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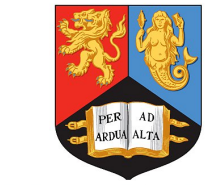

UNIVERSITY OF BIRMINGHAM

Which of the adaptations to a period of acclimatisation to chronic hypoxia are important in determining skeletal muscle performance and fatiguability?

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Abstract

Patients with oxygen-deficient diseases suffer from chronic hypoxia and exercise intolerance. However it is not clear whether this is due to muscular dysfunction or changes in oxygen delivery. Acclimation to chronic hypoxia involves increased haematocrit, angiogenesis and changes in skeletal muscle fibre-type. The objective of this study was to investigate the effect of changing oxygen delivery by isovolaemic haemodilution on muscle fatigue, in normoxic and chronic hypoxic rats.

Arterial blood pressure, femoral blood flow and tension in the extensor digitorum longus muscle were recorded in four groups of alfaxan-anaesthetised male Wistar rats. The peroneal nerve was stimulated for five successive 3 min periods at 15Hz. The treatments encompassed haemodilution, a total of 9ml sequestered between consecutive stimulations, in normoxic and hypoxic animals, acclimated to 12% O_2 for three weeks that inspired oxygen at 8% and 12%.

Consistent response profiles were generated by the time controls; contractions were associated with a ~1200g.s^{-1} muscle performance and ~40% fatigue. Haemodilution had no effect on these responses. The chronic hypoxic group (12%O_2) fatigued ~10% more than haemodilution controls at matched oxygen delivery. It is proposed that muscular adaptations evoked by 12%O_2 acclimation play a greater role in mechanisms limiting exercise tolerance than vascular adaptations.

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List of abbreviations

ABP	arterial blood pressure
AH	acute hypoxia
AMP/ATP	adenosine monophosphate / triphosphate
AMPK	AMP-dependent protein kinase
BG	blood gas
C:F	capillary to fibre ratio
CaO_2	total arterial oxygen content
CH	chronic hypoxia
CH(PaO_2~33)-HD	chronic hypoxic, 8% O_2
CH(PaO_2~47)-HD	chronic hypoxic, 12% O_2
CHF	chronic heart failure
COPD	chronic obstructive pulmonary disease
Cr	creatine
dH_20	distilled water
DO_2	oxygen delivery
EDL	extensor digitorum longus
FBF	femoral blood flow
FVC	femoral vascular conductance
Hct	haematocrit
HD	normovolaemic haemodilution
MFBF	mean femoral blood flow
MFCA	mean fibre cross-sectional area
N	normoxic

N- HD	normoxic haemodilution
N- TC	normoxic time controls
$PaCO_2$	partial pressure of carbon dioxide
PaO_2	partial pressure of oxygen
PBS	phosphate buffered saline
PCr	phosphocreatine
PG12	prostacyclin
PHK	Phosphorylase b kinase
Pi	phosphate ion
P_t	peak tension
R	recovery
S	stimulation
SNP	sodium nitroprusside
VO_2	oxygen consumption

1. Introduction

1.1 Overview

Chronic hypoxia (CH) is a term used to describe long periods of low oxygen delivery (DO_2) to the tissues, resulting from a decline in oxygen partial pressures and therefore oxygen content in the arterial blood (CaO_2). Chronic heart failure (CHF) and chronic obstructive pulmonary disease (COPD) are associated with skeletal muscle dysfunction and exercise intolerance (Lundby et al., 2009). These diseases are also linked with CH, a factor that may cause premature fatigue in patients. Remodelling of the skeletal muscles, vasculature and of metabolic pathways occurs in response to prolonged periods of reduced CaO_2 in order to maintain normal DO_2 (El-Khoury et al., 2012).

The mechanisms responsible for these phenotypic changes have been studied in detail, however very little is known about the roles of individual adaptations in skeletal muscle performance and fatiguability (Lundby et al., 2009). The objective of the present study was to separate the effects of DO_2 and the skeletal muscle adaptations to CH on muscle performance and fatiguability, by studying the response to stimulated extensor digitorum longus (EDL) muscle contraction in normoxia (N) and CH rats at the same DO_2.

1.2 Factors influencing skeletal muscle performance and fatigue

Fatigue is defined as a decline in the performance of a muscle during intense and prolonged activity. Several factors influence the tension generated by a skeletal muscle and how quickly the muscle performance deteriorates. Muscle fatigue has

been associated with the type of muscle fibres within the muscle itself, fast fibre types fatiguing more quickly than slow fibres (Westerblad et al., 2010). Fibre fatiguability is closely coupled with fibre energetics and is constrained by fast or slow protein expression, mitochondrial content, calcium (Ca^{2+}) handling and intramuscular energy stores (Allen et al., 1995). Metabolic by-products such as lactate, hydrogen (H^+) and phosphate (Pi) ions produced during contraction can have detrimental effects on efficient physiological functioning thereby promoting fatiguability (Spangenburg and Booth, 2003). However, release of potassium ions (K^+) promotes functional hyperaemia, an increase in blood flow to the muscle during contraction, which enhances muscle performance via glucose and DO_2. Functional hyperaemia corresponds with local vasodilatation, which is dependent on CaO_2 (Poucher, 1996). The amount of oxygen in the blood is influenced by the haematocrit (Hct) and the partial pressure of oxygen (PaO_2) (Roach et al., 1999), while DO_2 distribution varies with capillary density, fibre size and capillary to fibre ratio. These factors are discussed in greater detail below.

1.3 Skeletal muscle composition

Muscle performance can be characterised in terms of the force produced by a muscle or muscle group and the time during which the force is generated. The performance capability of human skeletal muscles is determined by the muscle fibre types, which comprise three types of myofibril: slow, intermediate and fast, enabling the muscle to cope with a range of activities from standing to ballistic movements.

