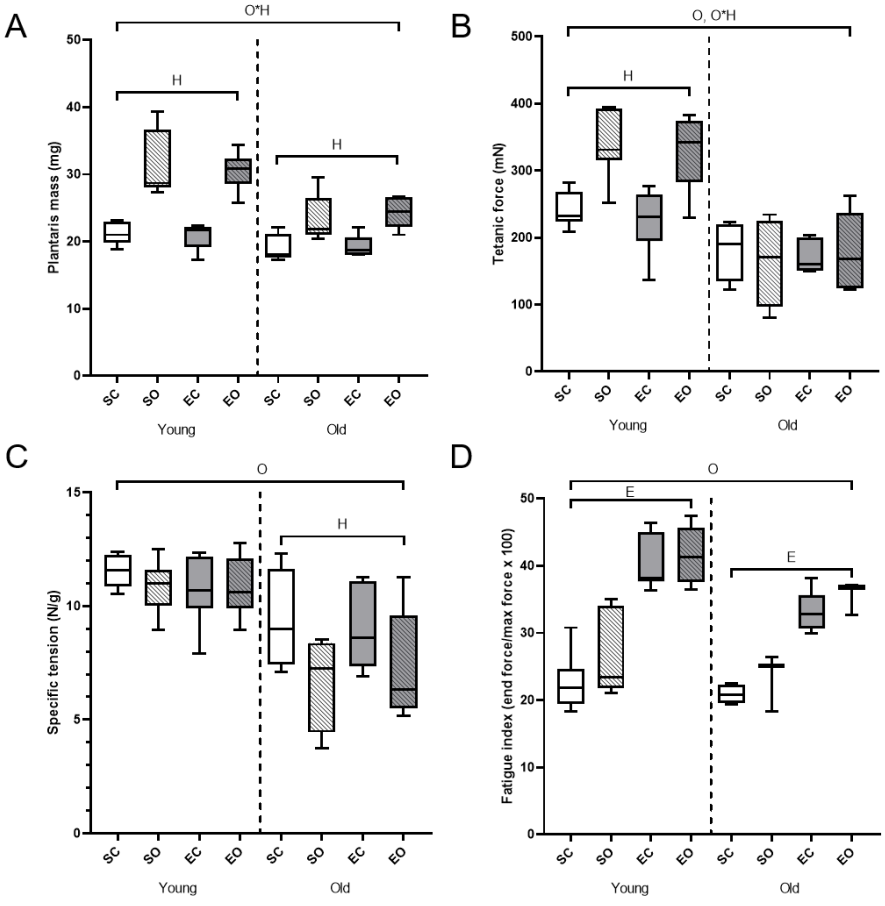

Figure 3. Effects of age, overload and exercise on plantaris muscle mass and contractile properties in C57BL/6J mice. A plantaris muscle mass B maximal tetanic force, C specific tension as tetanic force/muscle mass and D fatigue resistance. SC = sedentary control, SO = sedentary overload, EC = exercise control, EO = exercise overload. O: effect of age (p≤0.001), H: effect of overload-induced hypertrophy

*(p≤0.021), E: effect of exercise (p<0.001), O*H: old age*hypertrophy interaction (p≤0.03)*

Fiber type composition

Since the proportion of type I and I/IIa fibers was <1% in almost all muscle regions (Table 2), these fiber types were excluded from further analysis. The percentage of type IIa, IIa/IIx and IIb/IIx fibers was larger in overloaded compared to control muscles (p≤0.020) and the proportion of type IIb fibers was lower (p<0.001). The proportion of type IIx fibers was lower in muscles of exercised mice (p=0.037). The overload*exercise interaction (p=0.039) for the proportion of type IIb/IIx fibers was reflected by a lower proportion of type IIb/IIx in control exercised, but not in overloaded exercised, than in corresponding non-exercised muscles.

The proportions of type IIa and IIa/IIx fibers were greater in the oxidative than in the glycolytic region of the plantaris muscle (p<0.001, Table 2), while for type IIb and IIb/IIx fibers the opposite was found (p≤0.029). The higher proportion of type IIa/IIx fibers in the oxidative region when compared to the superficial region was more pronounced in muscles from old than from young-adult mice (age*region interaction p=0.047).

Table 2: Fiber type composition (%) and fiber cross sectional area (FCSA) (µm²) for different fiber types in the oxidative and glycolytic regions of plantaris muscles exposed to overload and endurance exercise in young and old C57BL/6J mice.

	Young				Old				Effects and interactions
	SC	SO	EC	EO	SC	SO	EC	EO	
Fiber type composition (%)									
Oxidative region									
Type I	0.1 ± 0.2	6.7 ± 4.8	0.2 ± 0.5	3.2 ± 4.4	0.3 ± 0.6	0.3 ± 0.6	0.6 ± 0.8	0.2 ± 0.5	
Type I/IIa	-	0.9 ± 1.1	-	0.1 ± 0.3	-	0.2 ± 0.4	-	-	
Type IIa	30.5 ± 9.3	32.4 ± 15.3	22.2 ± 17.8	40.1 ± 10.4	19.9 ± 9.4	37.8 ± 9.5	29.3 ± 11.9	44.9 ± 10.6	R***, H***
Type IIa/IIx	8.1 ± 6.0	10.0 ± 6.8	2.1 ± 1.8	12.3 ± 5.7	9.2 ± 7.2	12.6 ± 6.8	7.6 ± 2.0	12.1 ± 3.2	R***, H**, OR*
Type IIx	22.1 ± 7.2	18.0 ± 6.5	20.8 ± 7.3	14.6 ± 4.2	19.7 ± 6.5	25.1 ± 8.5	14.9 ± 3.3	17.4 ± 10.9	E*
Type IIb/IIx	3.8 ± 2.7	7.4 ± 6.5	2.6 ± 1.5	6.4 ± 5.5	9.4 ± 8.2	4.8 ± 2.4	4.5 ± 2.6	8.6 ± 8.2	R*, H*, OH*
Type IIb	35.4 ± 9.4	24.7 ± 12.0	52.2 ± 22.8	23.4 ± 6.8	41.4 ± 18.3	19.2 ± 8.6	43.1 ± 13.9	16.9 ± 17.5	R***, H***
Glycolytic region									
Type I	-	3.3 ± 3.7	0.5 ± 1.0	0.5 ± 0.9	-	-	-	-	
Type I/IIa	-	1.3 ± 1.1	-	-	-	-	-	-	
Type IIa	7.4 ± 3.7	23.9 ± 14.1	12.9 ± 16.2	26.2 ± 7.1	14.4 ± 10.1	16.2 ± 11.8	18.1 ± 8.7	27.6 ± 8.5	R***, H***
Type IIa/IIx	6.0 ± 4.1	8.3 ± 5.4	2.2 ± 2.0	8.8 ± 8.7	3.0 ± 2.1	6.3 ± 6.7	4.4 ± 1.7	10.2 ± 6.0	R***, H**, OR*
Type IIx	14.3 ± 5.7	20.7 ± 6.2	16.6 ± 9.6	16.2 ± 10.4	19.1 ± 8.6	24.4 ± 4.2	16.1 ± 4.2	15.0 ± 10.1	E*
Type IIb/IIx	5.4 ± 3.8	10.5 ± 5.8	3.0 ± 3.2	7.0 ± 7.4	13.6 ± 9.7	12.0 ± 7.6	4.2 ± 1.5	11.5 ± 3.7	R*, H*, OH*
Type IIb	67.0 ± 10.8	32.0 ± 12.5	64.8 ± 25.8	41.3 ± 9.9	49.3 ± 18.4	41.1 ± 14.4	57.1 ± 10.1	35.6 ± 13.7	R***, H***
FCSA (μm²)									
Oxidative region									
Type I	367 ± 127	1057 ± 205	706 ± 140	940 ± 329	619 ± 76	735 ± 23	617 ± 315	538 ± 12	
Type I/IIa	-	1102 ± 39	-	1364 ± 20	-	750 ± 59	-	-	
Type IIa	709 ± 195	1362 ± 364	654 ± 192	1237 ± 454	618 ± 151	1075 ± 262	533 ± 157	1092 ± 298	O**, R***, H*** b***, x***, ax***, bx***
Type IIa/IIx	1124 ± 193	1784 ± 357	1146 ± 192	1722 ± 571	805 ± 257	1506 ± 273	745 ± 221	1416 ± 375	O*, H*** a***, b***, x**, bx***
Type IIx	1283 ± 290	2109 ± 420	1121 ± 257	2090 ± 426	1021 ± 271	1742 ± 370	946 ± 252	1648 ± 469	O*, R***, H*** a***, b***,ax**, bx*
Type IIb/IIx	1577 ± 238	2429 ± 705	1454 ± 217	2199 ± 519	1127 ± 312	1687 ± 274	1221 ± 226	1767 ± 386	O***, H***, OH**, RE*, HE*** a***, x* ax***
Type IIb	1667 ± 285[a]	2809 ± 706[a]	1651 ± 388[a]	2566 ± 529	1344 ± 351	1863 ± 433	1228 ± 334	2071 ± 471	O***, H*** a***, x***, ax***
Glycolytic region									
Type I	-	1102 ± 147	6900 ± 69	814 ± 27	-	-	-	-	
Type I/IIa	-	1177 ± 191	-	-	-	-	-	-	
Type IIa	647 ± 123	1456 ± 509	574 ± 133	1011 ± 315	521 ± 126	895 ± 178	497 ± 145	773 ± 210	O**, R***, H*** b***, x***, ax***, bx***
Type IIa/IIx	867 ± 187	1855 ± 395	837 ± 134	1532 ± 238	663 ± 273	1506 ± 273	674 ± 102	1232 ± 272	O*, H*** a***, b***, x**, bx***
Type IIx	1065 ± 282	2253 ± 524	927 ± 195	1838 ± 356	844 ± 285	1401 ± 452	850 ± 196	1552 ± 437	O*, R***, H*** a***, b***,ax**, bx*
Type IIb/IIx	1327 ± 244	2906 ± 714	1366 ± 396	1871 ± 376	1006 ± 267	1769 ± 302	1232 ± 272	1679 ± 419	O***, H***, OH**, RE*, HE*** a***, x* ax***
Type IIb	1709 ± 370	3136 ± 631	1528 ± 338	2378 ± 579	1242 ± 322	1978 ± 473	1142 ± 266	1990 ± 475	O***, H*** a***, x***, ax***

*SC = sedentary control, SO = sedentary overload, EC = exercise control, EO = exercise overload. Data are expressed as means ± standard deviation. O: age effect, R: region effect, H: hypertrophy effect, E: exercise effect, OR: age*region interaction, OH: age*hypertrophy interaction, OE: age*exercise interaction, RH: region*hypertrophy interaction, RE: region*exercise interaction, HE: hypertrophy*exercise interaction, a: significantly different from type IIa, b: significantly different from type IIb, x: significantly different from IIx, ax: significantly different from type IIa/IIx hybrid, bx: significantly different from type IIb/IIx hybrid, *: p<0.05, **: p<0.01, ***: p<0.001*.

Fiber cross-sectional area

The pooled FCSA was lower in the plantaris muscles of old than young animals ($p<0.001$, Fig.4A) and was increased with overload in both age groups ($p<0.001$).

The pooled FCSA was smaller in the oxidative than the glycolytic region ($p<0.014$, Fig. 4B&C). Overload led to an increase in average FCSA in both regions of the muscle ($p<0.001$). Exercised mice had a lower pooled FCSA in the glycolytic, but not the oxidative, region of the muscle ($p=0.019$) (region*exercise effect $p=0.017$, Fig. 4B&C).

We also studied differences between fiber types, region, age, overload and exercise (Table 2). As with fiber type composition, type I and I/IIa fibers were excluded from statistical analyses due to their low prevalence in the plantaris muscle. Type IIa fibers were smaller than fibers of all other types ($p<0.001$), and type IIb and IIb/IIx were larger than fibers of all other types ($p\leq0.044$). Type IIa and IIx fibers were larger in the oxidative than in the glycolytic region ($p<0.001$).

While the FCSA was greater in overloaded muscles irrespective of fiber type ($p<0.001$), the hypertrophic response was less in type IIb/IIx fibers in old than young-adult muscles (age*hypertrophy interaction $p=0.007$). Exercise resulted in a decrease in the FCSA of type IIb/IIx fibers in the glycolytic, but not in the oxidative region of the muscle (region*exercise interaction $p=0.045$) and attenuated the hypertrophic response of type IIb/IIx fibers (overload*exercise interaction $p<0.001$).

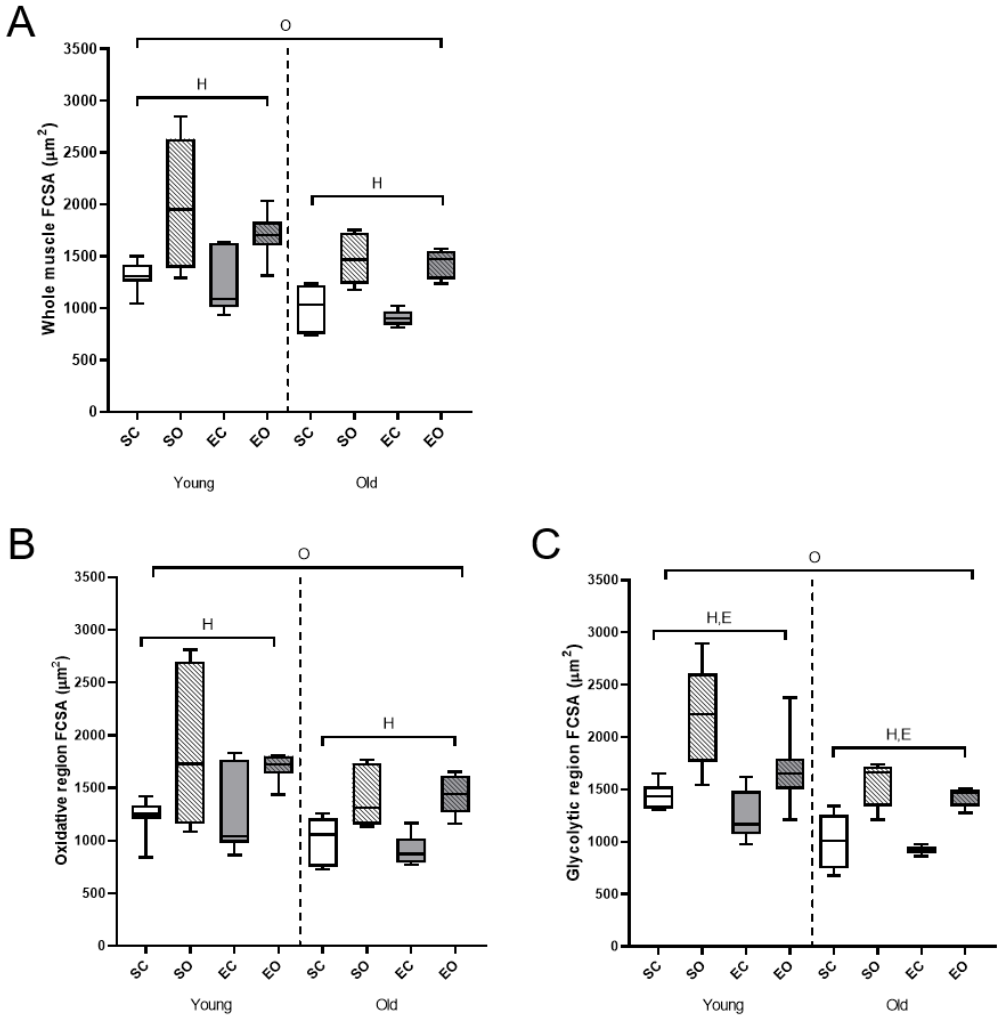

Figure 4. Effects of age, overload and exercise on fiber cross-sectional area (FCSA) C57BL/6J mice. A whole muscle, B oxidative region and C glycolytic region. SC = sedentary control, SO = sedentary overload, EC = exercise control, EO = exercise overload. O: effect of age (p<0.001), H: effect of overload-induced hypertrophy (p<0.001), E: effect of exercise (p=0.019)

Overall Capillarization

The oxidative region of plantaris muscles had greater C:F than the glycolytic region (Fig. 5A&B; p<0.001). Overload increased the C:F in the plantaris muscle (p<0.001, Fig. 5A-C), but less so in old than young-adult mice (age*overload interaction p=0.010). There was no significant effect of the exercise program on C:F (Fig 5A-C).

The oxidative region of the plantaris muscle had a higher CD compared to the glycolytic region (p<0.001, Fig. 5D&E). The plantaris muscles of old animals had a

higher CD than young animals ($p<0.001$, Fig. 5D-F). Overloaded muscles demonstrated a lower CD compared to control muscles in old ($p=0.005$), but not in young animals (age*hypertrophy interaction $p=0.028$).

The heterogeneity of capillary spacing (Log_RSD) was not significantly affected by region, age, overload or exercise (Fig. 5G-I).