Furthermore, muscle fibres can be broadly grouped into small ‘red’ slow twitch oxidative fibres and large ‘white’ fast twitch glycolytic fibres. More specifically, three

myofilament myosin subtypes (I, IIa and IIx) are used for classification of human skeletal myofibres. Rat muscle fibres express a fourth type of myosin, myosin IIb (Smerdu et al., 1994). The type of myosin determines the rate of cross-bridge formation and therefore the contractile speed of the fibre, the slowest oxidative fibres being types I and the fastest, glycolytic types IIx/b.

Fast-oxidative fibres exhibit a rapid contraction phenotype and are reasonably fatigue resistant. A small number of these fibres contain more than one myosin isoform. The transcription regulators, nuclear factor of activated T cells and myocyte specific enhancer factor 2 control the gene expression in this fibre type. Calcineurin has a role in fibre type regulation and is reported to activate a subpopulation of 'slow' genes such as myoglobin promoters and slow troponin I (Chin et al., 1998). CaN is insensitive to the rapid changes in Ca^{2+} during contraction which reflect patterns of fast muscle stimulation (Liu et al., 2001). Fast-glycolytic fibres fatigue more rapidly than other fibre types and can be identified by their larger size and tendency to group together. Regulating fibre type requires several co-ordinating pathways to control gene expression.

A muscle dominated by oxidative fibres is more fatigue resistant while generating less tension, than a muscle predominantly composed of powerful, fast glycolytic fibres. The majority of muscles contain a mixture of fibre types consistent with the performance requirements of the muscle, and as different fibre types fatigue at different rates the susceptibility of a particular muscle to fatigue is a function of its fibre composition. The characteristics of skeletal muscle fibre types are summarised in Table 1. However fibre typing is currently believed to be much more complex than

previously described with ‘slow’ and ‘fast’ proteins being expressed across all fibre types and Ca^{2+} transients altering the phenotype of a myofibril but not being restricted to corresponding fibre type or myosin isoform (Westerblad et al., 2010).

Table 1. Characteristics of skeletal muscle fibre type

Characteristic	Slow-twitch oxidative (I)	Fast-oxidative glycolytic (IIa)	Fast-twitch glycolytic (IIx)
Cross-sectional area	Small	Intermediate	Large
Tension	Low	Intermediate	High
Contractile velocity	Slow	Fast	Fast
Fatiguability	Low	Intermediate	High
Colour	Red	Pink	White
Myoglobin content	High	Low	Low
Capillary density	High	Intermediate	Low
Mitochondrial content	High	Intermediate	Low
Glycogen content	Low	High	High

Adapted from Martini (2006)

1.4 Energetic properties of skeletal muscle fibres

Myosin hydrolyses adenosine triphosphate (ATP) at different rates. The IIx fast myosin isoform breaks ATP down three fold faster than slow type I fibres in isometric conditions (Bottinelli and Reggiani, 2000). Intracellular ATP was found to decrease by ~40% in the whole muscle from the resting value after maximal isometric contractions. However, studies on individual myofibres suggest that ATP levels vary significantly between fibres post exercise. For example, ATP was reduced to a fifth of resting values in type IIx fast muscle fibres categorised according to their myosin isoform (Karatzaferi et al., 2001). ATPase activity also varies within fibres expressing

the same isoform, which is a field of on-going research. Due to low ATPase activity in type I fibres it is more efficient to use type I in isometric contractions than any other fibre type.

Rodent fibre types differ in terms of the sensitivity of contractile apparatus to Ca^{2+} sensitivity. Type I fibres have been found to have the highest Ca^{2+} sensitivity and differential sensitivity to Ca^{2+} contributes to decreased force generation in fatigue. Studies suggest that a decrease in pH during exercise also has adverse affects on Ca^{2+} sensitivity, reducing contractile tension over a prolonged period. Ca^{2+} transients in slow fibres reach a lower peak and decline more slowly than in fast fibres in rat skeletal muscle (Bottinelli and Reggiani, 2000). In vitro patch clamp experiments confirm similar findings in human muscle fibres (Delbono and Chu, 1995). The low calcium transient amplitude in slow fibre types is reflected in slow fibre directed single twitch components featuring longer contraction and relaxation phases post glycolytic fibre fatigue (Figure 1).

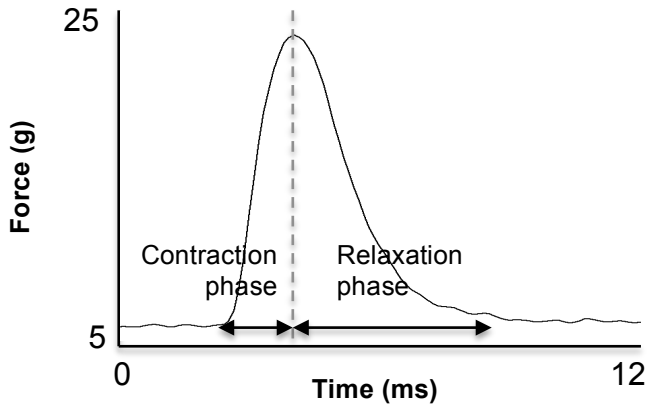

Figure 1. The constituents of a single EDL rat muscle twitch

The slow decline in calcium transients in slow fibres may be due to a reduction in the expression of SERCA (Bottinelli and Reggiani, 2000). Calcium transport mechanisms vary within fibre type as SERCA1 is preferentially expressed in fast fibres and SERCA2 in slow fibres (Warmington et al., 1996).

Mitochondrial numbers also vary between fibre types. Links between slow fibre types and oxidative enzyme activity, mitochondrial content and oxygen uptake are generally accepted. There is a 4% decrease in mitochondrial volume between fibre type I and type IIx (Bottinelli and Reggiani, 2000). This suggests that slow fibres have a higher aerobic metabolic capacity and fast fibres a higher glycolytic capability. Accordingly, glycogen content has been observed to be more than 30% higher in fast fibres (Vollestad et al., 1984).

Overall differentiation in myofibre fatigue is associated with significant alterations in metabolic rate, cellular Ca^{2+} handling and contractile velocity between fibre types (Bottinelli and Reggiani, 2000; Spangenburg and Booth, 2003).

1.5 Energy metabolism in skeletal muscle fibres

The amount of ATP required by SERCA and the myosin-actin cross-bridges depends on the type of contraction and the muscle mass engaged. Long periods of single twitch stimulation where muscle fibres are required to perform at their maximum cause the activation of the anaerobic metabolic pathway to maintain contractile function (Sahlin et al., 1998). Aerobic and anaerobic metabolisms are not independent as lactate generation occurs prior to the full utilisation of aerobic capacity (Jorfeldt et al., 1978). Furthermore, anaerobic metabolism is inefficient in

comparison to aerobic ATP production and decreases intracellular pH having a detrimental effect on the rate of fatigue.

1.5.1 Oxidative aerobic metabolism

Due to the lower ATP requirements and relative low metabolism of type I fibres, aerobic metabolism is maintained for longer during contraction, delaying the onset of fatigue. The oxygen dependent metabolism of lipids and carbohydrates are the most efficient pathways for the production of ATP (Spriet and Watt, 2003). However they often play a minor role during maximal exertion due to constraints in DO_2. Glycogen and extracellular glucose are the main carbohydrate substrates utilised in aerobic metabolism during submaximal and prolonged exercise. The rate of glucose uptake increases by fifty-fold during short-term maximal dynamic exercise, although glucose uptake has no impact on ATP production during this time due to limitations of hexokinase availability (Katz et al., 1991).

An insulin independent pathway mediates the control of glucose transport through the sarcolemma during exercise but the precise mechanism is not completely understood. Previous studies in rats have shown that blood glucose falls during electrical muscle stimulation due to AMP-dependent protein kinase (AMPK), which regulates metabolism in skeletal muscle and becomes activated by the exercise-induced depletion of phosphorylated substrates and the corresponding increase in adenosine monophosphate (AMP) (Helmreich et al., 1957). The increase in AMP is transient in nature and therefore there are likely to be additional mechanisms involved in the regulation of plasma glucose, such as an increase in the levels of reactive oxygen species mediated by AMPK signalling during submaximal exercise,

where extracellular glucose contributes to aerobic ATP production (Sandström et al., 2006).

Intramuscular triglycerol stores and plasma lipids are broken down to free fatty acids during lipid metabolism. The rate of lipid metabolism varies depending on the intensity of exercise. At an intensity requiring 60% oxygen consumption (VO_2), ATP production is at its most efficient. At higher levels of VO_2, fatty acid oxidation falls (Sahlin et al., 1998). In the absence of carbohydrate or lipid availability muscle protein is degraded to amino acids which are used in aerobic metabolism but add little overall to energy production. The concentration of activated branched chain amino acids is therefore raised in slow-twitch muscles (Henriksson, 1991).

1.5.2 Glycolytic anaerobic metabolism

Phosphocreatine (PCr) degradation coupled with glycogen breakdown is the central pathway in fast-twitch anaerobic metabolism utilised during bursts of maximum activity. Muscle fatigue corresponds with particular intramuscular metabolic alterations such as glycogen and PCr depletion and with lactate accumulation during prolonged exercise. Glycogen is broken down in the process of generating ATP, and by-products of anaerobic metabolism may cause a decline in contractile function enhancing fatigue (Karatzaferi et al., 2001).

PCr breakdown requires ADP and releases creatine (Cr) and ATP. PCr concentrations decline during periods of exercise when ATP is in high demand and there are increases in Cr and inorganic phosphate ion (Pi) levels. Previous research has suggested that an increase in Pi associated with PCr degradation is a factor causing fatigue. However, it was also noted that Cr supplements contribute to muscle

performance and Pi is therefore unlikely to enhance a declining contractile function (Casey et al., 1996). The recovery of PCr levels after exercise has been used to indirectly assess the rate of oxidative metabolism in patients suffering exercise intolerance (Mancini et al., 1992).

Glycogen degeneration is catalysed by phosphorylase b kinase (PHK), primarily activated by AMP, which phosphorylates glycogen phosphorylase at serine 14. PHK is primarily responsible for the accelerated glycogen breakdown due to the transient increase in AMP concentration during contraction. A single site phosphorylation acts to raise glycogen phosphorylase activity rapidly *in vivo* (Katz et al., 2003). Glycolysis converts phosphorylase-liberated glucose molecules from glycogen to pyruvate. Lactate dehydrogenase catalyses the production of NAD^+ kinase and lactate from nicotinamide adenine dinucleotide, H^+ ions and pyruvate (Jorfeldt et al., 1978). NAD^+ is available for an upstream glycolytic reaction that maintains glycolysis. Lactate formation is dependent on oxygen availability relative to energy consumption and can be responsible for 60% of ATP turnover in an anoxic isometric contraction (Katz et al., 1986). Severe acidosis, a drop in pH from 7.1 to 6.6, a result of lactate accumulation in the muscle during intense exercise, causes a reduction in enzyme and channel activity affecting both contraction and relaxation phases due to the reduced affinity of calcium for troponin and the blunting of SERCA (Lamb et al., 1992).

Theoretically, anaerobic lactate production generates three ATP units per glucosyl residue compared to thirty-eight in aerobic metabolism. A decrease in ATP availability results in fatigue, as intramuscular cross-bridge formation is reduced

resulting in a decline in the tension generated per contraction. In summary, fatigue is associated with the capacity of an individual fibre to accommodate aerobic or anaerobic endurance, while the depletion of metabolic reserves results in a cumulative decrease in the performance of the skeletal muscle as a whole.

1.6 Nutrient delivery to skeletal muscle

An insufficient nutrient supply to active muscle can limit fibre performance and increase fatigue. Muscle glucose uptake from the blood increases with exercise and is influenced by muscle blood flow, capillary distribution and glucose transporter type 4 expression (Wasserman et al., 2011). An increase in blood flow to the muscle during exercise ensures an increase in glucose delivery maintaining the extracellular glucose gradient at a sufficient level to support an increased metabolic rate. Blood flow also facilitates an increase in DO_2 required during exercise as VO_2 increases.