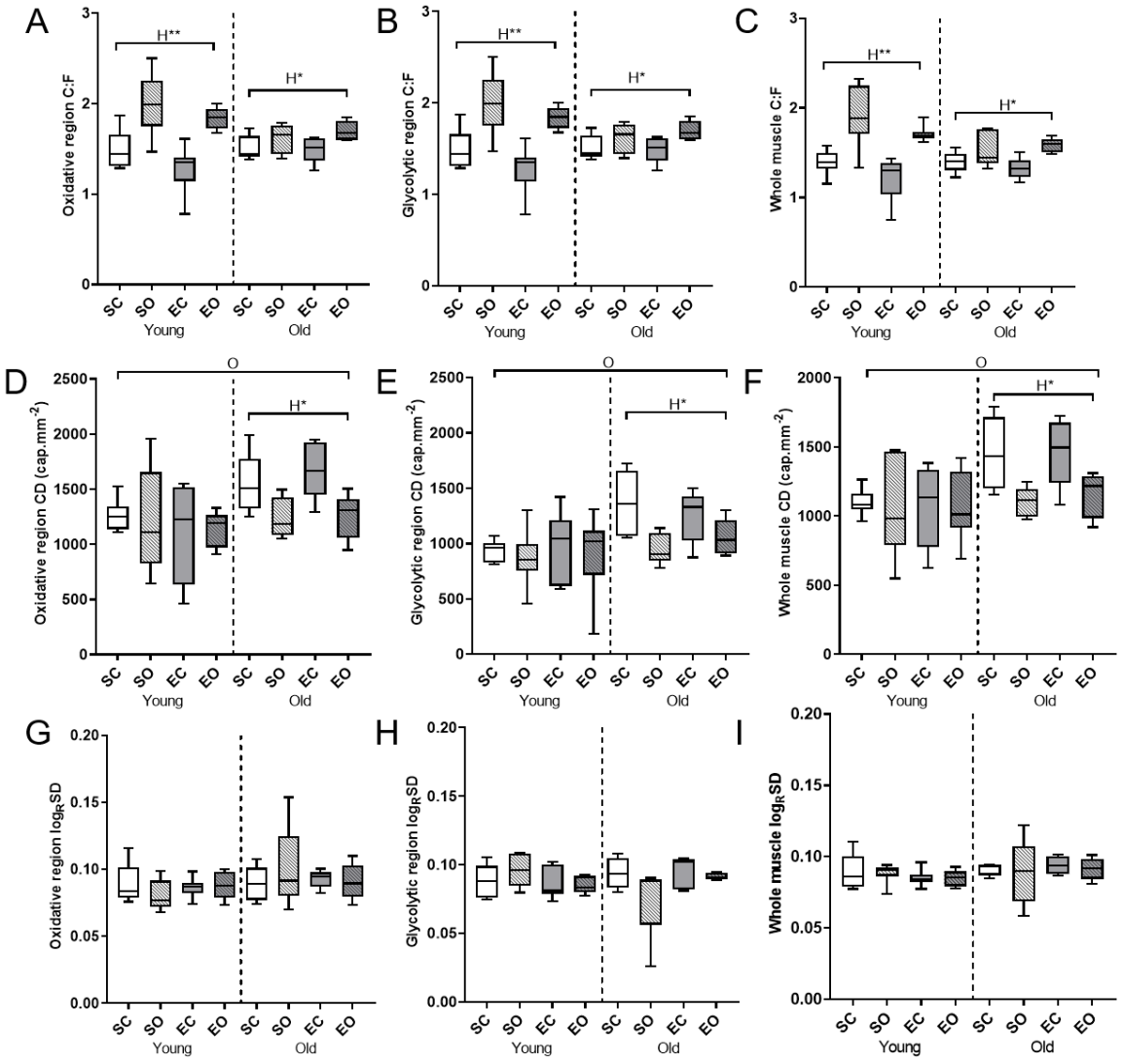

Figure 5 Effects of age, overload and exercise on skeletal muscle capillarization in C57BL/6J mice. A, B and C show capillary to fiber ratio (C:F) in the oxidative region (A), glycolytic region (B) and whole plantaris muscle (C). D, E and F show capillary density (CD) in the oxidative region (D), glycolytic region (E) and the whole muscle (F). G, H and I show the logarithmic standard deviation of the radius of the capillary domains (Log_RSD) in the oxidative region (G), glycolytic region (H) and whole muscle (I). SC = sedentary control, SO = sedentary overload, EC = exercise control, EO = exercise overload. O: effect of age ($p<0.001$), H: effect of overload-induced hypertrophy ($p<0.005$), H**: effect of overload-induced hypertrophy ($p<0.001$)*

Local capillary to fiber ratio

Supplementary Table 1 shows the LCFR data. Each fiber type had a greater LCFR in the oxidative than glycolytic region ($p \leq 0.044$) except for type IIa/IIx fibers. The LCFR of type IIb fibers from old muscles was lower than that in young-adult animals ($p=0.019$). The overload-induced increase in LCFR in all fiber types was less in old than young animals (age*hypertrophy interaction $p \leq 0.030$), except for type IIa/IIx fibers that demonstrated a similar increase in both age groups. The age*exercise interaction ($p \leq 0.033$) for type IIb and IIb/x fibers was reflected by an exercise-induced decrease in the LCFR of these fibers in young-adult, and an increase in old animals (Supplementary Table 1).

Capillary fiber density

In Supplementary Table 1 it can be seen that for all fiber types, except type IIb/IIx fibers, the CFD was greater in the oxidative than in the glycolytic region of the plantaris muscle ($p \leq 0.013$). The CFD was higher in old than young-adult animals, irrespective of fiber type ($p \leq 0.042$). Old animals demonstrated greater differences in type IIa/IIx CFD between regions than young animals (age*region interaction $p=0.004$).

Overloaded muscles had a lower CFD for all fiber types than control muscles ($p \leq 0.008$), with greater reductions in type IIa and IIa/IIx fibers of old than young-adult mice (age*overload interaction $p \leq 0.003$). The reduction in CFD of type IIa/IIx fibers after overload was less in the glycolytic than oxidative region of the muscle (region*hypertrophy interaction $p<0.001$). There was also a region*exercise interaction ($p \leq 0.047$) for type IIa/IIx and IIb/IIx fibers.

Succinate dehydrogenase

Old animals demonstrated lower average SDH OD than young-adult animals ($p<0.001$, Fig. 6A-C), which was the case for each fiber type, except type IIb fibers ($p \leq 0.033$, Supplementary Table 1). The SDH OD of type IIa and type IIa/IIx fibers was lower in overloaded muscle ($p \leq 0.010$), but this overload-induced reduction was less pronounced in old animals (age*hypertrophy interaction $p=0.008$). Additionally, the type IIa/IIx fibers of young animals showed an increase in SDH OD with exercise, whereas old animals did not (age*exercise interaction $p=0.020$). The region*overload ($p=0.004$) and region*exercise ($p=0.013$) interactions for the SDH

OD of type IIb/IIx fibers was difficult to interpret as there were no consistent patterns visible.

Supply:demand ratio

To assess differences in the matching of oxygen supply (LCFR) to demand (SDH-INT) of a fiber, the LCFR/SDH-INT was calculated as a measure of the supply:demand ratio (Fig. 6D-F). Old mice had a greater LCFR/SDH-INT than young-adult mice (p=0.038, Fig. 6A-C). The age*overload interaction (p=0.033) was reflected by a reduction in LCFR/SDH-INT in old (p<0.001), but not young-adult animals. There were also region*overload (p=0.003) and region*exercise (p=0.014) interactions.

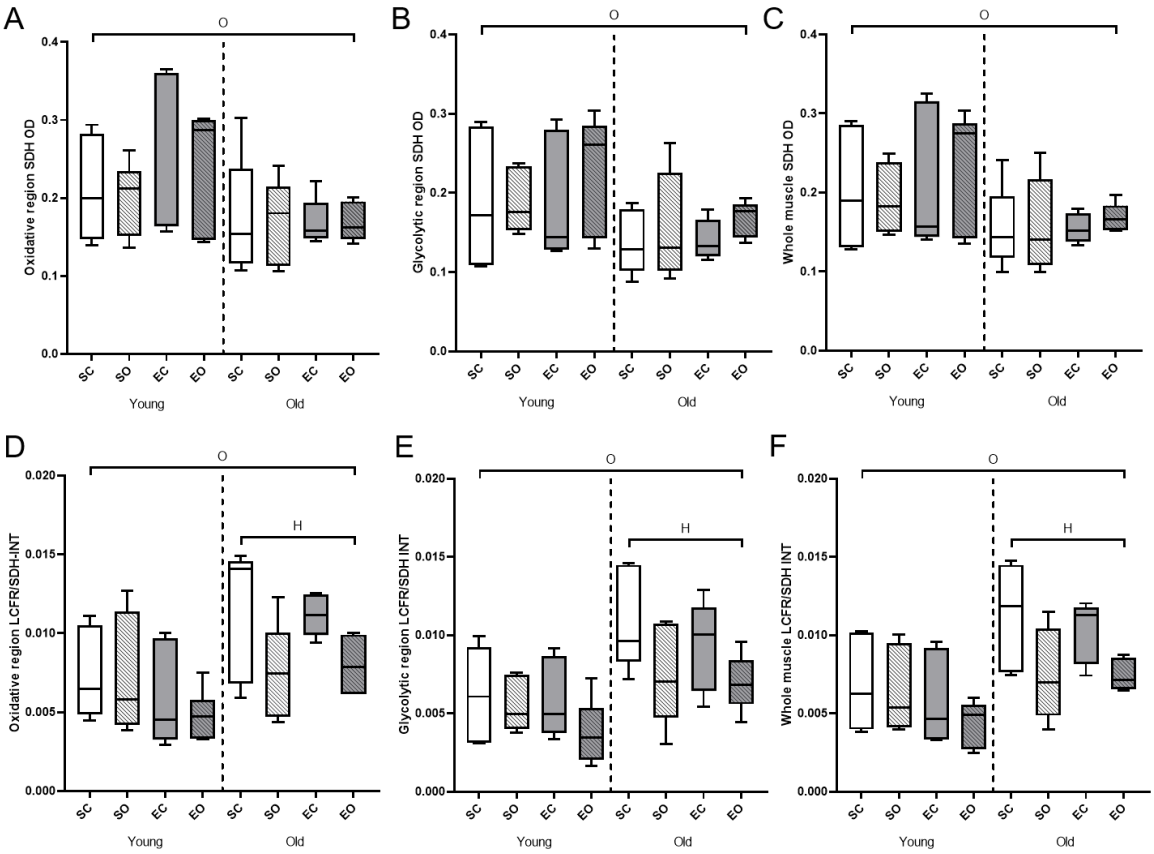

Figure 6 Effects of age, overload and exercise on succinate dehydrogenase optical density (SDH OD) and local capillary to fiber ratio (LCFR):integrated succinate dehydrogenase activity (SDH-INT) in C57BL/6J mice. A, B and C show SDH OD in the oxidative region (A), glycolytic region (B) and whole plantaris muscle (C). D, E and F show LCFR/SDH-INT in the oxidative region (D), glycolytic region (E) and the whole muscle (F). SC = sedentary control, SO = sedentary overload, EC = exercise control, EO = exercise overload. O: effect of age (p ≤ 0.038), H: effect of overload-induced hypertrophy (p<0.001).

Table 3 Local capillary to fiber ratio (LCFR) and capillary fiber density (CFD) for different fiber types in the oxidative and glycolytic regions of plantaris muscles exposed to overload and endurance stimuli in young and old C57BL/6J mice.

	Young				Old				Effects and interactions
	SC	**SO**	**EC**	**EO**	**SC**	**SO**	**EC**	**EO**	
Oxidative region									
Type IIa									
LCFR	1.06 ± 0.37	1.70 ± 0.39	0.93 ± 0.29	1.60 ± 0.31	1.20 ± 0.46	1.26 ± 0.17	1.16 ± 0.14	1.51 ± 0.33	R***, H***, OH**
CFD	1496 ± 196	1398 ± 479	1503 ± 502	1381 ± 238	1881 ± 429	1314 ± 268	2146 ± 163	1468 ± 299	O**, R***, H***, OH**
Type IIa/IIx									
LCFR	1.44 ± 0.39	2.17 ± 0.64	1.34 ± 0.80	2.08 ± 0.69	1.60 ± 0.69	1.36 ± 0.28	1.61 ± 0.26	1.95 ± 0.64	H*
CFD	1481 ± 158	1246 ± 373	1225 ± 188	1294 ± 244	1792 ± 374	1113 ± 234	2025 ± 225	1453 ± 363	O*, R***, H**, OR***, RH**, RE**
Type IIx									
LCFR	1.71 ± 0.58	2.33 ± 0.49	1.23 ± 0.33	2.56 ± 0.80	1.76 ± 0.57	1.84 ± 0.22	1.74 ± 0.34	1.86 ± 0.42	R**, H***, OH***
CFD	1346 ± 254	1217 ± 466	1200 ± 405	1168 ± 239	1598 ± 449	1220 ± 386	1830 ± 247	1204 ± 205	O*, R*, H**
Type IIb/IIx									
LCFR	1.59 ± 0.17	2.13 ± 0.46	1.43 ± 0.59	2.03 ± 0.36	1.62 ± 0.29	1.62 ± 0.28	1.62 ± 0.35	2.12 ± 0.29	R*, H***, OH*, OE*
CFD	1172 ± 294	956 ± 350	982 ± 446	987 ± 176	1578 ± 431	1112 ± 264	1440 ± 351	1359 ± 90	O*, H***, RE*
Type IIb									
LCFR	1.76 ± 0.18	2.56 ± 0.32	1.50 ± 0.44	2.50 ± 0.55	1.81 ± 0.68	1.73 ± 0.18	1.80 ± 0.53	1.93 ± 0.35	O*, R**, H***, OH*, OE*
CFD	1098 ± 147	1033 ± 439	1013 ± 396	952 ± 197	1401 ± 371	1005 ± 259	1487 ± 169	995 ± 274	O*, R***, H**
Glycolytic region									
Type IIa									
LCFR	0.73 ± 0.12	1.56 ± 0.71	0.71 ± 0.30	1.13 ± 0.27	1.06 ± 0.20	1.06 ± 0.33	0.91 ± 0.18	0.93 ± 0.17	R***, H***, OH**
CFD	1150 ± 230	1105 ± 242	1229 ± 364	1236 ± 303	1769 ± 334	1324 ± 110	1880 ± 262	1278 ± 126	O**, R***, H***, OH**
Type IIa/IIx									
LCFR	1.09 ± 0.31	1.77 ± 0.73	1.39 ± 1.15	1.46 ± 0.18	1.21 ± 0.54	1.67 ± 0.76	1.09 ± 0.20	1.56 ± 0.35	H*
CFD	1216 ± 73	1016 ± 199	1294 ± 244	1018 ± 124	1631 ± 438	1534 ± 410	1784 ± 89	1185 ± 127	O*, R***, H**, OR***, RH**, RE**
Type IIx									
LCFR	1.09 ± 0.19	2.00 ± 0.53	1.05 ± 0.33	1.89 ± 0.52	1.42 ± 0.14	1.41 ± 0.34	1.34 ± 0.29	1.86 ± 0.42	R**, H***, OH***
CFD	1062 ± 248	924 ± 244	1159 ± 239	1076 ± 302	1314 ± 403	1161 ± 278	1581 ± 155	1209 ± 155	O*, R*, H**
Type IIb/IIx									
LCFR	1.26 ± 0.47	2.24 ± 0.41	1.04 ± 0.51	1.62 ± 0.56	1.46 ± 0.24	1.79 ± 0.77	1.37 ± 0.47	1.64 ± 0.43	R*, H***, OH*, OE*
CFD	989 ± 189	768 ± 131	991 ± 553	830 ± 212	1445 ± 521	1123 ± 322	1221 ± 222	1052 ± 181	O*, H***, RE*
Type IIb									
LCFR	1.50 ± 0.24	2.53 ± 0.30	1.29 ± 2.5	1.70 ± 0.46	1.41 ± 0.16	1.81 ± 0.53	1.37 ± 0.28	1.92 ± 0.21	O*, R**, H***, OH*, OE*
CFD	899 ± 137	813 ± 170	879 ± 323	836 ± 247	1201 ± 446	1043 ± 186	1245 ± 140	997 ± 76	O*, R***, H**

*SC = sedentary control, SO = sedentary overload, EC = exercise control, EO = exercise overload. Data are expressed as means ± standard deviation. To signify significant differences: O= old age effect, R=region effect, H=hypertrophy effect, E=exercise effect. To signify interactions: OR=old age*region interaction, OH= old age*hypertrophy interaction, OR=old age*region interaction, OE=old age*exercise interaction, RH=region*hypertrophy interaction, RE= region*exercise interaction. To denote statistical significance: *denotes p<0.05, ** denotes p<0.01, ***denotes p<0.001.*

Table 4 Succinate dehydrogenase optical density (SDH OD) for different fiber types in the oxidative and glycolytic regions of plantaris muscles exposed to overload and endurance training in young and old C57BL/6J mice.