1.7 Functional hyperaemia

Functional hyperaemia (Figure 2) is defined as an increase in blood flow to active tissues caused by an increase in metabolic rate, which can only occur with an increase in arterial blood pressure (ABP), a vasodilatation or a combination of the two. Assuming ABP remains constant, an increase in vascular conductance (vasodilatation) is required to achieve an increase in blood flow. During exercise a rapid increase in DO_2 is achieved through an increase in blood flow, which in turn allows the VO_2 to increase. However skeletal muscle VO_2 and related muscle function are limited when DO_2 is no longer sufficient to maintain VO_2. A mismatch between DO_2 and VO_2 occurs when arterioles are maximally dilated.

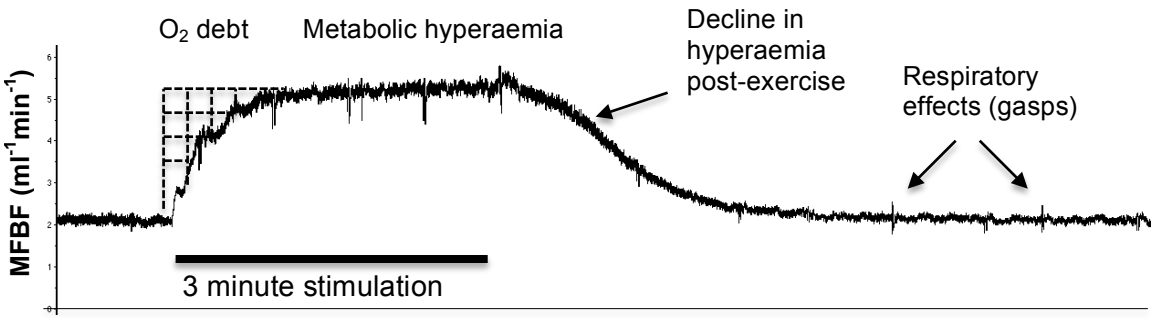

Figure 2. Functional hyperaemia
Mean femoral blood flow (MFBF), trace from the current study, recorded in rat femoral artery during high frequency EDL muscle contractions in a 3 min period adapted from Walloe and Wesche (1988). Time bar marks the stimulation period.

Vasodilation during low intensity and high intensity exercise in humans maintains DO_2 in excess of the VO_2 in the muscle fibres and vasculature (Jones et al., 2012). The magnitude of exercise hyperaemia observed in humans has been shown to relate to the training status of the muscle (Mortensen et al., 2012). Adequate muscle perfusion is critical for normal physiological function and requires a well controlled and suitably distributed O_2 supply (Ellsworth and Sprague, 2012). Increase in blood flow is proportional to exercise intensity (Tschakovsky et al., 2004). Therefore dilatation is also related to muscle activation.

1.7.1 Control of vascular tone

Vascular tone controls local blood flow and therefore regulates capillary recruitment and perfusion homogeneity. Tension within the smooth muscle of the tunica media, is an important prerequisite for vascular dilatation, and skeletal muscles which are capable of significant hyperaemia have a raised vascular tone at rest (Segal and Jacobs, 2001). The feed arteries to the EDL have a resting diameter of 75μm compared to 45μm in the soleus, despite the EDL feed arteries having a larger

capacity for dilatation (Williams and Segal, 1993). Proximal arterioles are highly reactive and are a major source of resistance to blood flow upstream of the microcirculation. Vascular tone is maintained in part by the sympathetic vasoconstrictor neurons but is also influenced by the intrinsic system of myogenic responses and vasodilator mediators, such as adenosine and nitric oxide (NO) (Clifford and Hellsten, 2004).

NO, is a mediator of flow-induced dilatation, released by endothelium in response to shear stress. Protein kinase B, activated by shear stress, stimulates endothelial NO synthase release. The dilatation of terminal arterioles decreases the total vascular resistance increasing both blood flow through feed arteries and capillary perfusion in a propagated response (Williams and Segal, 1993). NO is not involved in the maintenance of functional hyperaemia. Experimental local microiontophoretic applications of acetylcholine have shown vasodilatation of conduit arteries upstream of local dilatation caused by conducted hyperpolarisation of endothelium and vascular smooth muscle (Segal and Jacobs, 2001).

1.7.2. Control of functional hyperaemia

Skeletal muscle fibres in the EDL increase metabolic rate during exercise triggering a corresponding exercise hyperaemia. Hyperaemia is induced by the diffusion of vasodilator mediators from active metabolic myofibrils, smooth muscle myocytes and endothelium into the interstitium. Several factors such as lactic acid, potassium ions, Pi, local exercise related tissue hypoxia and adenosine contribute to the vasodilatation in the active muscle (Clifford and Hellsten, 2004).

Adenosine originates from two sources: active muscle tissue and endothelial cells. AMP is released into the interstitium of the active muscle tissue, which is hydrolysed by AMP 5'nucleotidase ectoenzyme into adenosine and Pis. Adenosine manipulates downstream protein kinase cascades initiating vasodilatation via the stimulation of A_1, A_{2A} and A_{2B} receptors. The A_{2A}-receptor is located on vascular myocytes and has been implicated in exercise hyperaemia activating adenylate cyclase. These receptors have been found responsible for ~14% of the hyperaemia induced by five minutes of supramaximal isometric twitch contractions and a quarter of the blood flow evoked by tetanic contractions stimulated at 4 and 40Hz respectively (Ray and Marshall, 2009). Hyperaemia is not fully attenuated with adenosine antagonists and is therefore an indicator that other vasoactive mediators are involved in the blood flow response.

Interstitial K+ concentrations increase during exercise from 4mM to 9mM causing vascular myocyte hyperpolarisation and vasodilatation. K+ transients in early exercise may be responsible for the rapid increase in blood flow observed immediately after the first contraction (Lott et al., 2001). Active myofibres also release inorganic Pis into the interstitium following the metabolism of Cr phosphate. In addition, the COX pathway is involved in the regulation of functional hyperaemia producing endothelium derived hyperpolarising factors such as prostacyclin (PGI2) (Cocks et al., 1985).

ATP release from erythrocytes in the presence of oxygen unloading has also been found to cause vasodilatation. The erythrocyte has been identified as an oxygen sensing mechanism, targeting DO_2 to hypoxic exercising tissues. It is the decrease in

oxygen tension and associated conformational change in the haemoglobin molecules that facilitate oxygen unloading, and the binding of PGI2 and β-adrenergic receptors that stimulate ATP release in metabolically active tissues (Ellsworth and Sprague, 2012; González-Alonso, 2012). ATP binds to purinergic receptors stimulating an endothelium-derived hyperpolarisation (Ellsworth and Sprague, 2012).

There are several mechanisms which control hyperaemia in active muscle including the conducted, flow-mediated, metabolic and myogenic responses.

1.8 Distribution of oxygen delivery

Functional hyperaemia, capillary distribution and recruitment in the skeletal muscle determine oxygen distribution. A high capillary density facilitates a short oxygen diffusion distance maintaining a homogeneous oxygen tension across fibres, thereby increasing myoglobin reserves and reducing fatiguability (Baldwin et al., 1972). Ascending dilatation, a component of functional hyperaemia, increases the capillary exchange capacity via a decrease in terminal arteriole resistance. Fibre size and capillary number are known to follow a medial-lateral gradient with the largest fibres and lowest number of capillaries located distally from the bone (Deveci et al., 2001). Consequently the majority of fast glycolytic fibres are located laterally in the muscle where DO_2 is low (Baldwin et al., 1972).

1.9 Reductions in arterial oxygen content

The CaO_2 of the blood is determined by the quantity of oxyhaemoglobin and the small amount of oxygen dissolved in the plasma. Both the total oxygen content and the oxygen tension are thought to be important signals for the regulation of blood

flow. Humans that have adapted to high altitude can maintain CaO_2 values similar to those at sea level during maximal exercise (Calbet et al., 2009). Therefore several investigations have attempted to evaluate the roles of arterial oxygen tension and its contents by experimentally reducing haemoglobin concentrations, haemoglobin saturation or PaO_2 via hypoxia, the administration of carbon monoxide (CO) or artificially induced anaemia. A decrease in CaO_2 is generally associated with an increase in leg blood flow and a corresponding increase in femoral vascular conductance (FVC) during submaximal exercise which do not change with alterations in PaO_2 (Gonzalez-Alonso et al., 2001; Roach et al., 1999). These findings support the role of the red blood cells in the regulation of DO_2 to local hypoxia in active skeletal muscle. In addition, the authors also suggested that oxygen extraction is also 'sensed' due to the high venous oxyhaemoglobin saturation in studies using CO to reduce CaO_2 (Gonzalez-Alonso et al., 2001).

Normovolaemic haemodilution (HD) in human participants, that had a fifth of their Hct removed, did not result in an elevation of blood flow during moderate knee extensor exercise, despite a large reserve capacity available in the vascular bed (Roach et al., 1999). Peak aerobic power is determined by matching DO_2 to oxygen offloading in the tissues. A lack of compensatory response in blood flow and a severe drop in CaO_2 during periods of AH is associated with a decline in efficient physiological function (Calbet et al., 2009).

1.10 Adaptations to chronic hypoxia

CH causes skeletal muscle remodelling and vascular adaptations. Skeletal muscle fibre composition has been reported to change from a slow to a fast phenotype.

However, much research has also described the opposite transition and no changes in structure (El-Khoury et al., 2012). Angiogenesis and arteriolar remodelling are also controversial findings in hypoxically challenged tissues (Smith and J M Marshall, 1999), whereas an increase in Hct in hypoxia is generally well supported in both rodents and humans (Faucher et al., 2005; Lundby et al., 2006).

1.11 Influence of chronic hypoxia adaptations on muscle performance and fatigue

CH has been found to increase fibre homogeneity across the muscle (Mian and Marshall, 1991). Faucher et al. (2005) noted that maximum twitch force decreased in the EDL after a four-week acclimation to 10% hypoxia. They attributed this to a significant decrease in intermediate IIx fibres and suggested an increase in fast IIb fibres may be the cause. The associated reduction in myoglobin concentration and oxidative capacity decreased the EDL's resistance to fatigue (Faucher et al., 2005), whereas a different study at a 12% hypoxic intensity for 5 weeks found an increase in IIx fibres (Sillau and Banchero, 1977).

CH induced remodelling causes capillaries to meander more, have increased length and a decreased resting tone that result in improved oxygen distribution within tissues (Deveci et al., 2001). Improved oxygen distribution in skeletal muscle increases fatigue resistance. Increased capillary density has been reported in skeletal muscles in the posterior compartment of the rodent hindlimb over a five-week period of acclimatisation to high altitude (Cassin et al., 1971). However several studies have found no significant alteration in capillary to fibre ratio (CF) and FCSA (Lundby et al., 2009). Findings in support of the adaptation may be influenced by low

body weight and fibre atrophy caused by a decrease in protein expression, which result in a decrease in mean fibre cross-sectional area (FCSA) and a corresponding false positive in the capillary count (Deveci et al., 2001). The extent of skeletal muscle adaptation is most likely linked to stimulus duration and intensity as exercise training can enhance the affects of hypoxia induced remodelling (Lundby et al., 2009).

Hypoxia-inducible factor-1 (HIF-1) has a central role in oxygen homeostasis through the control of the glycoprotein erythropoietin. Iron metabolism is increased in the bone marrow in prolonged hypoxia to support erythropoiesis, which can evoke systemic iron deficiency (Lundby et al., 2009). Increased erythropoiesis in severe hypoxia can also stimulate intramuscular iron storage. Enhanced erythropoiesis increases Hct by 14% in animals acclimated to 450mmHg hypobaric hypoxia for six-weeks (El-Khoury et al., 2012). Increased Hct aids in the maintenance of CaO_2 compensating for the fall in PaO_2.