	Young SC	SO	EC	EO	Old SC	SO	EC	EO	Effects and interactions
SDH OD									
Deep									
Type I	-	0.196 ± 0.038	0.320 ± 0.000	0.232 ± 0.054	0.131 ± 0.000	-	0.156 ± 0.028	0.192 ± 0.000	-
Type I/IIa	-	0.2182 ± 0.061	-	-	-	0.160 ± 0.000	-	-	-
Type IIa	0.284 ± 0.092	0.256 ± 0.073	0.413 ± 0.146	0.320 ± 0.099	0.232 ± 0.103	0.188 ± 0.055	0.236 ± 0.045	0.208 ± 0.051	O**, H**
Type IIa/IIx	0.300 ± 0.140	0.231 ± 0.081	0.407 ± 0.201	0.251 ± 0.087	0.209 ± 0.058	0.178 ± 0.056	0.186 ± 0.02	0.145 ± 0.011	O*, H**, OH**, OE**
Type IIx	0.203 ± 0.052	0.195 ± 0.074	0.290 ± 0.089	0.230 ± 0.077	0.178 ± 0.055	0.170 ± 0.062	0.160 ± 0.043	0.152 ± 0.069	O*
Type IIb/IIx	0.166 ± 0.054	0.155 ± 0.062	0.269 ± 0.173	0.161 ± 0.049	0.151 ± 0.055	0.140 ± 0.028	0.129 ± 0.008	0.118 ± 0.011	O*, RH*, RE**
Type IIb	0.122 ± 0.028	0.107 ± 0.020	0.139 ± 0.029	0.117 ± 0.033	0.113 ± 0.035	0.125 ± 0.045	0.112 ± 0.015	0.109 ± 0.011	
Superficial									
Type I	-	0.205 ± 0.05	0.140 ± 0.080	0.337 ± 0.000	-	-	-	-	-
Type I/IIa	-	0.318 ± 0.115	-	-	-	-	-	-	-
Type IIa	0.300 ± 0.107	0.258 ± 0.058	0.385 ± 0.104	0.319 ± 0.118	0.210 ± 0.088	0.196 ± 0.065	0.218 ± 0.045	0.239 ± 0.058	O**, H**
Type IIa/IIx	0.260 ± 0.140	0.249 ± 0.079	0.385 ± 0.148	0.252 ± 0.091	0.193 ± 0.114	0.220 ± 0.124	0.158 ± 0.027	0.163 ± 0.022	O*, H**, OH**, OE**
Type IIx	0.2712 ± 0.124	0.191 ± 0.060	0.275 ± 0.091	0.244 ± 0.105	0.179 ± 0.053	0.183 ± 0.097	0.144 ± 0.018	0.170 ± 0.062	O*
Type IIb/IIx	0.209 ± 0.077	0.135 ± 0.029	0.222 ± 0.194	0.171 ± 0.057	0.134 ± 0.032	0.152 ± 0.060	0.115 ± 0.011	0.124 ± 0.008	O*, RH*, RE**
Type IIb	0.128 ± 0.038	0.111 ± 0.012	0.122 ± 0.022	0.122 ± 0.034	0.104 ± 0.033	0.125 ± 0.054	0.109 ± 0.020	0.104 ± 0.027	

*SC = sedentary control, SO = sedentary overload, EC = exercise control, EO = exercise overload. Data are expressed as means ± standard deviation. To signify significant differences: O= old age effect, R=region effect, H=hypertrophy effect, E=exercise effect. To signify interactions: OR=old age*region interaction, OH= old age*hypertrophy interaction, OR=old age*region interaction, OE=old age*exercise interaction, RH=region*hypertrophy interaction, RE= region*exercise interaction. To denote statistical significance: *denotes p<0.05, ** denotes p<0.01, ***denotes p<0.001.*

DISCUSSION

The main observations of the present study are that in both age groups overload-induced hypertrophy was not blunted by regular endurance exercise and regular endurance exercise-induced increases in fatigue resistance were not impaired by overload. The attenuated hypertrophic response to overload in old mice was associated with a reduced specific tension and impaired angiogenesis. These data indicate that 1) combination of endurance exercise and overload elicit the benefits of both types of exercise without compromising specific adaptations and 2) that the attenuated hypertrophic response in old age may be attributable to impaired angiogenesis.

Effects of ageing on muscle function and morphology

Ageing has detrimental effects on skeletal muscle morphology, plasticity and function (Larsson et al., 2019). Many studies have used rodents to investigate the mechanisms of musculoskeletal ageing and they are indeed useful models for human muscle ageing, provided that the correct age groups are chosen (Ballak et al., 2014b). For instance, we found an age-related decrease in fatigue resistance in 28-month-old mice, while in a previous study using 25-month-old mice, similar to early ageing (60-70 years) in humans (Ballak et al., 2014b), no significant decrease in fatigue resistance was found (Ballak et al., 2016). The age-related decrement in fatigue resistance may largely be due to the lower oxidative capacity found in the old animals, something also seen in elderly humans (Conley et al., 2000, Proctor et al., 1995), as well as to impaired blood flow (Irion et al., 1987).

In this study we found a lower type II FCSA in the old mice (type I fibers were too few in the plantaris muscle), similar to the age-related type II atrophy observed in humans (Lexell et al., 1988, Coggan et al., 1992). Although this was not seen in 25-month-old mice (Ballak et al., 2014a), the specific tension was already lower at that age, which is in agreement with the reduced specific force found in humans from the 6th decade of life (early ageing) (Ballak et al., 2014a, Jubrias et al., 1997). While CD was greater in the old mice studied here, this is likely due to the lower FCSA in old muscle (Larsson et al., 2019), as indeed reflected by the similar C:F in the non-overloaded (control) plantaris muscles from young and old animals. The decrease in LCFR for type IIb fibers with age found here is comparable to the reduction in type

II fiber capillarization seen in the muscle of elderly humans (Proctor et al., 1995, Nederveen et al., 2016).

Effects of regular endurance exercise

In the present study, we showed that endurance exercise induced an improved fatigue resistance. While fatigue resistance is positively related to the density of capillary network (Tickle et al., 2020) and oxidative capacity (Burke et al., 1973), there was no concomitant increase in capillarization or oxidative capacity. It has been shown that slower myosin isoforms are more efficient during isometric contractions (Stienen et al., 1996) and therefore the reduction in the proportion of type IIx fibers with endurance exercise may contribute to greater fatigue resistance during repeated isometric contractions. In addition, improvements in vasodilatory function and maximal blood flow after regular endurance exercise (Snell et al., 1987) may contribute to the improved muscle performance.

The absence of endurance exercise-induced increases in C:F, CD, or SDH-OD was unexpected, as this exercise program has been reported to induce endurance exercise adaptations in muscles from C57BL/6J mice in a previous study (Savage and McPherron, 2010). The discrepancy between their work and our observation of an absence an exercise-induced increase in oxidative capacity (reflected by SDH-OD) may be related to a differential response between muscles. In fact, in line with our observation, they observed no exercise-induced increase in the citrate synthase activity in the plantaris muscle. We therefore suggest that this protocol did not provide enough intensity or volume to induce angiogenesis and increases in oxidative capacity in the plantaris muscle.

Effects of overload by synergist inactivation

Overload of the plantaris muscle through denervation of the gastrocnemius and soleus induced hypertrophy and in young-adult mice only, a proportional rise in maximal isometric tetanic force, similar to that seen previously (Ballak et al., 2016). The hypertrophy was in both age groups accompanied with angiogenesis, as reflected by the increase in C:F and LCFR. This adaptation is well documented in the literature (Tickle et al., 2020, Ballak et al., 2016) and illustrates the coupling between fiber size and capillary supply (Bosutti et al., 2015), where it has been shown that the overload-induced hypertrophy and angiogenesis even follow a similar time course (Plyley et al., 1998).

Based on the inverse relationship between FCSA and SDH OD (oxidative capacity), we expected the hypertrophy to be accompanied with a reduction in SDH OD. Yet, we found no significant decrease in SDH OD (van der Laarse et al., 1998), something also seen before during overload-induced hypertrophy (Ballak et al., 2016).

Effects of endurance exercise on overload-induced hypertrophy

In the present study, we demonstrated that overload-induced hypertrophy was not impaired by endurance exercise, suggesting that there was no trade-off between the adaptations to each training modality used in the current study. Although the regular endurance exercise in our study did not induce an increase in SDH OD, previous studies have shown an amplified hypertrophy and an undiminished increase in SDH activity in rats subject to both treadmill running and synergist ablation (Gollnick et al., 1981, Riedy et al., 1985). Perhaps even more significant is that the hypertrophic response was not even attenuated when combined with chronic electrical stimulation that elicited a significant rise in SDH activity (Frischknecht and Vrbova, 1991). This and the higher oxidative capacity of fibers than expected for their size in hypertrophied muscles (Ballak et al., 2016) challenge the concept that there is a trade-off between fiber size and oxidative capacity (Degens, 2012, van der Laarse et al., 1998, van Wessel et al., 2010) and add to mounting evidence that acute cellular interference effects do not necessarily translate to impaired adaptations to exercise (Murach and Bagley, 2016).

Impact of overload on endurance exercise-induced increases in fatigue resistance

According to van Wessel et al. (2010) the inverse relationship between fiber size and oxidative capacity suggests that combining endurance and resistance exercise impairs adaptations to endurance exercise and attenuates the hypertrophic response to resistance training. Similar to the absence of attenuated hypertrophy during concomitant endurance exercise and overload discussed above, the endurance exercise-induced increase in fatigue resistance was not diminished by concomitant overload. The proportional increase in C:F and fiber size, as reflected by the maintained CD and CFD, during hypertrophy prevented an increase in the average diffusion distance from capillaries to mitochondria, and hence minimized any hypertrophy-associated diffusion limitations of oxygen to the working mitochondria (Degens, 2012). Angiogenesis may thus explain a maintained, but not

an increased fatigue resistance. As discussed above, another factor is the type IIb to IIa fiber type transition that occurs irrespective of concomitant overload that will reduce the ATP demand during isometric contractions.

Responses to overload and endurance exercise in old mice

The absence of blunted hypertrophy and fatigue resistance when both endurance exercise and overload were combined was seen in both young-adult and old mice. However, the increase in muscle mass in response to overload was attenuated in old mice, something seen also in 'early ageing' mice (Ballak et al., 2016) and in response to loaded voluntary wheel running (Soffe et al., 2016). Some have even found no hypertrophy with overload at this age (Lee et al., 2016). The smaller increase in muscle mass in old animals is not due to differing proportions of connective tissue in the two age groups and it appeared that the percentage increase in FCSA was also similar between young and old. The blunted increase in muscle mass with overload in spite of similar fiber hypertrophy to that with young animals is comparable to other examples of discrepancies in measurement of hypertrophy noted elsewhere (Haun et al., 2019b). Regardless of this, our data show the adaptive response to overload is diminished in old muscle.

Part of the diminished hypertrophic response may be attributable to a lower LCFR for type IIb fibers in old mice, as in elderly men with lower type II capillarization demonstrated less muscle fiber growth with resistance training than those with higher type II capillarization (Snijders et al., 2017a). However, the overall capillary density of the muscle was even higher in old than young mice, and following the argument above, should lead to an enhanced, rather than a diminished hypertrophic response. Here we propose like Ballak et al. (2016), that the impaired hypertrophic response is at least partly due to the impaired angiogenic response, as hypertrophy has a similar time course to capillary growth (Plyley et al., 1998, Egginton et al., 2011).

It has been reported previously in rats that at very advanced age (33 and 36 months) overload may not result in hypertrophy and even cause a reduction in the specific tension (Degens and Alway, 2003, Blough and Linderman, 2000). Combining our current data with previous data (Ballak et al., 2015), the pattern emerges that also in mice overload in early ageing is accompanied with a commensurate increase in force generating capacity, while the specific tension is reduced with overload in older mice. Also in humans, some studies report a similar hypertrophy in older people and

young-adults (Cannon et al., 2007, Kryger and Andersen, 2007), but others report attenuated increases in thigh muscle cross-sectional area (Kraemer et al., 1999), attenuated fiber size (Slivka et al., 2008) or even no hypertrophy at all in very old (83-94 year old) people (Karlsen et al., 2019). Whatever the cause of these discrepant observations between human studies, it seems that the hypertrophic response deteriorates with age, where the muscle has potentially already maximally activated its regeneration potential (Edstrom and Ulfhake, 2005), not being able to cope with any additional challenges. Therefore chronic mechanical tension such as overload may be detrimental given the impaired muscle recovery response in old animals (Brooks and Faulkner, 1990).

The LCFR divided by the SDH INT (FCSA * SDH OD) gives an indication of the oxygen supply to demand ratio of a muscle fiber. Here we showed, as even seen in early ageing in previous studies (Hepple and Vogell, 2004, Messa et al., 2019) that there was a capillary supply in excess of the oxygen demand in old age. The oxygen supply to demand ratio was, however, reduced with overload in old animals only as a consequence of the impaired angiogenesis. Nevertheless, fatigue resistance was still elevated after exercise even in the overloaded muscles. This again indicates that the muscle fatigue resistance in our test is probably more determined by the ATP demand than the aerobic ATP generating capability of the muscle fibers.

Limitations

The endurance training may have been of too low volume and intensity to induce capillary growth and increases in oxidative capacity. As a consequence, the overload and endurance exercise stimuli may have been insufficient to challenge the trade-off between fiber size and oxidative capacity. Nevertheless, they do show that these types of exercise can be combined to gain the benefits of each exercise modality. In fact, the reduction in specific tension in the old mice after our overload stimulus may well be an indication that we were already close to the capability of the muscle to adapt to a new challenge, and there is danger that longer and more intense training stimuli would result in damage rather than gains in muscle function. The extent of hypertrophy induced by overload via denervation of synergists is much greater than that in response to resistance training. Rather than a weakness, this is a strength of the present study as the large hypertrophic response is more likely to reveal limits of any trade-off between fiber size and oxidative capacity than the hypertrophy during conventional resistance exercise. The stress associated with the

use of a shock grid to encourage running may have attenuated any extra hypertrophy that could be induced by exercise (Conner et al., 2014), but this effect is likely limited as the hypertrophic response was not diminished with exercise. Although this study only used male mice and results may differ in females, this is unlikely as the trade-off between fiber size and oxidative capacity is a function of diffusion limitations, a physical property that is independent of sex. Additionally, previous work in female rats showed similar levels of hypertrophy to that seen in the male mice in this study (Degens et al., 1993a). These are different species however, and future studies may systematically investigate differences in the hypertrophic response to overload in males and females.

Conclusion

Our data indicate that combining endurance exercise and overload induces the benefits of both stimuli without diminishing the adaptations of either and that the smaller increases in muscle mass found in old mice may be due to impaired angiogenesis with overload. The muscles of older animals might have been at the limits of their adaptive capacity as indicated by the reduction in specific tension after overload.

Prelude to Chapter 6

In chapter 5 effects of hypertrophic and endurance stimuli were studied in young and old mice. In the final experimental chapter, the same interventions were used in three mouse strains of different baseline fibre size. According to the size principle it is expected that animals with larger fibres would experience less hypertrophy than those with smaller fibres due to greater diffusion distances.

Chapter 6

ENDURANCE EXERCISE PLUS OVERLOAD INDUCES FATIGUE RESISTANCE AND SIMILAR HYPERTROPHY IN MICE IRRESPECTIVE OF MUSCLE MASS

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ABSTRACT

Previous studies have demonstrated that fibre cross-sectional area (FCSA) is inversely related to oxidative capacity, which is thought to be determined by diffusion limitations of oxygen, ADP and ATP. Consequently, it is hypothesised that 1) when endurance training is combined with a hypertrophic stimulus the response to each will be blunted, and 2) that muscles with a smaller FCSA will show a larger hypertrophic response than those with a large FCSA.

To investigate this, we combined overload with endurance exercise in 12-month-old male mice from three different strains with different FCSA: Berlin High (BEH) (large fibres), C57BL/6J (C57) (normal-sized fibres) and Berlin Low (BEL) (small fibres). The right plantaris muscle was subjected to overload through denervation of synergists with the left muscle acting as an internal control. Half the animals trained 30 min per day for 6 weeks.

The overload-induced hypertrophy was not blunted by endurance exercise, and the exercise-induced increase in fatigue resistance was not impaired by overload. All strains demonstrated similar absolute increases in FCSA, although the BEH mice with more fibres than C57 demonstrated the largest increase in muscle mass (13.4 mg ± 2.1) and BEL mice with fewer fibres the smallest increase in muscle mass (4.38 mg ± 1.9).

This study suggests that the endurance exercise and hypertrophic stimuli can be combined without attenuating adaptations to either modality, and that increases in FCSA are independent of baseline fibre size.

INTRODUCTION

In isolated muscle fibres an inverse relationship between fibre cross-sectional area (FCSA) and maximal oxygen uptake or succinate dehydrogenase activity – dubbed the 'size principle of striated muscle' - has been observed, that was explicable by oxygen diffusion limitations (van der Laarse et al., 1998). Such potential limitations to fibre size may not only be limited by oxygen diffusion limitation, but also by limitations in the diffusion of ATP and ADP, and the size of the myonuclear domain (van Wessel et al., 2010, Degens, 2012, Kinsey et al., 2007).

Corresponding with the concept that diffusion constraints limit fibre size is the positive relationship between the size and capillary supply to a fibre (Bosutti et al., 2015). During overload, the similar time course of angiogenesis and increases in FCSA (Plyley et al., 1998, Egginton et al., 2011) may well serve to mitigate diffusion limitations associated with hypertrophy. Nevertheless, capillaries are only found at the perimeter of muscle fibres and the distance from the capillary to the centre of the fibre increases with hypertrophy and may thus impose a limit on increment in FCSA, no matter the amount of angiogenesis (Degens, 2012). This limit may be alleviated by a reduction in oxidative capacity (van Wessel et al., 2010).

While concurrent training is commonly used to gain the benefits of both resistance and endurance exercise, the combination of both training modalities is thought to lead to diminished responses to each stimulus (van Wessel et al., 2010). For instance, a reduction in oxidative capacity to alleviate potential diffusion limitations of fibre growth during overload runs counter to adaptations from endurance training and *vice versa* (van Wessel et al., 2010). It is therefore expected that overload-induced increases in FCSA are attenuated by endurance exercise, and increases in oxidative capacity in response to endurance exercise are dampened when accompanied by a hypertrophic stimulus.