VO_2 in skeletal muscle in CH increases due to an enhanced endothelial metabolism and increased vasoactivity of the smooth muscle in the vasculature (Marshall and Davies, 1999), which is consistent with a 10% increase in oxygen extraction during hypoxia acclimation noted by Roach et al. (1999).

1.12 Present study

1.12.1 Physiological properties of the EDL muscle

The EDL was indirectly stimulated via the peroneal nerve in the current study. The fast-twitch EDL muscle is recruited during high intensity exercise and is therefore a

natural candidate for studies into muscle performance and fatigue. The EDL muscle is capable of rapidly increasing metabolic rate and energy consumption to more than 100 fold resting rates to accommodate high frequency contractions during prolonged periods of strenuous exercise (Sahlin et al., 1998).

The maximal stimulation of the muscle and the recruitment of a majority of muscle fibres described in this study is dissimilar to innate muscle stimulation where muscles are inherently stimulated in an intermittent manner with the recruitment of fewer muscle fibres in isotonic contractions. Intermittent stimulation was not utilised to fatigue the muscle, as this would have required a protocol of considerable duration and alterations in twitch dynamics could not have been investigated. Isometric single twitch contractions were used in preference to tetanic contractions to ensure hindlimb blood flow was not restricted by intramuscular pressure, maximal ATP consumption and achievable twitch dynamics.

The EDL muscle is easily accessible being the most lateral muscle of the anterior compartment and has comparable metabolic profiles with the other muscles in the hindlimb due to its composition of 56% oxidative and 42% glycolytic fibres (Ray and Marshall, 2009). Total blood flow during the third minute of exercise has been shown to be similar to flow in the rest of the leg during locomotion at 181ml.min^{-1}.100g^{-1} (Armstrong and Laughlin, 1983).

1.12.2 Hypotheses

In order to determine the extent to which skeletal muscle or vascular adaptations to CH influence skeletal muscle performance and fatiguability, the following hypotheses were proposed and formed the basis of this study:

Hypothesis 1: An incremental decrease in CaO_2 and DO_2 by a staggered reduction in Hct by HD progressively increases the fatiguability of the EDL in N rats.

Hypothesis 2: Phenotypic adaptations in the CH animals maintain a fatigue profile comparable to that of N animals.

Hypothesis 3: HD in hypoxic animals results in a similar increase in fatiguability to that seen in HD in N rats.

2. Materials and methods

2.1 Animals

Three-month-old male albino rats of the strain Wistar and species *Rattus norvegicus* weighing between 300g and 369g were evaluated. Rats were housed in the biomedical services unit (University of Birmingham) maintained in a controlled environment, under a standard light cycle (07:00-19:00) and a stable temperature of (21±1°C) until required for the experiment. Rat chow and water were available *ad libitum* until 16:00 on the day preceding the experiment when food was limited to 10g, the equivalent to fasting from ~midnight. All animals were acquired from Charles River, Laboratories International, Margate, UK.

CH animals were housed in a normobaric hypoxic chamber (Oxycycler, model A84XOV, Biospherix) with an ambient oxygen concentration controlled at 12% oxygen (6kPa), the equivalent to an altitude of 4000m, for a period of three weeks prior to the experiment. Inspired air remained at 8 or 12% oxygen for the duration of the protocol. All other rats breathed 21% oxygen in the period leading up to and during the *in vivo* set up.

Testing and handling of the animals were conducted in accordance with the Animals (Scientific Procedures) Act 1986.

2.2 Surgical procedure

The animals (n=19) were anaesthetised using volatile inhalable anaesthetic Isoflurane 4% in oxygen at 4L.min^{-1} (General Anaesthetic Services Ltd, Adjustable Veterinary Trolley, 013406 and Harvard Apparatus Ltd, Veterinary Fluosorber). The rat was placed in a supine position on a homeothermic blanket and a rectal temperature probe (Harvard Apparatus Ltd) maintained the animals' temperature at 37ºC (*Figure 3*).

Anaesthesia was maintained with Alfaxalone (Alfaxan® Vétoquinol). The jugular was cannulated with translucent silicone tubing, 0.5mm bore x 0.5mm wall (Sterilin Ltd) to deliver 5mg.ml^{-1} of the injectable steroid anaesthetic Alfaxan® in 0.9% saline (Sigma-Aldrich Co. LLC.) at an infusion rate of 0.9-1.2ml.hr^{-1} via a 50ml syringe driver (B.Braun Medical Ltd, Perfusor® secura FT) resulting in a dose ranging between 13.5-18.0mg.kg^{-1}.hr^{-1}. Anaesthetic boluses of 0.05 - 0.1ml were delivered through a 1ml syringe as needed to maintain the appropriate depth of anaesthetic determined by the absence of the pedal withdrawal reflex (Coney & Marshall, 2007). A tracheotomy with a T-shaped stainless steel cannula maintained airway patency throughout the experiment and allowed inspired gases to be adjusted as required.

The right brachial artery was isolated from the brachial plexus and cannulated with fine bore polyethylene tubing, 0.4mm bore x 0.8mm wall (Smith Medical ASD Inc) containing heparinised 0.9% saline (20 U.ml $^{-1}$ (LEO Pharma GmbH)). The right femoral vein and artery were isolated from the femoral sheath, distal to the inguinal ligament, and cannulated with translucent silicone tubing, 0.5mm bore x 0.5mm wall (Sterilin Ltd) and fine bore polyethylene tubing, 0.58mm bore x 0.96mm wall (Smith

Medical ASD Inc) respectively containing heparinised 0.9% saline (20 $U.ml^{-1}$ (LEO Pharma GmbH)). The left femoral artery was isolated and the abdominal wall above the inguinal ligament was held away from the fat pad with suture.