Based on the 'size principle' one might expect that muscles with smaller fibres will show a larger hypertrophic response, particularly when combined with endurance exercise, than muscles with larger fibres. In line with this, in human training studies individuals with lower muscle thickness and smaller FCSA experienced greater hypertrophy with resistance training than those with greater muscle thickness and larger FCSA (Mobley et al., 2018, Haun et al., 2019a). While many studies of concurrent training have been completed in both humans and rodents (Hickson, 1980, Methenitis, 2018, Castoldi et al., 2013), the effect of baseline muscle

mass/FCSA on the response to combined hypertrophic and endurance stimuli is yet to be studied. Therefore, the aim of the present study was to determine the effects of combining a hypertrophic stimulus with endurance exercise on FCSA, muscle mass, oxidative capacity, capillarisation and contractile properties in three mouse strains with a 5-fold difference in plantaris muscle mass: Berlin High (BEH) and Berlin Low (BEL) and C57BL/6J (C57) (Kilikevicius et al., 2016). The Berlin High (BEH) mouse strain was derived from a heterogeneous population of mice for its large body mass and high protein content at the age of 60 days (Bunger et al. 2001) and has been used to study effects of myostatin dysfunction on muscle growth and. This strain has a large muscle mass and FCSA, due to myostatin dysfunction and long-term selection for a large muscle mass (Lionikas et al., 2013b), while the Berlin Low (BEL) strain was selected for its low body mass and low protein content (Bunger et al. 2001) and has a small muscle mass and small fibres (Lionikas et al., 2013b). The muscle mass and FCSA of the C57 mouse strain is in between that of the BEL and BEH mice (Lionikas et al., 2013b) and it is well studied in muscle physiology and serves as a control between the BEL and BEH strains (Ballak et al. 2016; Soffe et al. 2016; Brooks and Vaulkner, 1990). Additionally, mice homozygous for the mutated myostatin *compact* allele such as the BEH mice, have been reported to have a lower number of capillaries per fibre and capillary density (CD) than wild type mice, which may further attenuate the hypertrophic response in these mice (Rehfeldt et al., 2005).

The minimal genetic variation between individuals within each inbred mouse strain means that fewer animals are required than would be needed in a human study and the overload method used here induces hypertrophy at a much faster rate than resistance training in humans (Alway et al. 2005). In contrast, the chronic nature of the overload stimulus differs to the repeated short bouts of resistance training used in humans (Lowe and Alway, 2002) and the signal which triggers growth (stretch or tension) is not yet known (Murach et al. 2020). Also, the genetic homogeneity of the mouse models used may mean that conclusions from this study may have limited application to humans as their responses to exercise vary greatly (McPhee et al. 2011; Erskine et al. 2010).

We hypothesise that 1) muscles from BEH mice will demonstrate the smallest increase in FCSA of the three strains, whereas muscles from BEL mice will experience the largest increase. Particularly in BEH mice 2) endurance training will

lead to an attenuated hypertrophic response and 3) overload will blunt improvements in fatigue resistance.

MATERIALS AND METHODS

Animals

Thirty-six 12-month-old male mice (14 C57BL/6J (C57), 10 Berlin high (BEH) and 12 Berlin low (BEL)) were kept in individual cages at 22 °C, on a reversed 12-hour light/dark cycle with *ad libitum* access to standard chow (LabDiet5001: protein 28.7%, carbohydrate 57.9%, fat 13.4%) and water at the Lithuanian Sports University. All animal procedures were approved by the Lithuanian State Food and Veterinary Service and Manchester Metropolitan University (SE171810). For a diagrammatic overview of the study design see Figure 1.

Surgery to overload the plantaris muscle

To induce compensatory hypertrophy of the plantaris muscle of the right leg, its synergists (the gastrocnemius and soleus) were denervated by cutting the medial and lateral branches of the tibial nerve to the gastrocnemius and soleus, as detailed previously (Degens and Alway, 2003). Sections of these nerve branches were removed to prevent reinnervation. Surgical procedures were performed under aseptic conditions and mice were anaesthetised with an intraperitoneal injection of a mix of ketamine (100 $mg \cdot kg^{-1}$) (Ketamidor, Richter Pharma AG, Wels, Austria) and xylazine (16 $mg \cdot kg^{-1}$) (Sedaxylan, Eurovet, Bladel, Netherlands) in saline. After surgery, the wound was closed with suture and the pain killer ketoprofen (2.5 $mg \cdot kg^{-1}$) (Rifen, Eurovet, Bladel, Netherlands) was administered immediately and again 24 hours later. The plantaris muscle of the intact contralateral limb served as the internal control as Baldwin et al. (1977) found no differences in enzyme activity and muscle mass between the hindlimb muscles of sham operated animals and contralateral limbs in animals subject to overload surgery.

Endurance exercise

One week after the overload surgery mice were either allocated to a sedentary group or to an endurance exercise group. The endurance exercise protocol consisted of running at a speed of 12 $m \cdot min^{-1}$, 30 $min \cdot day^{-1}$, 5 days per week on a treadmill (Savage and McPherron, 2010) for six weeks. A shock-grid was used to

encourage running. Any mice that refused to run during the first 5 exercise sessions were removed from the exercise group and replaced with an animal from the sedentary group. While this maintained the numbers of animals in each group, this introduced a selection bias into the study as mice unwilling to run were included in the sedentary group and therefore group allocation was not entirely random. Exercise was undertaken under the supervision of a researcher in the dark with a red light.

In situ m. plantaris contractile properties

Seven weeks after the initial surgery, the mice were anaesthetised as detailed above. Since the terminal experiment required a longer period of anaesthesia than the denervation surgery, the pedal withdrawal reflex was tested every 10 minutes and 20 % of the original dose was administered to the animal when needed. The gastrocnemius of one hindlimb was excised to expose the sciatic nerve and weighed. The animal was then moved to the *in-situ* force measurement apparatus where its body temperature was kept at 36.5 °C. The distal plantaris tendon was cut and attached to a force transducer (Dual-Mode Muscle Lever Systems 305C-LR Aurora Scientific, Ontario, Canada) using 3-0 silk suture.

The sciatic nerve was cut proximally and then stimulated with 0.2-ms pulses incrementally every 30 s until maximal twitch force was achieved. After this, the stimulating current was increased by 10 % to ensure supramaximal stimulation. The muscle was then stimulated every 30 s to determine optimal length, defined as the muscle length at which maximal active twitch force was produced.

The maximal tetanic force was then determined by stimulating the muscle twice, 5 min apart, for 300 ms at 200 Hz. Five minutes after the final tetanus, the muscle was stimulated with a series of 330 ms 40-Hz trains once every second for 4 min to assess fatigue resistance. A fatigue index was then calculated as the force of the last contraction divided by the force of the strongest contraction of the series.

The same process was repeated on the contralateral limb. The overloaded and control plantaris muscles were stimulated in random order. After the contractile properties were determined for both plantaris muscles, the animal was euthanised with carbon dioxide. The plantaris muscles were then removed, weighed and mounted in OCT embedding medium (Thermo Scientific, Loughborough, UK) in liver, frozen in liquid nitrogen and stored at -80°C.

Muscle morphology

A cryostat was used to cut serial 10-µm sections from the mid-belly of each plantaris muscle at –20 °C and three sections were mounted per slide.

Two slides were blocked with 10 % goat serum (Vector Laboratories, USA) in PBS for 60 minutes. Then one slide was incubated for 2 hrs in an antibody cocktail containing monoclonal antibodies BAD-5 (1:600), SC-71 (1:600) and 6H1 (1:50) in blocking solution (Developmental Studies Hybridoma Bank, USA) to identify types I, IIa and IIx fibres, respectively. The second slide was incubated for 2 hrs with BF-F3 (1:100) and biotinylated *Griffonia simplicifolia* lectin I, (5µL.mL^{-1}) (Vector Laboratories, USA) in blocking solution to identify type IIb fibres and capillaries, respectively. After three 5-min PBS washes the first slide was incubated in secondary antibodies Alexa Fluor 350IgG2b for type I (1:500), Alexa Fluor 488 IgG1 for type IIa (1:500) and Alexa Fluor 555 IgM for type IIx (1:500) (Thermofisher Scientific, USA). The second slide was incubated in Alexa Fluor 555 IgM for type IIb (1:500) and Streptavidin, Alexa Fluor 350 conjugate (1:200) (Thermofisher Scientific) for capillaries. After three further 5-min washes the slides were mounted with ProLong Diamond Antifade mountant (Thermofisher Scientific, USA).

Digital images of both the oxidative and glycolytic regions (deep and superficial, respectively) of the plantaris muscle were taken using a fluorescent microscope (Zeiss Axio Imager Z1, Zeiss, Germany) at 20x magnification. For each digital image, BTablet (BaLoH Software, Ooij, Netherlands) was used to manually trace and type fibres and record capillary coordinates. Using this data, AnaTis was used to calculate FCSA, capillary to fibre ratio (C:F), capillary domain size, Local capillary to fibre ratio (LCFR), capillary fibre density (CFD) and an index of the heterogeneity of capillary spacing (Log_RSD).

Succinate dehydrogenase optical density

A third serial slide from each plantaris muscle was stained for succinate dehydrogenase (SDH) activity as described previously (Ballak et al., 2016). The optical density (OD) of the stain has been shown to give a quantitative indication of oxidative capacity (van der Laarse et al., 1989). Photomicrographs of stained sections were taken on a light microscope with an AxioCam ICMI camera (Göttingen, Germany) and a 660-nm filter. For each sample, a series of filters with known OD were used to create a calibration curve to adjust for variation in background staining and lighting between sections. ImageJ (Rasband, W.S.,

ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.net/Downloads) was used to determine the OD of the stain for each fibre. Integrated SDH activity (SDH-INT) was calculated as the SDH-OD multiplied by the FCSA.

Statistics

Statistical analyses were completed with IBM SPSS version 25. Data were tested for normal distribution with Shapiro-Wilk tests with strain, overload and exercise as factors, and where applicable fibre type and region. Non-normally distributed data were transformed logarithmically. To study differences in responses to overload and exercise a repeated-measures ANOVA with as within factors muscle region and overload, and between factors strain (3 levels: C57, BEH and BEL), exercise and fibre type (5 levels: IIa, IIb, IIx, IIa/IIx, IIb/IIx) was performed. In addition to main effects, two-way interactions were considered. To determine the nature of two-way interactions with strain, the analysis was performed in each group separately. Effects and interactions were considered significant at $p<0.05$. Bonferroni correction was applied to correct for the effect of multiple testing.

RESULTS

Body mass and muscle masses

The BEH had the highest body mass, followed by C57 and then BEL mice ($p<0.001$; Table 1). The strain * time interaction ($p=0.047$) was reflected by a significant decrease in the body mass of C57 mice during the period of overload ($p=0.003$), but not in the other strains (Table 1). Like the differences in body mass, the BEH had the highest *m. gastrocnemius* mass, followed by C57 and then BEL mice ($p<0.001$; Table 1). A similar pattern was seen in the severity of atrophy of the denervated *m. gastrocnemius* mass in the limb subjected to surgery to overload the *m. plantaris* (strain * overload interaction $p<0.001$).

The mass of the sedentary control *m. plantaris* mass was largest in the BEH and smallest in the BEL mice, with that of the C57 mice in between (one-way ANOVA $p<0.001$, Fig. 2A). These differences were also found when all plantaris masses were included in a repeated measures ANOVA ($p<0.001$). In each strain, overload induced an increase in *m. plantaris* mass ($p<0.001$), which was most pronounced in the BEH and least in the BEL mice, with the increase in muscle mass in C57 mice

in between (strain * overload interaction $p<0.001$, Bonferroni post-hoc $p\leq0.014$, Fig. 2A).

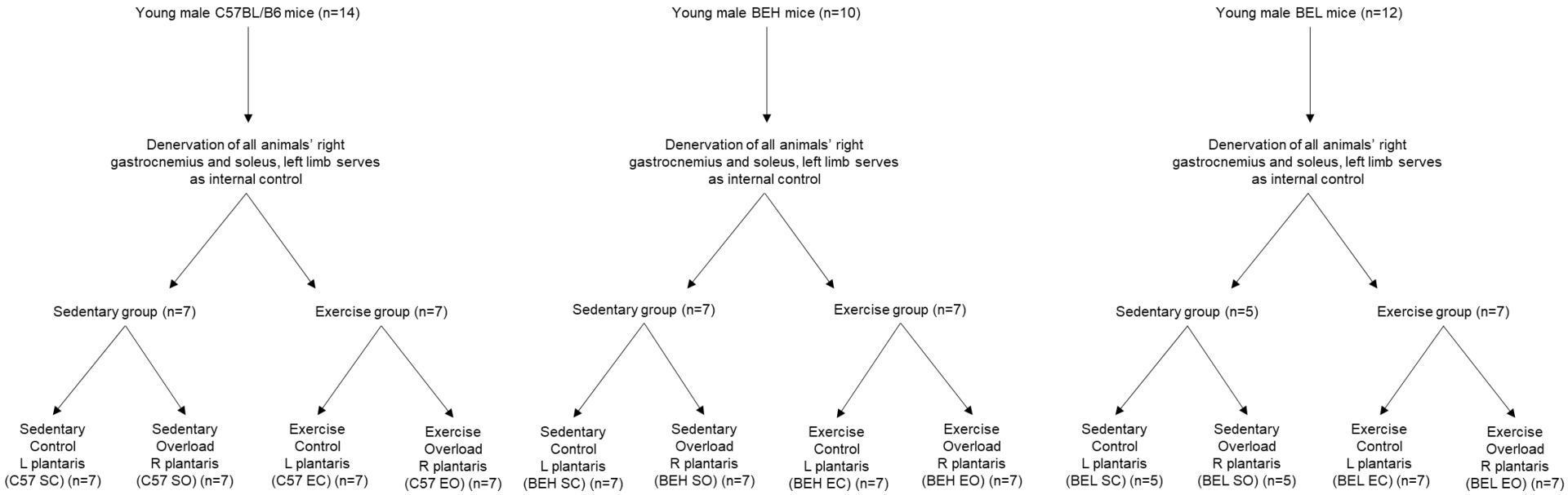

Figure 1. Diagrammatic overview of the study design. Thirty-six young male C57BL/B6 (n=14), BEH (n=10) and BEL (n=12) mice were subject to denervation surgery in which the plantaris muscles of the right hindlimb were overloaded through denervation of the gastrocnemius and soleus muscles. The left plantaris served as an internal control. Mice of each strain were then split into sedentary and exercise groups, the latter undergoing 30 minutes of treadmill running 5 days·week^{-1}. The sedentary mice had a sedentary control plantaris muscle (SC) and an overloaded plantaris muscle (SO) while the exercised mice had an exercised control plantaris muscle (EC) and an exercised overloaded plantaris muscle (EO).

Table 1: *Body mass and gastrocnemius muscle mass in young adult C57BL/6J (C57), Berlin high (BEH) and Berlin Low (BEL) mice exposed to overload with or without exercise.*

	C57				**BEH**				**BEL**				
	Sed pre	**Sed post**	**Ex pre**	**Ex post**	**Sed pre**	**Sed post**	**Ex pre**	**Ex post**	**Sed pre**	**Sed post**	**Ex pre**	**Ex post**	**Effects and interactions**
Body mass (g)	38.1 ± 3.2	37.5 ± 4.8	41.5 ± 7.3	34.8 ± 5.8	66.0 ± 10.4	63.6 ± 6.7	57.8 ± 5.7	58.7 ± 2.2	24.6 ± 3.9	23.9 ± 3.8	21.2 ± 3.7	21.4 ± 2.6	S*** T* ST*
	SC (n=7)	**SO (n=7)**	**EC (n=7)**	**EO (n=7)**	**SC (n=5)**	**SO (n=5)**	**EC (n=5)**	**EO (n=5)**	**SC (n=5)**	**SO (n=5)**	**EC (n=7)**	**EO (n=7)**	
Gastrocnemius mass (mg)	129 ± 11	74 ± 11	133 ± 15	74 ± 10	181 ± 13	88 ± 7	185 ± 13	97 ± 25	68 ± 10	41 ± 8	67 ± 14	40 ± 11	S*** O*** SO***

*Sed = sedentary, Ex = exercise, SC = sedentary control, SO = sedentary overload, EC = exercise control , EO = exercise overload. S=effect of strain, T= effect of time, O=effect of overload surgery ST = strain*time interaction SO = strain*overload interaction. *: p<0.05, **: p<0.01, ***: p<0.001.*

Contractile properties and fatigue resistance

The maximal isometric tetanic force was lower in BEL mice compared to that of the other strains ($p<0.001$; Fig. 2B). Overload induced a significant increase in maximal tetanic force ($p<0.001$), but the strain * overload interaction ($p=0.046$) indicates that this response differed between strains; in Fig. 2B it can be seen that BEH had a greater increase in tetanic force than BEL with overload (Bonferroni post-hoc $p=0.013$). Specific tension (force per g muscle mass) was lower in BEH than in the other two strains ($p<0.001$; Fig. 2C) and did not change significantly with overload in any of the strains.

The fatigue resistance, reflected by the fatigue index, was highest in the C57 and lowest in the BEL mice, with that of the BEH mice in between ($p<0.001$; Fig. 1D). Exercised muscles had greater fatigue resistance compared to those of sedentary animals in all strains, but the strain * exercise interaction ($p=0.005$) was reflected by a smaller effect of exercise in BEH mice when compared to C57 and BEL (Bonferroni post-hoc $p<0.05$).

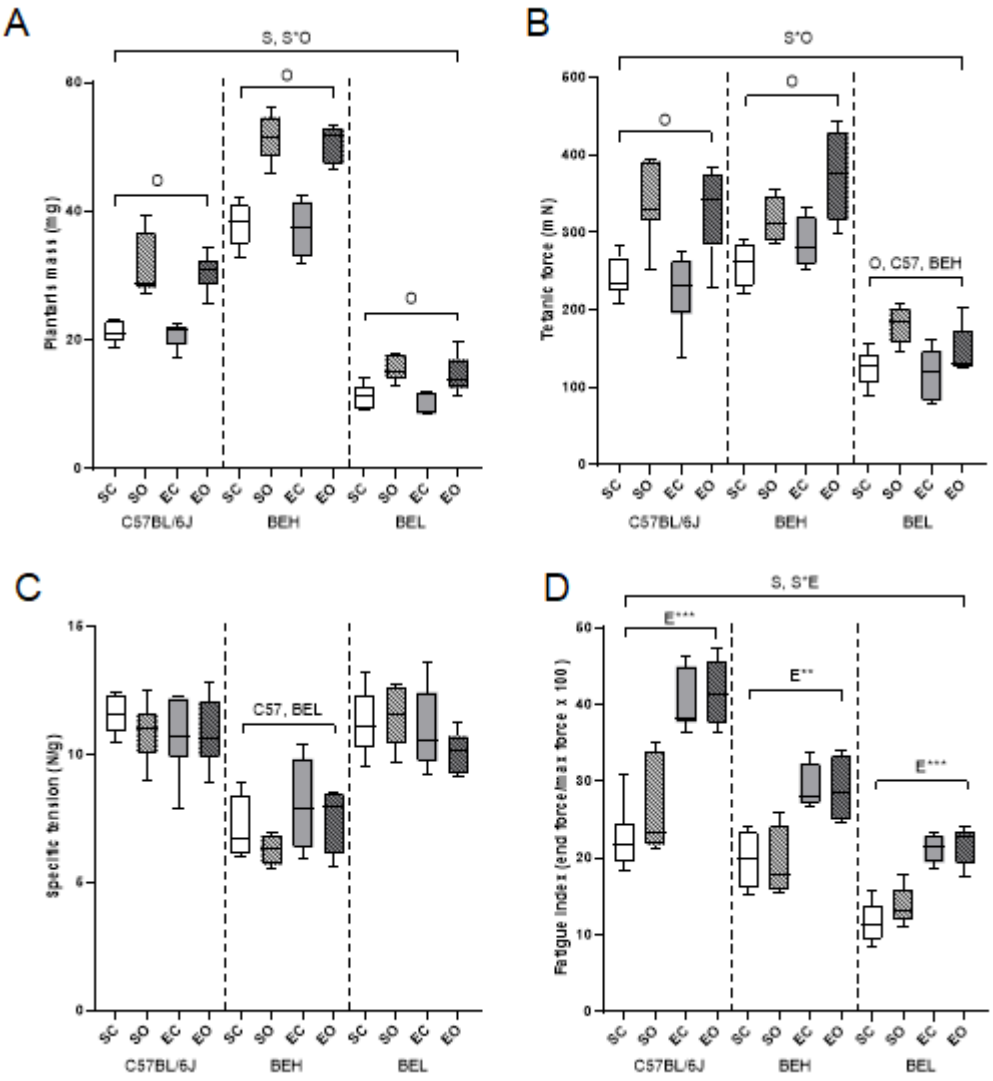

*Figure 2. Effects of overload and exercise on plantaris muscle mass and contractile properties in C57BL/6J (C57), Berlin high (BEH) and Berlin low (BEL) mice. A plantaris muscle mass B maximal tetanic force, C specific tension as tetanic force/muscle mass D fatigue index. SC = sedentary control, SO = sedentary overload, EC = exercise control, EO = exercise overload. S = effect of strain (p<0.001), O = effect of overload (p<0.001), E** = effect of exercise (p<0.01), E*** = effect of exercise (p<0.001). S*O = strain*overload interaction (p≤0.046) S*E = strain*exercise interaction (p=0.05). C57 = significantly different to C57 (p<0.001), BEH = significantly different to BEH, BEL = significantly different to BEL (p<0.001)*

Fibre composition and fibre cross-sectional area

BEH mice had a greater proportion of type IIb fibres compared to BEL mice ($p=0.001$). The glycolytic region of *m. plantaris* contained a lower proportion of type IIa fibres than the oxidative regions ($p<0.001$) and this difference was most pronounced in C57 mice (strain*region interaction $p=0.043$; Table 2).

The proportion of type I and type IIa fibres was higher ($p<0.001$), while the percentages of type IIb and IIx fibres were lower in overloaded muscles ($p<0.001$) regardless of exercise status. BEL mice demonstrated a greater increase in the percentage of type I fibres (strain*overload interaction $p=0.003$) and BEH mice demonstrated a greater increase in type IIa fibre proportion with overload (strain*overload interaction $p=0.021$). Since fewer than 2.5% of the fibres in all regions were type I/IIa (Table 2), they were omitted from further analysis.

Table 2: *Fibre type composition (%) for different fibre types in the oxidative and glycolytic regions of plantaris muscles exposed to overload with or without exercise in young adult C57BL/6J, BEH and BEL mice.*

	C57 SC	SO	EC	EO	BEH SC	SO	EC	EO	BEL SC	SO	EC	EO	Effects and interactions
Fibre type composition (%)													
Oxidative region													
Type I	0.1 ± 0.2	6.7 ± 4.8	0.2 ± 0.5	3.2 ± 4.4	-	0.4 ± 0.8	-	2.3 ± 1.6	0.3 ± 0.4	7.5 ± 9.0	0.7 ± 1.2	13.1 ± 11.3	S** O*** SO**
Type I/IIa	-	0.9 ± 1.1	-	0.1 ± 0.3	-	0.4 ± 1.0	-	-	-	0.3 ± 0.7		2.1 ± 5.1	-
Type IIa	30.5 ± 9.3	32.4 ± 15.3	22.2 ± 17.8	40.1 ± 10.4	15.7 ± 5.0	35.4 ± 17.6	10.8 ± 1.5	57.7 ± 8.0	19.9 ± 15.9	36.7 ± 20.7	30.1 ± 23.6	29.3 ± 12.3	R*** O*** SR* SO*
Type IIa/IIx	8.1 ± 6.0	10.0 ± 6.8	2.1 ± 1.8	12.3 ± 5.7	2.3 ± 4.4	5.4 ± 5.5	4.7 ± 4.0	18.7 ± 10.4	15.8 ± 15.5	14.2 ± 2.2	5.3 ± 2.8	12.2 ± 4.3	S* R** O*** SE* OE**
Type IIx	22.1 ± 7.2	18.0 ± 6.5	20.8 ± 7.3	14.6 ± 4.2	31.2 ± 11.1	19.5 ± 6.0	20.6 ± 3.9	13.9 ± 4.1	32.3 ± 3.3	21.1 ± 10.5	28.8 ± 7.9	21.6 ± 7.2	S** O**
Type IIb/IIx	3.8 ± 2.7	7.4 ± 6.5	2.6 ± 1.5	6.4 ± 5.5	3.2 ± 1.5	5.4 ± 4.9	3.2 ± 1.5	-	5.1 ± 2.2	4.4 ± 3.6	3.6 ± 3.0	5.0 ± 4.9	SO*
Type IIb	35.4 ± 9.4	24.7 ± 12.0	52.2 ± 22.8	23.4 ± 6.8	47.6 ± 9.7	33.5 ± 23.4	57.6 ± 6.9	7.4 ± 3.4	26.6 ± 26.6	15.7 ± 16.1	31.4 ± 20.2	16.7 ± 14.9	S** R*** O***
Glycolytic region													
Type I	-	3.3 ± 3.7	0.5 ± 1.0	0.5 ± 0.9	0.4 ± 0.9	2.0 ± 2.3	0.2 ± 0.4	3.1 ± 3.6	-	7.1 ± 8.0	0.6 ± 1.3	13.1 ± 14.1	S** O*** SO**
Type I/IIa	-	1.3 ± 1.1	-	-	-	-	-	-	-	1.2 ± 2.2	-	2.3 ± 5.4	-
Type IIa	7.4 ± 3.7	23.9 ± 14.1	12.9 ± 16.2	26.2 ± 7.1	7.6 ± 4.0	28.8 ± 17.8	10.6 ± 4.6	37.3 ± 15.1	20.9 ± 20.9	33.0 ± 17.8	16.4 ± 12.2	23.2 ± 10.6	R*** O*** SR* SO*
Type IIa/IIx	6.0 ± 4.1	8.3 ± 5.4	2.2 ± 2.0	8.8 ± 8.7	0.8 ± 1.3	4.6 ± 5.2	3.7 ± 2.6	10.1 ± 5.8	11.8 ± 13.9	11.8 ± 3.3	4.3 ± 3.9	13.9 ± 7.3	S* R** O*** SE* OE**
Type IIx	14.3 ± 5.7	20.7 ± 6.2	16.6 ± 9.6	16.2 ± 10.4	16.0 ± 7.1	19.6 ± 10.0	18.3 ± 3.3	20.8 ± 9.0	26.2 ± 12.4	24.6 ± 10.9	26.8 ± 4.0	21.5 ± 6.5	S** O**
Type IIb/IIx	5.4 ± 3.8	10.5 ± 5.8	3.0 ± 3.2	7.0 ± 7.4	4.5 ± 4.2	4.9 ± 3.9	5.8 ± 4.8	5.7 ± 4.4	3.2 ± 2.9	4.2 ± 2.3	5.4 ± 2.9	5.8 ± 4.8	SO*
Type IIb	67.0 ± 10.8	32.0 ± 12.5	64.8 ± 25.8	41.3 ± 9.9	70.7 ± 13.9	40.1 ± 29.0	61.4 ± 8.8	22.9 ± 14.5	37.9 ± 38.8	18.2 ± 14.0	46.4 ± 17.6	20.1 ± 13.0	S** R*** O***

*SC = sedentary control, SO = sedentary overload, EC = exercise control, EO = exercise overload. S: strain effect, R: region effect, O: overload effect, SR: strain*region interaction, SO: strain*overload interaction, SE: strain*exercise interaction, OE: overload*exercise interaction, *: p<0.05, **: p<0.01, ***: p<0.001.*

The FCSA in the sedentary control *m. plantaris* was greater in BEH compared to C57 mice, which in turn was greater than that of BEL ($p<0.05$). When all muscles were included in a repeated measures ANOVA, FCSA was larger in the plantaris muscle of BEH than BEL mice (Bonferroni post-hoc $p=0.004$) with no significant difference between C57 and the other two strains (Fig. 3A-C). Overload induced similar increases in FCSA in all three strains ($p<0.001$ Fig. 3A-C). As there was a region*strain interaction for FCSA ($p=0.014$; Fig. 3B and C), repeated measures ANOVAs were completed for each strain separately to determine the nature of this interaction. In the C57 mice there was a region effect ($p=0.014$) and a region*exercise interaction ($p=0.007$) for FCSA that was reflected by an endurance exercise-induced reduction in the FCSA in the glycolytic, but not in the oxidative region of the muscle (Fig. 3B and C). In the BEH mice a region*overload interaction was found ($p=0.037$), which was evident as a lower hypertrophic response in the oxidative than the glycolytic region of the muscle (Fig. 3B and C). FCSA for different fibre types can be seen in supplemental data table 1.

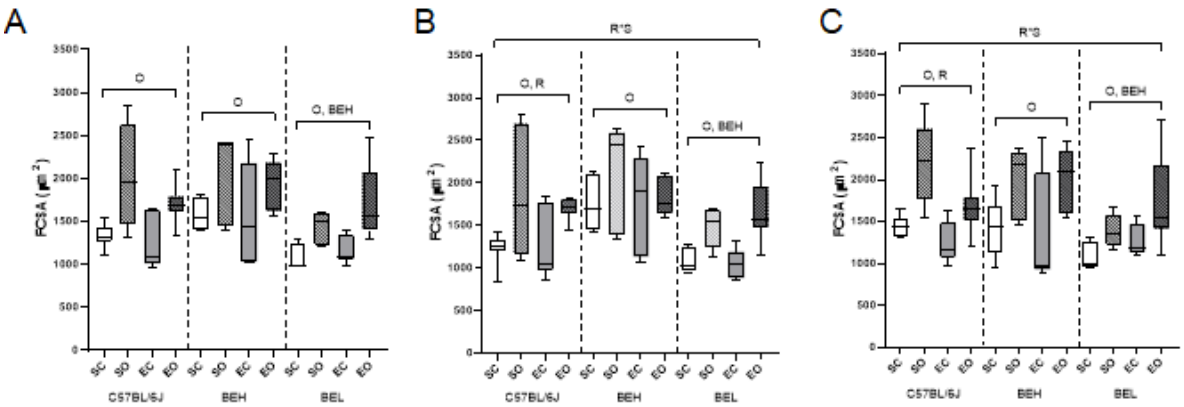

*Figure 3. Effects of age, overload and exercise on fibre cross-sectional area (FCSA) in C57BL/6J (C57), Berlin high (BEH) and Berlin low (BEL) mice. A whole plantaris muscle, B oxidative region, C glycolytic region. SC = sedentary control, SO = sedentary overload, EC = exercise control, EO = exercise overload. R = effect of region ($p=0.026$), O = effect of overload ($p<0.001$), R*S = region*strain interaction ($p=0.014$,) BEH = significantly different to BEH ($p=0.004$)*

Overall capillarisation

BEH mice had a higher C:F in the plantaris muscle than C57 mice ($p=0.045$ Fig. 4A-C). The oxidative region of the plantaris muscles had a greater C:F than glycolytic region in C57 and BEH mice, but not in the BEL mice (strain * region interaction $p=0.017$). Overload led to an increase in C:F ($p<0.001$). An overload * exercise interaction was also found, which was reflected by a larger increase in C:F in exercised overloaded than non-exercised overloaded muscles, and a lower C:F in exercised than non-exercised control muscle ($p=0.024$).

BEL mice had greater CD compared to C57 and BEH mice ($p\leq0.025$; Fig. 4D-F). In C57 mice only, the CD was lower in the glycolytic than the oxidative region of the plantaris muscle (strain * region interaction $p=0.030$).

The heterogeneity of capillary spacing index, Log_RSD, was higher in the glycolytic than oxidative region of the plantaris muscle in the BEL mice only (strain*region interaction $p=0.027$ Fig. 4G-I). An overload*exercise interaction ($p=0.011$) was reflected by an increased in Log_RSD with overload in non-exercised muscles and a decrease in exercised muscles (Fig. 4G-I). LCFR and CFD for different fibre types can be seen in supplementary data table 2.

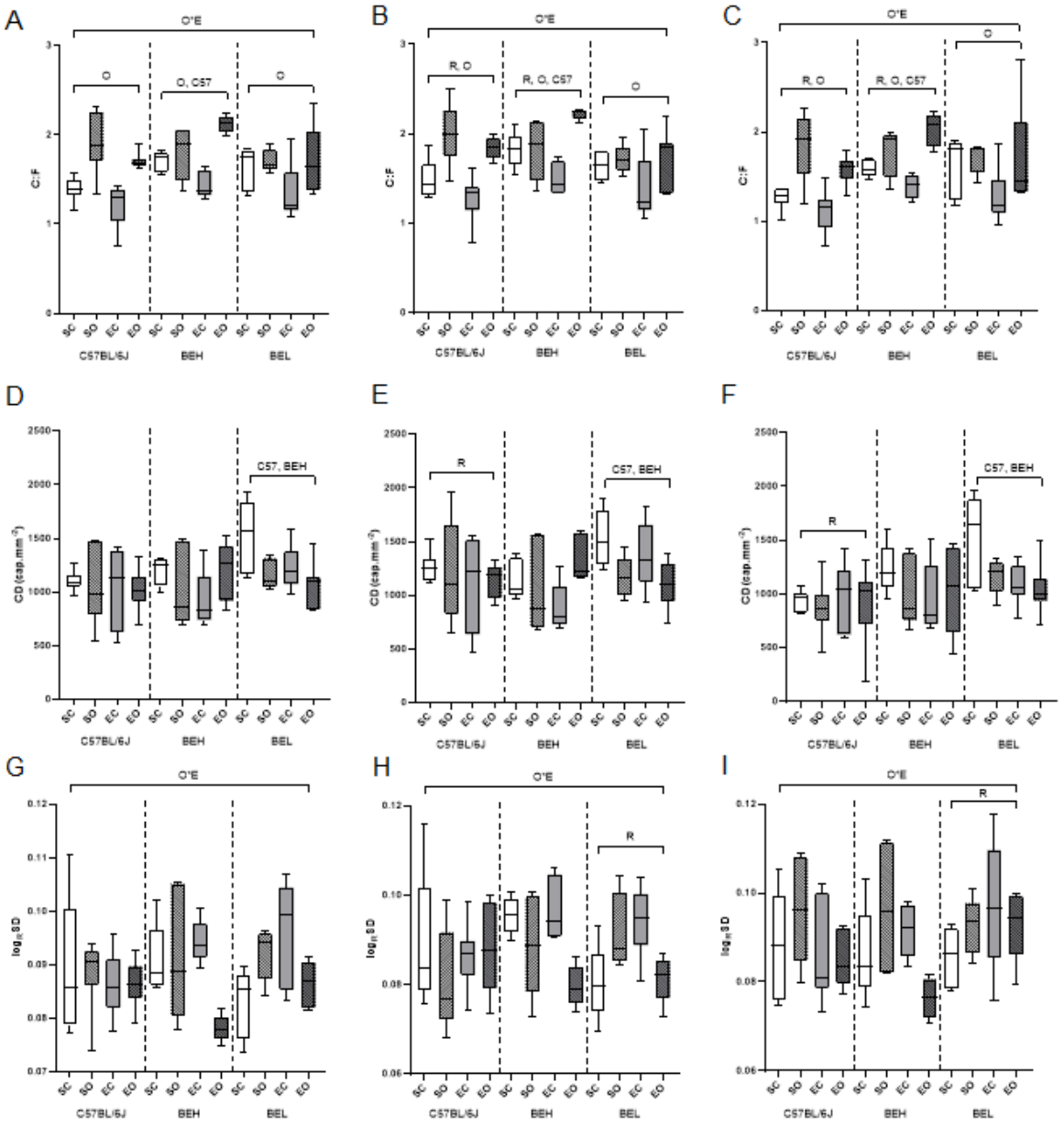

*Figure 4. Effects of age, overload and exercise on skeletal muscle capillarisation in C57BL/6J (C57), Berlin High (BEH) and Berlin Low (BEL) mice. A, B and C show capillary to fibre ratio (C:F) in the whole plantaris muscle (A), oxidative region region (B) and glycolytic region (C). D, E and F show capillary density (CD) in the whole muscle (D), oxidative region (E) and the glycolytic region (F). G, H and I show the logarithmic standard deviation of the radius of the capillary domains (Log_RSD) in the whole muscle (G), oxidative region (H) and the glycolytic region (I). SC = sedentary control, SO = sedentary overload, EC = exercise control, EO = exercise overload. R = effect of region ($p \leq 0.013$), O = effect of overload ($p < 0.001$). O*E =overload*exercise interaction ($p \leq 0.024$). C57 = significantly different to C57 ($p \leq 0.016$), BEH = significantly different to BEH ($p = 0.025$).*

Oxidative capacity and oxygen supply to demand

Succinate dehydrogenase optical density

The fibres in the oxidative region of the muscle had greater SDH OD than those in the glycolytic region ($p<0.001$ Fig. 5B & C). SDH OD for different fibre types can be seen in supplementary data table 3.