The EDL tendon was isolated in the right hindlimb and disengaged after the maximum length was drawn from the foot. 4.0 U.S.P. non-distensible suture (Angiotech) was tied tightly around the anterior section of the tendon in proximity to the muscle and the free-end of the tendon was looped underneath and secured with 6.0 suture to avoid slip during muscle contraction. Liquid paraffin (BDH Ltd.) was dabbed on the tendon and surrounding tissue to keep both moist and maintain motility throughout the experimental protocol.

The deep branch of the common peroneal nerve was exposed and isolated in the lateral groove between the TA and the plantaris and a handmade electrode of Teflon insulated 0.125mm annealed silver wire (Advent Ltd) further insulated by PP10 polyethylene tubing (Portex Ltd) was hooked onto the nerve and the electrode wires were separated and held in place with silicone elastomer (WPI Inc).

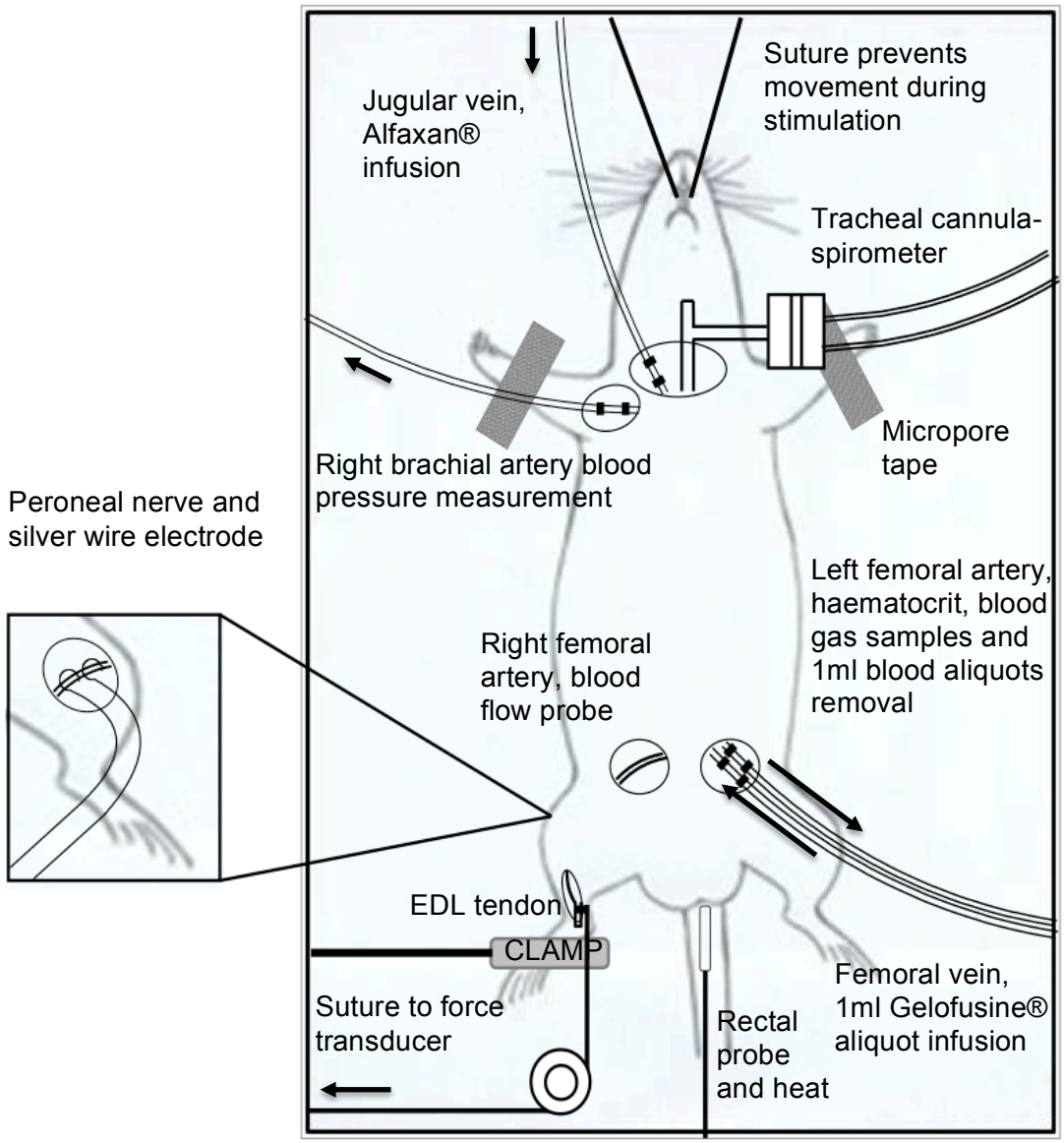

Figure 3. A diagram indicating the location of surgical intervention in an instrumented rat

2.3 Experimental setup

The animal was held in position by tying suture around the teeth and securing the anterior limbs with micropore surgical tape (3M Micropore^{TM}). The animal's right paw was tightly secured in a paw clamp and the suture previously tied to the isolated EDL tendon was attached to a calibrated 50g isometric force transducer and dual bridge pod (MLTF050/ST, ADInstruments) via a pulley reel at 90°. Suture placed around the teeth aided in the maintenance of a stable 5g baseline tension on the EDL muscle as it prevented the rat being pulled forward by the twitch contractions (*Figure 4*).

A blood pressure transducer and bridge pod were pre-calibrated using a sphygmomanometer (Accoson) prior to attachment of the brachial artery cannula. A flow probe (0.7V, Transonic Systems Inc) was coated with ultrasound jelly (Surgilube®, Fougera®) to maintain acoustic coupling and placed around the right femoral artery to measure blood flow via a small pre-calibrated animal blood flow meter (T106, Transonic Systems Inc) adjusted to zero. A calibrated spirometer was attached to the side-arm of the tracheal cannula and a screw was used to redirect air flow through the spirometer.