Supply:demand ratio

To assess differences in the matching of oxygen supply (LCFR) to oxygen demand (SDH-INT) for a fibre, the LCFR/SDH-INT was calculated (Fig. 5D-F). An overload effect was found ($p=0.020$), but there was also a region*overload interaction ($p=0.010$). Post-hoc analyses revealed that only in the oxidative region of BEL mice overload induced a significant decrease in this ratio ($p=0.001$).

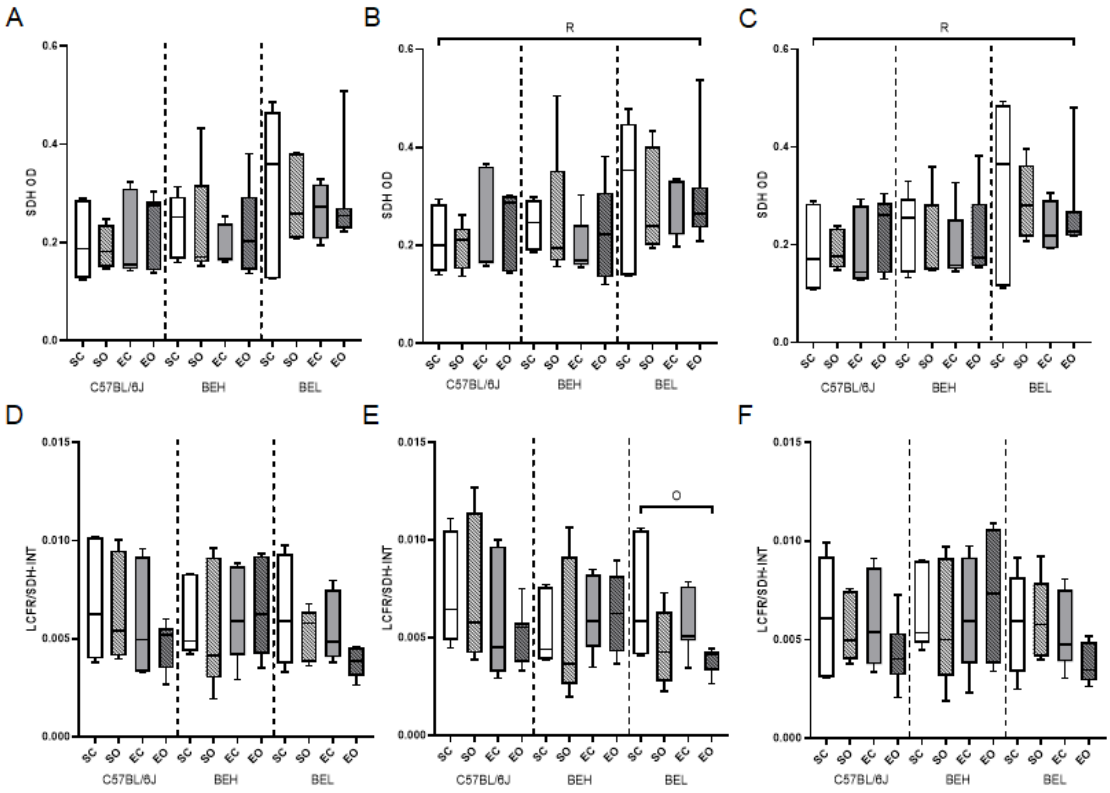

Figure 5. Effects of age, overload and exercise on succinate dehydrogenase optical density (SDH OD) and local capillary to fibre ratio (LCFR):integrated succinate dehydrogenase activity (SDH-INT) in C57BL/6J (C57), Berlin high (BEH), Berlin low (BEL) mice. A, B and C show SDH OD in the whole muscle (A), oxidative region (B) and glycolytic region (C). D, E and F show LCFR/SDH-INT in the whole plantaris muscle (D), oxidative region (E) and glycolytic region (F). SC = sedentary control,

SO = sedentary overload, EC = exercise control, EO = exercise overload. R = effect of region ($p \leq 0.013$), O = effect of overload-induced hypertrophy ($p < 0.001$).

Table 3 Fibre cross sectional area (µm²) for different fibre types in the oxidative and glycolytic regions of plantaris muscles exposed to overload with or without exercise in young adult C57BL/6J, BEH and BEL mice.

	C57				**BEH**				**BEL**				**Effects and interactions**
	SC	**SO**	**EC**	**EO**	**SC**	**SO**	**EC**	**EO**	**SC**	**SO**	**EC**	**EO**	
FCSA (µm²)													
Oxidative region													
Type I	3677 ± 127	1057 ± 205	706 ± 140	940 ± 329	-	921 ± 0	-	876 ± 448	781 ± 28	718 ± 271	783 ± 339	1107 ± 290	-
Type I/IIa	-	1102 ± 39	-	1364 ± 20	-	410 ± 0	-	-	-	454 ± 0	-	1145 ± 330	-
Type IIa	709 ± 195	1362 ± 364	654 ± 192	1237 ± 454	928 ± 108	1161 ± 376	828 ± 315	1203 ± 129	551 ± 128	970 ± 255	603 ± 180	1270 ± 404	R** O*** b*** x*** ax* bx***
Type IIa/IIx	1124 ± 193	1784 ± 357	1146 ± 192	1722 ± 571	1450 ± 8	1745 ± 613	1057 ± 443	1952 ± 231	754 ± 160	1239 ± 397	770 ± 254	1726 ± 739	S* R*** O** a* b*** ax*** bx***
Type IIx	1283 ± 290	2109 ± 420	1121 ± 257	2090 ± 426	1591 ± 259	1935 ± 859	1425 ± 621	2386 ± 707	1094 ± 253	1235 ± 253	1050 ± 292	1926 ± 608	S* R** O*** SR* OE* a*** b*** ax***
Type IIb/IIx	1577 ± 238	2429 ± 705	1454 ± 217	2199 ± 519	1983 ± 359	1755 ± 404	1639 ± 520	-	1358 ± 208	1481 ± 416	1213 ± 433	2428 ± 959	a*** ax***
Type IIb	1667 ± 285[a]	2809 ± 706[a]	1651 ± 388[a]	2566 ± 529	2238 ± 541	1934 ± 981	2165 ± 681	2045 ± 696	1674 ± 88	1914 ± 610	1585 ± 390	2399 ± 615	O*** SO* a*** x*** ax***
Glycolytic region													
Type I	-	1102 ± 147	690 ± 69	814 ± 27	3240	1459 ± 410	5200	1252 ± 588	-	974 ± 119	1246 ± 0	1036 ± 208	-
Type I/IIa	-	1177 ± 191	-	-	-	-	-	-	-	1089 ± 408	-	1172 ± 102	-
Type IIa	647 ± 123	1456 ± 509	574 ± 133	1011 ± 315	614 ± 61	1173 ± 244	921 ± 638	1510 ± 141	619 ± 46	1070 ± 185	589 ± 133	1206 ± 357	R** O*** b*** x*** ax* bx***
Type IIa/IIx	867 ± 187	1855 ± 395	837 ± 134	1532 ± 238	6130 ± 250	1954 ± 456	9020 ± 286	1996 ± 529	751 ± 150	8580 ± 585	873 ± 356	1514 ± 547	S* R*** O** a* b*** ax*** bx***
Type IIx	1065 ± 282	2253 ± 524	927 ± 195	1838 ± 356	1093 ± 276	2195 ± 697	1350 ± 677	2391 ± 242	1069 ± 288	1522 ± 260	1041 ± 267	2171 ± 1007	S* R** O*** SR* OE* a*** b*** ax***
Type IIb/IIx	1327 ± 244	2906 ± 714	1366 ± 396	1871 ± 376	1163 ± 318	2688 ± 1223	1631 ± 440	2960 ± 857	1104 ± 222	1941 ± 1110	1418 ± 613	2411 ± 717	a*** ax***
Type IIb	1709 ± 370	3136 ± 631	1528 ± 338	2378 ± 579	1652 ± 436	2421 ± 557	1953 ± 807	2864 ± 709	1896 ± 479	2330 ± 858	1709 ± 263	2634 ± 1095	O*** SO* a*** x*** ax***

*SC = sedentary control, SO = sedentary overload, EC = exercise control, EO = exercise overload. S: strain effect, R: region effect, O: overload effect, SR: strain*region interaction, SO: strain*hypertrophy interaction, OE: hypertrophy*exercise interaction, a: significantly different from type IIa, b: significantly different from type IIb, x: significantly different from IIx, ax: significantly different from type IIa/IIx hybrid, bx: significantly different from type IIb/IIx hybrid, *: p<0.05, **: p<0.01, ***: p<0.001.*

Table 4 Local capillary to fibre ratio (LCFR) and capillary fibre density (CFD) for different fibre types in the oxidative and glycolytic regions of plantaris muscles exposed to overload with or without endurance stimuli in young C57BL/6J, BEH and BEL mice.

	C57				BEH				BEL				Effects and interactions
	SC	SO	EC	EO	SC	SO	EC	EO	SC	SO	EC	EO	
Oxidative region													
Type IIa LCFR	1.06 ± 0.37	1.70 ± 0.39	0.93 ± 0.29	1.60 ± 0.31	1.38 ± 0.24	1.34 ± 0.46	1.07 ± 0.32	1.82 ± 0.27	1.10 ± 0.31	1.16 ± 0.37	1.01 ± 0.40	1.46 ± 0.44	S* R* O*** SO* RO*
CFD	149 6 ± 196	139 8 ± 479	150 3 ± 502	138 1 ± 238	142 1 ± 437	105 0 ± 503	141 2 ± 366	124 1 ± 214	195 5 ± 148	101 0 ± 70	167 8 ± 248	119 7 ± 226	SR* SO*
Type IIa/IIx LCFR	1.44 ± 0.39	2.17 ± 0.64	1.34 ± 0.80	2.08 ± 0.69	1.84 ± 0	1.68 ± 0.89	1.48 ± 0.37	2.57 ± 0.47	1.53 ± 0.42	1.41 ± 0.67	1.07 ± 0.41	1.92 ± 0.75	O*
CFD	148 1 ± 158	124 6 ± 373	122 5 ± 188	129 4 ± 244	133 0 ± 0	846 ± 472	172 0 ± 547	101 4 ± 98	190 4 ± 434	105 2 ± 202	155 6 ± 249	111 6 ± 222	
Type IIx LCFR	1.71 ± 0.58	2.33 ± 0.49	1.23 ± 0.33	2.56 ± 0.80	1.89 ± 0.31	1.70 ± 0.65	1.52 ± 0.55	2.78 ± 0.78	1.82 ± 0.58	1.38 ± 0.29	1.51 ± 0.51	1.88 ± 0.56	R** O*** SO**
CFD	134 6 ± 254	121 7 ± 466	120 0 ± 405	116 8 ± 239	127 7 ± 252	834 ± 568	117 5 ± 203	104 9 ± 363	163 5 ± 163	979 ± 131	142 4 ± 214	102 3 ± 240	O** SE*
Type IIb/IIx LCFR	1.59 ± 0.17	2.13 ± 0.46	1.43 ± 0.59	2.03 ± 0.36	2.36 ± 0.26	2.04 ± 1.27	1.76 ± 0.10	-	1.90 ± 0.31	1.00 ± 0.30	1.59 ± 1.02	1.74 ± 0.13	O*
CFD	117 2 ± 294	956 ± 350	982 ± 446	987 ± 176	119 1 ± 355	956 ± 91	118 3 ± 305	-	800 ± 742	827 ± 324	122 9 ± 254	963 ± 212	
Type IIb LCFR	1.76 ± 0.18	2.56 ± 0.32	1.50 ± 0.44	2.50 ± 0.55	2.14 ± 0.31	1.48 ± 0.17	1.80 ± 0.23	2.20 ± 0.27	2.15 ± 0.29	1.58 ± 0.09	1.63 ± 0.48	2.35 ± 1.07	R* O** RE*
CFD	109 8 ± 147	103 3 ± 439	101 3 ± 396	952 ± 197	104 3 ± 206	729 ± 439	872 ± 191	667 ± 50	126 2 ± 69	700 ± 193	111 0 ± 184	883 ± 204	O***
Glycolytic region													
Type IIa LCFR	0.73 ± 0.12	1.56 ± 0.71	0.71 ± 0.30	1.13 ± 0.27	0.98 ± 0.15	1.65 ± 0.31	0.88 ± 0.14	2.07 ± 0.58	1.43 ± 0.09	1.44 ± 0.12	0.98 ± 0.39	1.38 ± 0.50	S* R* O*** SO* RO*
CFD	115 0 ± 230	110 5 ± 242	122 9 ± 364	123 6 ± 303	166 1 ± 319	127 8 ± 285	152 9 ± 589	144 3 ± 378	193 3 ± 632	145 2 ± 58	159 2 ± 280	125 5 ± 291	SR* SO*
Type IIa/IIx LCFR	1.09 ± 0.31	1.77 ± 0.73	1.39 ± 1.15	1.46 ± 0.18	0.99 ± 0.06	1.80 ± 0.48	1.09 ± 0.32	2.98 ± 1.87	1.33 ± 0.40	1.72 ± 0.12	1.31 ± 0.42	1.61 ± 0.69	O*
CFD	121 6 ± 73	101 6 ± 199	129 4 ± 244	101 8 ± 124	179 9 ± 648	925 ± 91	131 3 ± 461	120 9 ± 334	188 0 ± 163	135 3 ± 161	137 4 ± 215	113 6 ± 255	
Type IIx LCFR	1.09 ± 0.19	2.00 ± 0.53	1.05 ± 0.33	1.89 ± 0.52	1.50 ± 0.32	1.93 ± 0.63	1.31 ± 0.58	2.58 ± 0.47	1.73 ± 0.72	1.98 ± 0.16	1.21 ± 0.54	1.88 ± 0.86	R** O*** SO**
CFD	106 2 ± 248	924 ± 244	115 9 ± 239	107 6 ± 302	139 4 ± 254	102 5 ± 393	120 8 ± 275	114 1 ± 254	159 8 ± 348	130 7 ± 157	112 9 ± 207	102 2 ± 257	O** SE*
Type IIb/IIx LCFR	1.26 ± 0.47	2.24 ± 0.41	1.04 ± 0.51	1.62 ± 0.56	1.59 ± 0.15	2.03 ± 0.19	1.16 ± 0.35	3.14 ± 1.24	1.57 ± 0.65	1.78 ± 0.01	1.42 ± 0.36	1.81 ± 0.57	O*
CFD	989 ± 189	768 ± 131	991 ± 553	830 ± 212	136 1 ± 445	103 8 ± 349	749 ± 138	951 ± 146	138 5 ± 269	127 9 ± 71	938 ± 210	890 ± 188	
Type IIb LCFR	1.50 ± 0.24	2.53 ± 0.30	1.29 ± 2.5	1.70 ± 0.46	1.81 ± 0.16	1.79 ± 0.54	1.58 ± 0.17	3.02 ± 1.13	2.35 ± 1.1	2.32 ± 0.98	1.59 ± 0.60	2.21 ± 1.26	R* O** RE*
CFD	899 ± 137	813 ± 170	879 ± 323	836 ± 247	121 0 ± 237	834 ± 313	936 ± 336	106 9 ± 193	118 6 ± 264	969 ± 60	905 ± 173	805 ± 192	O***

*SC = sedentary control, SO = sedentary overload, EC = exercise control, EO = exercise overload. S: strain effect, R: region effect, O: overload effect, SR: strain*region interaction, SO: strain*overload interaction, SE: strain*exercise interaction, RO: region*overload effect, RE: region*exercise interaction, HE: hypertrophy*exercise interaction, *: p<0.05, **: p<0.01, ***: p<0.001.*

Table 5 Succinate dehydrogenase optical density (SDH OD) for different fibre types in the oxidative and glycolytic regions of plantaris muscles exposed to overload with or without endurance training in young C57BL/6J, BEH and BEL mice.

	C57 SC	SO	EC	EO	BEH SC	SO	EC	EO	BEL SC	SO	EC	EO	Effects and interactions
SDH OD													
Oxidative region													
Type I	-	0.196 ± 0.038	0.320 ± 0.000	0.232 ± 0.054	-	-	-	0.142 ± 0.044	-	0.125 ± 0.025	0.521 ± 0	0.322 ± 0.108	-
Type I/IIa	-	0.218 ± 0.061	-	-	-	0.165 ± 0	-	-	-	-	-	0.515 ± 0	-
Type IIa	0.284 ± 0.092	0.256 ± 0.073	0.413 ± 0.146	0.320 ± 0.099	0.386 ± 0.068	0.287 ± 0.154	0.347 ± 0.160	0.210 ± 0.107	0.374 ± 0.154	0.284 ± 0.149	0.441 ± 0.143	0.369 ± 0.143	O**
Type IIa/IIx	0.300 ± 0.140	0.231 ± 0.081	0.407 ± 0.201	0.251 ± 0.087	0.416 ± 0	0.183 ± 0.082	0.283 ± 0.030	0.201 ± 0.091	0.393 ± 0.159	0.296 ± 0.203	0.457 ± 0.157	0.293 ± 0.060	O** SR** RE*
Type IIx	0.203 ± 0.052	0.195 ± 0.074	0.290 ± 0.089	0.230 ± 0.077	0.297 ± 0.085	0.194 ± 0.144	0.276 ± 0.113	0.160 ± 0.109	0.303 ± 0.120	0.274 ± 0.147	0.339 ± 0.148	0.337 ± 0.156	S* O** SO*
Type IIb/IIx	0.166 ± 0.054	0.155 ± 0.062	0.269 ± 0.173	0.161 ± 0.049	0.200 ± 0.012	0.131 ± 0.029	0.176 ± 0.017	-	0.200 ± 0.064	0.197 ± 0.072	0.272 ± 0.117	0.245 ± 0.023	
Type IIb	0.122 ± 0.028	0.107 ± 0.020	0.139 ± 0.029	0.117 ± 0.033	0.151 ± 0.018	0.102 ± 0.013	0.115 ± 0.007	0.083 ± 0.001	0.115 ± 0.026	0.107 ± 0.017	0.143 ± 0.020	0.140 ± 0.018	
Glycolytic region													
Type I	-	0.205 ± 0.05	0.140 ± 0.080	0.337 ± 0.000	0.533 ± 0	0.460 ± 0.162	-	0.128 ± 0.018	-	0.228 ± 0.027	-	0.310 ± 0.117	-
Type I/IIa	-	0.318 ± 0.115	-	-	-	-	-	-	-	-	-	0.327 ± 0	-
Type IIa	0.300 ± 0.107	0.258 ± 0.058	0.385 ± 0.104	0.319 ± 0.118	0.417 ± 0.170	0.309 ± 0.168	0.274 ± 0.017	0.246 ± 011 4	0.379 ± 0.182	0.367 ± 0.129	0.375 ± 0.116	0.357 ± 0.162	O**
Type IIa/IIx	0.260 ± 0.140	0.249 ± 0.079	0.385 ± 0.148	0.252 ± 0.091	0.499 ± 0.184	0.232 ± 0.095	0.298 ± 0.072	0.281 ± 0.154	0.441 ± 0.148	0.373 ± 0.188	0.275 ± 0.060	0.286 ± 0.062	O** SR** RE*
Type IIx	0.2712 ± 0.124	0.191 ± 0.060	0.275 ± 0.091	0.244 ± 0.105	0.323 ± 0.121	0.204 ± 0.045	0.209 ± 0.020	0.188 ± 0.072	0.337 ± 0.134	0.306 ± 0.118	0.306 ± 0.103	0.300 ± 0.080	S* O** SO*
Type IIb/IIx	0.209 ± 0.077	0.135 ± 0.029	0.222 ± 0.194	0.171 ± 0.057	0.289 ± 0.064	0.154 ± 0.002	0.213 ± 0.084	0.202 ± 0.027	0.180 ± 0.027	0.261 ± 0.074	0.215 ± 0.072	0.206 ± 0.054	
Type IIb	0.128 ± 0.038	0.111 ± 0.012	0.122 ± 0.022	0.122 ± 0.034	0.161 ± 0.050	0.136 ± 0.034	0.120 ± 0.022	0.128 ± 0.032	0.142 ± 0.049	0.124 ± 0.011	0.137 ± 0.027	0.154 ± 0.029	

*SC = sedentary control, SO = sedentary overload, EC = exercise control, EO = exercise overload. S: strain effect, O: overload effect, SR: strain*region interaction, SO: strain*overload interaction, SE: strain*exercise interaction, RE: region*exercise interaction, *: p<0.05, **: p<0.01, ***: p<0.001.*

DISCUSSION

The main observation of the present study is that in mouse strains with a 3-fold difference in muscle mass the response to regular endurance exercise with or without concomitant overload is similar. In addition, in none of the strains did the combination of overload and endurance training attenuate the hypertrophic response to overload or blunt the exercise-induced increases in fatigue resistance.

Differences between strains

Although smaller than the difference found in the soleus muscle (Lionikas et al., 2013b) we found a 3-fold range in body mass, gastrocnemius mass and plantaris mass between the BEL (smallest) and BEH (largest) mice. In a sedentary non-overloaded state, FCSA of m. plantaris was largest in BEH mice and smallest in BEL with C57 in between. The size of the differences in FCSA, however, evidenced that contrasted muscle mass between these strains were also partly due to differences in number of fibres, which is line with previous findings of both larger fibre number and FCSA in animals with myostatin dysfunction when compared to wild-type mice (Omairi et al., 2016).

The maximal isometric tetanic force of the plantaris muscle was lower in the BEL than C57 and BEH mice, which is explained by their smaller muscle size as the specific tension was similar to that of C57 mice. As a consequence of the lower specific tension in BEH mice, seen before in mice with myostatin dysfunction (Amthor et al., 2007, Mendias et al., 2011, Omairi et al., 2016), the maximal isometric force was similar to that of C57 mice, despite the larger muscle mass.

Similar to previous observations in the extensor digitorum longus muscle (EDL) of myostatin knock-out mice (Mouisel et al., 2014, Savage and McPherron, 2010) we observed that the fatigue resistance of the plantaris muscle of BEH mice was lower than that of C57 mice, with that of BEL mice in between. One possible explanation is the larger proportion of supposedly glycolytic type IIb fibres in the plantaris muscle of the BEH than other strains. However, we found no significant difference in SDH OD in the pooled fibre population between strains, and perhaps the lower fatigue resistance in BEH than control mice is more related to the lower ratio of ATP consumption to isometric tension of type I than type IIb fibres (Stienen et al., 1996). The lowest fatigue resistance in BEL mice was rather unexpected given they had the highest CD, which has been found to have a positive relationship with fatigue

resistance (Tickle et al., 2020). One explanation is that the mass-specific metabolic cost of repolarisation is proportionally larger in small than large fibres, as illustrated during maturational muscle growth in the lobster (Jimenez et al., 2011). The larger C:F in the BEH relative to the other strains was unexpected given the lower capillarisation found in the rectus femoris (Rehfeldt et al., 2005), but this disparity may be attributed to the different muscles studied here.

Response to regular endurance exercise

In contrast to previous observations in myostatin null mice (Matsakas et al., 2012), we did not see an exercise-induced increase in tetanic force or specific tension. In the study by Matsakas et al. (2012) the improvement in specific tension in the myostatin null mice was accompanied by a reduction in fibre size, which we also did not see. The discrepancy between that and our study may be related to the higher training volume and intensity in that study, although an identical training programme as we used did induce a reduction in plantaris mass in myostatin null mice (Savage and McPherron, 2010). Another possible explanation is that the other studies used myostatin null mice (Savage and McPherron, 2010, Matsakas et al., 2012), whereas BEH mice are homozygous for the *compact* allele (Lionikas et al., 2013a). Whatever the cause of the discrepancies between studies, the endurance exercise did not induce a significant change in muscle mass, fibre size, force or specific tension in any of the strains studied.

The endurance exercise did increase, however, the fatigue resistance of the plantaris muscle in all three strains. Although fatigue resistance is positively related to CD (Tickle et al., 2020) and oxidative capacity (Burke et al., 1973), we did not see improvements in either of these measures with endurance exercise, even though others, using an identical programme, showed an increase in oxidative capacity (citrate synthase activity) in myostatin null mice (Savage and McPherron, 2010). Given the absence of angiogenesis and increased oxidative capacity in our study, the exercise-induced increase in fatigue resistance must be attributable to other factors, such as improvements in vasodilation and maximal blood flow (Snell et al., 1987). The lesser improvement in fatigue resistance with endurance exercise in BEH than the other mice may be due to their high proportion of type IIb fibres that are less economical than slower isoforms during isometric contractions (Stienen et al., 1996).

Response to overload by synergist muscle inactivation

In contrast to our prediction that BEH mice would demonstrate the smallest increase in FCSA with overload, the increase in fibre size found in this strain was similar to that of the other strains. BEH mice demonstrated a similar increase in FCSA when compared to BEL, therefore suggesting that baseline FCSA is not predictive of increases in fibre size. This is similar to the findings in mice that baseline muscle mass is not predictive of hypertrophic response (Kilikevicius et al., 2016) but is in contrast to findings in humans (Haun et al., 2019a).

The largest increase in muscle mass with overload in BEH and the smaller increase in muscle mass found with overload in BEL mice are likely due to the greater and lower numbers of fibres found in the muscles of these strains, respectively (Lionikas et al., 2013b) as they demonstrated similar increases in FCSA. The similar growth in FCSA despite the larger diffusion distances in BEH mice may be due to the greater C:F, which would blunt diffusion distances for the delivery of oxygen, preventing an anoxic core of the muscle fibre, and supplying growth factors and amino acids to facilitate hypertrophy (Hendrickse and Degens, 2019, Degens, 2012).

Overload of the plantaris muscle led to a similar angiogenic response in all three strains that was evident in the increased C:F found with hypertrophied muscle. The muscle stretch induced by overload is thought to induce capillary growth independent of changes in blood flow (Egginton et al., 2001) and has been demonstrated in multiple studies (Tickle et al., 2020, Ballak et al., 2016). Interestingly, LCFR/SDH-INT was lower after overload in the oxidative region of BEL mice, suggesting that oxygen supply does not match demand after the muscle fibres have hypertrophied in this region.

Effects of regular endurance exercise on the hypertrophic response to overload

Here we demonstrate that increases in muscle mass and FCSA with overload were not impaired by endurance exercise. This indicates that there was no trade-off in this adaptation when both stimuli were used and suggests that interference in signalling pathways found in some acute studies that combine hypertrophic stimuli and endurance exercise may not manifest in blunted increases in FCSA when aerobic exercise volume is moderate (Murach and Bagley, 2016). However, it may

be that greater endurance exercise volume increases the risk of diminishing the hypertrophy and strength adaptations in response to overload (Methenitis, 2018).

The maintenance of SDH OD with hypertrophy seems to challenge the concept of an inverse relationship between fibre size and oxidative capacity (van der Laarse et al., 1998, van Wessel et al., 2010). This has also been found elsewhere (Campbell et al., 1996, Ballak et al., 2016) and indicates a proportional increase in fibre size and mitochondrial biogenesis. Perhaps the apparent contradiction of the size principle is overcome by decreases in type IIb and IIx fibres and increases in type I and IIa fibres we observed, the latter of which have greater concentrations of subsarcolemmal mitochondria compared to the homogeneous distribution found in glycolytic fibres (Wust et al., 2009), that would reduce the oxygen diffusion limitation. Such relocation of mitochondria to the periphery of the fibre has indeed been observed during an 8-fold increase in FCSA during maturational growth in the blue crab (Hardy et al., 2009). A potential complication is that it would result in greater diffusion distances for ATP from these mitochondria to the myofibrils in the centre of the fibre (Kinsey et al., 2007, Degens, 2012).

Effect of overload on endurance exercise-induced improvements in fatigue resistance

The inverse relationship thought to exist between fibre size and oxidative capacity (van Wessel et al., 2010) would indicate that combining hypertrophic and endurance stimuli would blunt adaptations to endurance training in addition to attenuating the increase in FCSA with overload. Similar to the maintained hypertrophic response in overloaded muscle when endurance exercise was used, the increase in fatigue resistance with endurance exercise was not impaired by overload. This adds further weight to the suggestion that adaptations to hypertrophic and endurance stimuli can be achieved simultaneously. The commensurate increase in C:F which occurs with fibre hypertrophy, evident by the maintenance of CD and CFD, likely prevented the increase in average diffusion distance from capillaries to mitochondria, thus blunting any diffusion limitations associated with increased FCSA (Degens, 2012). The oxidative fibre type shift which occurred with overload may have contributed to the similar fatigue resistance in exercised overloaded muscles when compared to those only subjected to regular endurance exercise as this would make the fibres more resistant to fatigue in spite of increases in FCSA (Stienen et al., 1996).

Limitations

Although muscle performance was improved by endurance exercise, the volume used was insufficient to induce significant increases in oxidative capacity or capillarisation of skeletal muscle. Regardless, the study demonstrated that adaptations to both hypertrophic and endurance stimuli can still occur to their full-scale when both modalities are used concurrently.

Perspectives

Despite different increases in muscle mass, which can be attributed to differences in fibre number, the three strains studied demonstrated similar increases in FCSA and capillarisation in response to overload. The hypertrophic response to overload was not impaired by concomitant endurance exercise training, and improvement in fatigue resistance after running training was not blunted with simultaneous overloading the muscles. This suggests that adaptations to hypertrophic and endurance stimuli are maintained when combined, regardless of baseline muscle mass and fibre size.

Chapter 7 GENERAL DISCUSSION

Skeletal muscle size, strength and endurance are crucial for maintaining quality of life. It has long been known that capillarisation is necessary for the supply of oxygen and nutrients to muscle and for the removal of metabolites and carbon dioxide, but the roles of the microcirculation in performance and plasticity are yet to be fully elucidated. The aim of this thesis was therefore to investigate, in rodent models and human subjects, the function of capillarisation in skeletal muscle fatigue resistance and hypertrophy in health, disease (chronic heart failure (CHF) in particular), ageing and at different baseline muscle mass.

Chapter 1 is a literature review on the current understanding of the contribution of the microcirculation to muscle performance and hypertrophy in health, and in CHF and ageing (**chapter 1**). In **chapter 2,** a method of capillary occlusion using microspheres (MS) was applied to determine the effect of impaired microcirculation on skeletal muscle fatigue resistance and the restorative function of overload-dependent angiogenesis. Furthering this, in **chapter 3,** a model of abdominal aortic banding in rats was used to induce CHF by compensatory cardiac hypertrophy and assess the role that angiogenic stimuli (voluntary running and overload) had on maintaining and restoring muscle performance, respectively. In **chapters 4 and 5** resistance-trained human subjects and overloaded mouse muscle were used to determine the role of concurrent hypertrophic and endurance stimuli in young and old individuals. In **chapter 6** the response to hypertrophic and endurance stimuli in mice strains with different baseline muscle masses was determined.

Microcirculation and fatigue resistance

The dense network of capillaries in skeletal muscle is useful for the delivery of oxygen and nutrients and the removal of waste products, both of which are necessary for maintaining muscle performance. In accordance with this, skeletal muscle with experimentally increased capillary density demonstrate improved fatigue resistance (Hudlicka et al., 1977). Additionally, reduced capillarity is found in numerous diseases, including CHF (Duscha et al., 1999), whose sufferers experience impaired exercise capacity (Rogers, 2001), suggesting that capillary rarefaction contributes to poor exercise tolerance in these conditions. While the improved fatigue resistance with greater capillarisation and the poor exercise tolerance with capillary rarefaction suggest that loss of capillaries contributes to a

reduction in fatigue resistance, it has never been investigated separately from other co-occurring changes in skeletal muscle, such as atrophy, shifts in fibre type composition and loss of oxidative capacity with disease. If it is shown that capillary rarefaction *per se* reduces muscle fatigue resistance, it will justify further study of the role of the microcirculation muscle performance in humans, potentially leading to the capillary bed as a therapeutic target in diseases which affect the skeletal muscle capillarisation.

In **chapter 2** this was tested using a novel unbiased method of MS injection into the femoral artery to block arterioles supplying otherwise healthy muscle to determine the effects of up to 70% loss of functional capillaries on muscle performance. The use of FITC-labelled dextran to reveal functional capillaries demonstrated that there was a dose response reduction in functional capillaries with the dose of MS injected. By measuring the fatigue resistance of the EDL muscle at different acute MS doses it was demonstrated that the fatigue resistance of a muscle is closely coupled with functional capillary density as there was an acute MS dose-dependent reduction in muscle performance. In Chapter 2 it was also found that long term reductions in functional capillary density in the absence of other pathologies led to impaired muscle fatigue resistance and that this performance can be restored through overload induced angiogenesis.

Chronic MS exposure did not impair the hypertrophic response when compared to control animals subject to overload. While unexpected, given the importance of capillaries for the delivery of growth factors and amino acids necessary for hypertrophy (Timmerman et al., 2010, Fry and Rasmussen, 2011), it may be that the reduction in functional capillarisation was insufficient to impair overload remodelling.

Capillary function in chronic heart failure

Exercise intolerance is a feature of CHF that persists even after left ventricular ejection fraction is restored, which suggests that peripheral factors are instrumental in the impaired exercise performance with this disease (Rogers, 2001). Alongside fibre atrophy and a fibre type shift from oxidative to glycolytic fibres, capillary rarefaction occurs in both experimentally induced and clinical CHF. It is unknown if angiogenic therapies can restore muscle performance.

After having determined that fatigue resistance is closely linked to functional capillary density in rat muscle (**chapter 2**), **Chapter 3** studied the effect of

angiogenic stimuli on muscle fatigue resistance in a rat model of chronic heart failure. Using the established technique of abdominal aortic banding to induce compensatory cardiac hypertrophy, it was seen that inclusion of endurance exercise, as an angiogenic stimulus, at the onset of banding, maintained muscle fatigue resistance and that inclusion of overload reversed impaired fatigue resistance in rats subject to 4 weeks of aortic banding. These findings indicate that the use of both blood flow dependent and independent angiogenic stimuli may be useful to mitigate exercise intolerance at the onset, and after the onset, of diseases affecting the microcirculation including CHF and warrant further study into the role of angiogenic stimuli in alleviating exercise intolerance in human CHF patients.

While this study shows that these angiogenic stimuli are beneficial in restoring or maintaining fatigue resistance in these animals, it needs to realised that this is a rat model of compensatory cardiac hypertrophy, and it remains to be seen whether these observations also apply during overt cardiac failure or in a similar study using human participants.

Although overload improved fatigue resistance in rats with CHF due to angiogenesis, increases in muscle mass were blunted when compared to overloaded control muscle. This is in contrast to chronic MS in **chapter 2**, and may be due to increased inflammatory cytokine levels in these animals (Sun et al., 2007), which can suppress mTOR activity (Schiaffino et al., 2013). Another interesting observation is that despite increases in muscle mass, FCSA is not significantly increased with overload in control or aortic-banded animals. Such a discrepancy between muscle mass and FCSA is well-documented (Haun et al., 2019b, Jorgenson and Hornberger, 2019). Early overload-induced mass increase may be attributed to increased muscle length, rather than increased FCSA, which manifests later after 4 weeks of overload (1-2 months)(Jorgenson and Hornberger, 2019).

The size principle of striated muscle

The work of van der Laarse et al. (1998) on the inverse relationship between fibre size and oxidative capacity suggests that one of these characteristics of muscle increases at the expense of the other. This helps to explain the so called “interference effect” first described by Hickson (1980); adaptations to resistance training are blunted when endurance training is also included and suggests that the response to endurance training (oxidative capacity) is dampened by resistance

training. This can be explained by the following formula described by van der Laarse et al. (1998):

$FCSA*VO_{2max} \leq 4\pi*\alpha_{M*} DO_2*PO_2$

in which VO_{2max} is the maximum rate of oxygen consumption (in nmol mm^{-3} s^{-1}), FCSA is the cross-sectional area of the muscle fibre (in mm^2), α_M is the solubility of oxygen in muscle (in mM $mmHg^{-1}$), DO_2 is the diffusion coefficient for oxygen in sarcoplasm (in mm^2 s^{-1}) and PO_2 is the interstitial oxygen tension (in mmHg). If the product at the left of the formula exceeds that of the right, oxygen needs of the muscle fibre will not be met, resulting in an anoxic core. Therefore, in order to maintain this, an increase in oxidative capacity (reflected in a greater VO_{2max}) needs to be accompanied by a reduction in FCSA, provided that the right side of the equation remains constant. In order to maintain, or even increase, oxidative capacity of a fibre while FCSA is increased, oxygen transport to the fibre can be increased through, flattening the shape of the fibre increasing myoglobin, capillary density and/or haematocrit. Alternatively, oxygen diffusion distances from capillary to mitochondria could be reduced through the relocation of mitochondria to the sarcolemma, although this would induce greater ADP and ATP diffusion limitations (Hardy et al., 2009, Degens, 2012).

Based on these assumptions, the combination of hypertrophic and endurance stimuli would result in a trade-off between the adaptations to each, resulting in a blunted response to both modalities (van Wessel et al., 2010). This may reflect the inverse relationship between fibre size and oxidative capacity due to oxygen diffusion limitations (Degens, 2012, van der Laarse et al., 1998, van Wessel et al., 2010), but these predictions have not been tested previously. The aim of **Chapters 4, 5** and **6** was to determine the effects of hypertrophic and endurance stimuli in young and old highly resistance trained men, ageing mice and mice of different baseline muscle size.

Ageing and the size principle of striated muscle

In accordance with the size principle of striated muscle, the incorporation of endurance training into the training programmes of young and old highly resistance trained men in **Chapter 4** results in reductions in FCSA and increases in capillarisation and oxidative capacity. Given that that the angiogenic response has been found to be impaired in elderly mice already during early ageing (Ballak et al., 2016) and the emerging importance of capillarisation in hypertrophic response to

resistance training (Snijders et al., 2017a), even greater reductions in FCSA were predicted in the older subjects. Surprisingly, FCSA and thigh ACSA were maintained after 10 weeks of superimposed endurance training and oxidative capacity and the number of capillaries around type I fibres were increased. Therefore, it seems that the increased fibre capillarisation may allow oxidative capacity to increase without a concomitant decrease in FCSA, thus reducing the diffusion distances and preventing the development of an anoxic core.

It has been shown that endurance exercise can induce hypertrophy and an increase in oxidative capacity (McPhee et al., 2011), but the absence of a resistance-training only group did not allow us to make a firm conclusion of any further hypertrophy that would have been obtained without endurance exercise. The highly trained status of the participants (a minimum of 5 years of resistance training in the young group and 20 years in the old) makes it, however, unlikely that the endurance training would have to experience notable increases in FCSA, ACSA and/or knee extension iMVC in this 10-week period as anabolic intracellular signalling and increases in strength in response to resistance training are diminished in resistance-trained individuals (Coffey et al., 2006, Peterson et al., 2005). It may be that a greater endurance training volume would further increase the oxidative capacity and lead to atrophy as reflected by an extreme pattern of endurance training -chronic electrical stimulation- in rat muscle induced marked reductions in muscle mass with concomitant increases in oxidative capacity compared to those of control limbs (Jarvis et al., 1996). The absence of an increase in muscle oxidative capacity in our resistance-trained individuals may indicate that they were close to the trade-off limit between fibre size and oxidative capacity. That this may be the case is reflected by the diminished hypertrophic response in electrically-stimulated overloaded rat muscles (Frischknecht and Vrbova, 1991).

The effects of combining overload with regular endurance exercise in young and old mice were studied in **chapter 5**. Similar to **chapter 4**, in accordance with the size principle it was predicted that inclusion of overload and endurance exercise leads to impaired hypertrophy, the endurance exercise-induced increase in fatigue resistance is attenuated by overload and responses to endurance exercise and overload are blunted in old mice due to lower capillary density and impaired angiogenesis. It was demonstrated in **Chapter 5** that this was not the case, and the hypertrophic response to overload was not blunted by endurance exercise while improvements in fatigue resistance from endurance exercise were not reduced by

overload. The proportional increase in C:F with hypertrophy in overload may have prevented an increase in diffusion distance, thus allowing for endurance exercise adaptations to be similar to those in non-overloaded animals. This study suggests that hypertrophic and endurance stimuli can be combined without diminishing the adaptations of either. The concurrent training effect may occur with increased volume endurance exercise volume however, as our exercise intervention may have been of too low volume and intensity to cause an increase in oxidative capacity and challenge the trade-off between fibre size and oxidative capacity.

It was also shown that old mice demonstrated less hypertrophy than young mice, along with impaired angiogenesis. Given the importance of capillarisation in the response to hypertrophic stimuli (Snijders et al., 2017a), it may be that the smaller hypertrophic response in old mice is in part due to this blunted angiogenesis found in ageing as the time course of hypertrophy is linked to capillary growth (Egginton et al., 2011, Plyley et al., 1998). This is supported by the previous work of Ballak et al. (2016), who also found smaller increments in muscle mass and less capillary growth with overload in old mice.

Baseline muscle size and the size principle

According to the size principle, fibres with a large FCSA would experience less hypertrophy than those with smaller FCSA due to greater diffusion distances. In **chapter 6** overload and endurance exercise were combined in three different mouse strains with different FCSA, one with large fibres (BEH), one with medium sized fibres (C57) and one with small fibres (BEL). Similar to **chapter 5**, it was found that overload-induced hypertrophy was not blunted by endurance exercise and increases in fatigue resistance with endurance exercise were not impaired by overload. Interestingly, all strains demonstrated similar increases in FCSA with overload, and different gains in muscle mass between the strains were attributed to differing numbers of fibres. This suggests that increases in FCSA are independent of baseline fibre size, in contrast to findings in human subjects (Haun et al., 2019a).

As in **chapter 5** increases in C:F proportionate to hypertrophy with overload may have prevented increases in diffusion distance and allowed for similar increases in fatigue resistance with endurance exercise to the non-overloaded limb. The oxidative shift in fibre type which occurs with overload would make fibres more resistant to fatigue (Degens and Veerkamp, 1994) in spite of increases in FCSA. This shift towards more oxidative fibres is also accompanied by greater

subsarcolemmal concentrations of mitochondria (Wust et al., 2009), which would further decrease the oxygen diffusion distance. This redistribution of mitochondria would, however, incur a cost in increased ATP diffusion distances (Degens, 2012, Kinsey et al., 2007). Therefore, according to the size principle, in order to accommodate the increase in FCSA, an anoxic core is overcome by augmenting oxygen delivery through increased numbers of capillaries per fibre and decreasing the diffusion distance from capillary to mitochondria by shifting the mitochondria to the subsarcolemmal region of the fibre (van Wessel et al., 2010). Further fibre growth may be limited however; although the angiogenesis which accompanies hypertrophy blunts the increase in diffusion distances, capillaries are always situated on the exterior of a fibre and hypertrophy may reach a point that increased capillarisation can no longer prevent an anoxic core (Degens, 2012). Similarly, mitochondrial concentration changes with oxidative shifts may result in larger ATP diffusion distances and limit the ATP supply to myofilaments in the centre of the fibres. As these distances increase, the time dependent reduction in diffusion coefficient gets larger due to the intracellular barriers such as the contractile apparatus and sarcoplasmic reticulum (Kinsey et al., 2011).

One limitation of **chapters 5** and **6** is that the endurance exercise used was of insufficient intensity and/or volume to induce increases in oxidative capacity necessary to incur a trade-off in adaptations to hypertrophic and endurance stimuli. Frischknecht and Vrbova (1991) demonstrated large increases in SDH activity when overloaded muscles were electrically stimulated for long periods at low frequencies and hypertrophy was blunted when compared to muscle subject to overload only.

Additionally, while hypertrophy demonstrated by removal of synergists via denervation is much greater than that found resistance training conducted over the same period of time, it is much less than that experienced by overload induced by synergist ablation (Degens, 2012). Thus, the use of a more extreme hypertrophic stimulus such as synergist ablation combined with a more challenging aerobic stimulus could reveal the fibre size principle proposed by van der Laarse et al (1998).

Practical implications and future research

Impaired exercise tolerance is a hallmark of multiple conditions which affect the microcirculation, including chronic heart failure. Chapter 2 demonstrated that muscle fatigue resistance is closely coupled to functional capillary density and that

lower muscle performance as a result of capillary loss can be reversed through an angiogenic stimulus. Using the model described above, skeletal muscle capillarisation was isolated as a target for improvements in exercise therapy for rehabilitation in these diseases. Further to this, angiogenic stimuli were shown to be able to maintain muscle performance at the onset of CHF and rescue fatigue resistance after 4 weeks of aortic constriction in a rat model. This supports the suggestion that the microcirculation is a valuable target in improving exercise tolerance in CHF, and may be worth studying in other diseases which affect skeletal muscle capillarisation.

The study of combining hypertrophic and endurance stimuli in chapters 4, 5 and 6 demonstrates that both modalities can be used without affecting the adaptations of the other, irrespective of age and baseline fibre size. The outcomes of these chapters add to other evidence which supports the use of resistance and endurance exercise concomitantly without detriment to muscle size or performance which could be applied in athletes which require the benefits of both modalities (Murach and Bagley., 2016). Still, it may be that greater exercise volume may lead to increased interference in the long-term responses to training, something which warrants exploration. Future study could determine if greater endurance and/or resistance training volume leads to a concurrent training effect and thus provide more detailed guidance for maximising the benefits of concurrent training.

The findings of chapter 5 also add further weight to observations of impaired angiogenesis with ageing and the implications for muscle plasticity (Ballak et al., 2016). It remains to be seen however, that increasing capillary number through exercise or other angiogenic means would improve the hypertrophic response to resistance training. This could be studied by comparing the hypertrophic response to resistance training in individuals that have undergone an endurance training programme to that of a previously untrained group.

The positive findings of chapters 2 and 3 on the benefits of capillary growth on muscle performance in CHF prompt further study in other models of disease. As mentioned in the literature review, skeletal muscle microcirculatory function is also negatively affected by type II diabetes. In a rat model of diabetes, such as the Goto-Kakizaki rat, a similar study could be done using overload and wheel running as angiogenic stimuli to determine their effect on muscle performance (Padilla et al. 2006).

The findings of chapter 2 prompt further study of the role the microcirculation plays in skeletal muscle fatigue resistance. While evident in the rat model used that functional capillary density is closely coupled with muscle performance, a similar acute study could be performed in humans to demonstrate this relationship is found in both species. This would be possible with the use of soluble microspheres with a short half-life. If a similar relationship between microsphere dose and fatigue resistance is found in humans this would identify the skeletal muscle microvasculature as a therapeutic target for exercise intolerance.

Furthermore, the findings that angiogenic stimuli can prevent and reverse decrements in muscle fatigue resistance from impaired functional capillary density could be applied to a training study in humans with mild symptoms of CHF. The use of blood flow restricted resistance training would allow for low intensity loading which can be tolerated by patients with CHF (Groennebaek et al., 2019) whilst providing an angiogenic stimulus (Nielsen et al. 2020).

The proposed study of angiogenic stimuli in a diabetic rat model could also be translated into a human study with diabetic participants with the use of angiogenic stimuli, taking care to accommodate for the poor exercise tolerance of many people with diabetes (Nesti et al., 2020) and providing encouragement during the trial. This could be done with a tolerable exercise modality such as blood flow restriction (Saatmann et al., 2020) or muscle stimulation (Theriault et al., 1996) and biopsies and functional measurements could determine the effects on the capillary bed and fatigue resistance.

Along with these proposed studies in human participants the work outlined in this thesis has the potential to influence the treatment of exercise intolerance in disease characterised by capillary rarefaction.

This thesis aimed to elucidate the roles of capillarisation in skeletal muscle function and plasticity. Here evidence is provided for the importance of functional capillarisation in muscle fatigue resistance and the angiogenic response in hypertrophy. Further study is required to determine whether expanding the microvascular bed can improve exercise tolerance in disease and augment the response to resistance training in health and in ageing. The findings detailed in this thesis add to previous evidence indicating the importance of the microcirculation in muscle function and hypertrophic response, and should stimulate development of

interventions in humans to improve muscle performance in health, disease and ageing.

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PUBLICATIONS AND PRESENTATIONS

Articles in peer-reviewed journals:

- *'An evaluation of common markers of muscle denervation in denervated young-adult and old rat gastrocnemius muscle'.* **Hendrickse P**, Galisnka M, Hodson-Tole E, Degens H. Experimental Gerontology 2018 Jun;106:159-164

- *'The role of the microcirculation in muscle function and plasticity'.* **Hendrickse PW**, Degens H. Journal of Muscle Research and Cell Motility 2019 40:127–140

- *'Impaired skeletal muscle performance as a consequence of random functional capillary rarefaction can be restored with overload-dependent angiogenesis'.* Tickle P**, Hendrickse PW,** Degens H, Egginton S. The Journal of Physiology 2020 Mar;598(6):1187-1203

- *'Regular endurance exercise of overloaded muscle of young and old male mice does not attenuate hypertrophy and improved fatigue resistance'.* **Hendrickse PW**, Krusnauskas R, Hodson-Tole E, Venckunas T, Degens H. GeroScience Jul; doi: 10.1007/s11357-020-00224-x

- *'Endurance exercise plus overload induces fatigue resistance and similar hypertrophy in mice irrespective of muscle mass'.* **Hendrickse P**, Krusnauskas R, Hodson-Tole E, Venckunas T, Degens H. Experimental Physiology Nov;105(12):2110-2122

Oral presentations

- Northern Vascular Biology Forum, 2017 – The Role of the Microcirculation in Skeletal Muscle
- Manchester Metropolitan University Science and Engineering Symposium, 2018 – The Role of Microcirculation in Skeletal Muscle
- Manchester Metropolitan University Musculoskeletal Science and Sports Medicine Annual General Meeting, 2019 – The Role of Microcirculation in Skeletal Muscle Function and Plasticity

Conference abstracts

- *'The role of the microcirculation in skeletal muscle.'* **Hendrickse P**, Tickle P, Ferris G, Egginton S, Degens H. Northern Vascular Biology Forum, Liverpool, 2017. Talk five.
- *'The effect of acute and chronic changes in microvascular perfusion on skeletal muscle performance.'* Tickle P, **Hendrickse P**, Degens H, Egginton S. Future Physiology, 2017. Leeds, 65, 2017.
- *'Role of the microcirculation in muscle wasting and plasticity'.* Degens H, **Hendrickse P**. 12th International Conference on Cachexia, Sarcopenia & Muscle Wasting, 2019, 99