All instruments were linked to a PowerLab (4/25, ADInstruments) and data was recorded at a frequency of 100-1000Hz on an Apple iMac computer using LabChart 7.2 software (ADInstruments). Eight channels recorded force, ABP, FBF, HR and calculated maximum force, MFBF, MAP and FVC (MFBF/MAP).

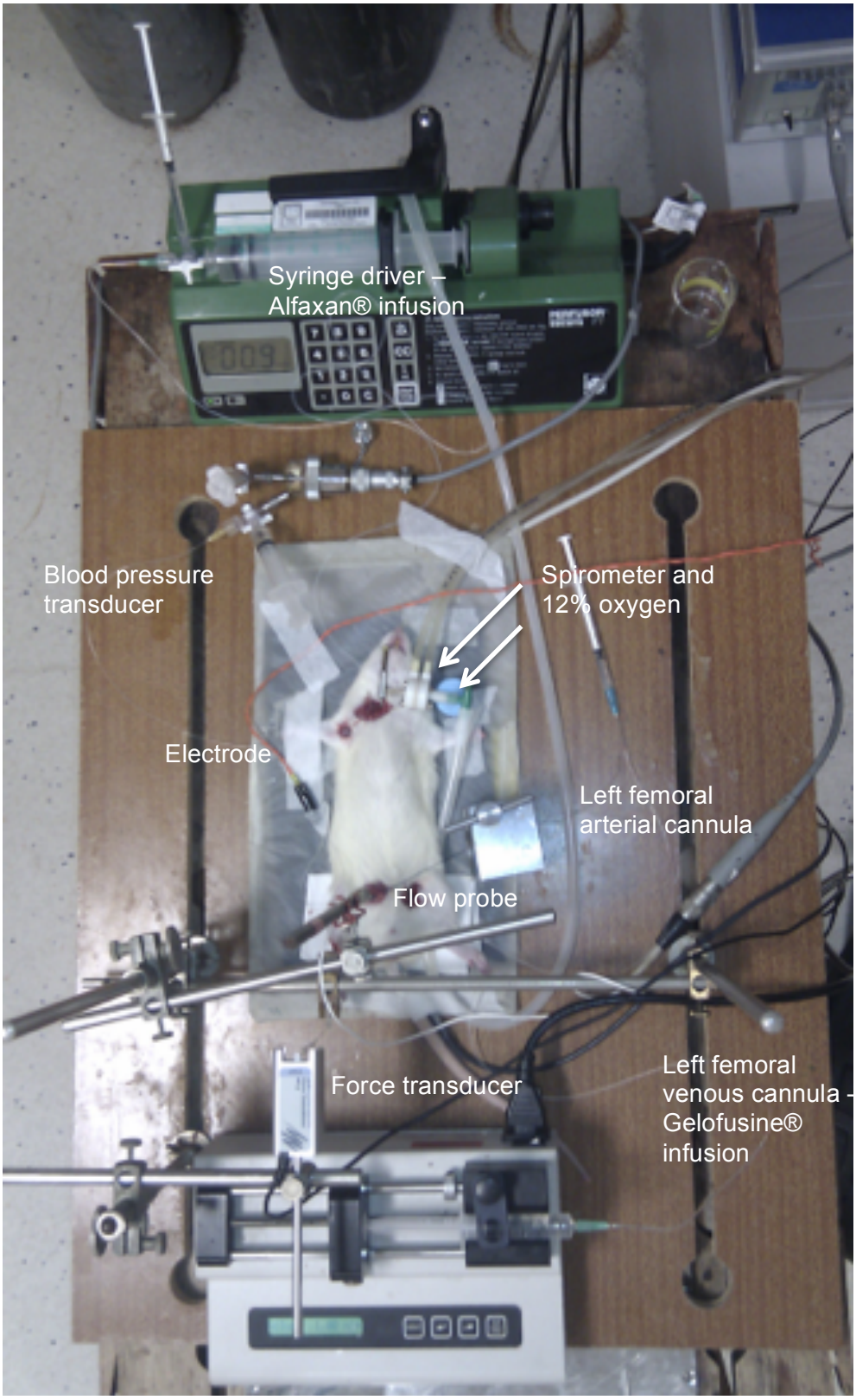

Figure 4. A photo of an instrumented rat

2.4 Experimental protocol

After setup the animals were allowed to stabilise for 30 minutes and baselines were recorded.

2.4.1 Group 1: Normoxic- time controls (N-TC)

The peroneal nerve was stimulated with increasing voltage (1-9 V) to determine the maximum single twitch of the EDL. Five periods of 3min isometric single twitch contractions were evoked with a supramaximal stimulus (usually 3-5V) at 15Hz (0.15 ms pulse duration, 2700 pulses) with a 30min recovery interval between each period.

Hct and blood gas (BG) analysis were carried out prior to each period of stimulation and after the fifth stimulation. Hct and BG samples were collected from the femoral artery in 75µl and 150µl glass tubes respectively (Hawksley & Sons Ltd) and either spun down in a micro-centrifuge (MSE UK) ready for measurement (*Figure 5*) or aspirated into a gas analysis system GEM4000 (Instrument laboratory) for analysis of PaO_2, partial pressure of carbon dioxide ($PaCO_2$), and pH.

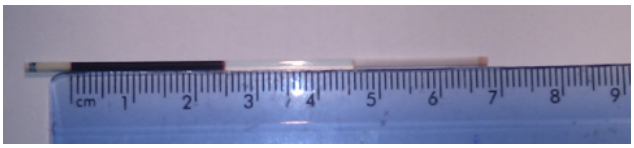

Figure 5. Haematocrit measurement after centrifugation

The maximal vasodilatation was determined by infusing sodium nitroprussside (SNP) (Sigma-Aldrich Co. LLC.) via a 5ml syringe driver (KD Scientific) at 0.68mg.kg.hr^{-1} in 0.9% saline with an infusion rate of 75ml/hr on completion of the five periods of stimulation.

The EDL was removed under anaesthesia for histological assessment. The muscle was weighed and the mid section cut, mounted on cork in a coating of optimal cutting temperature embedding matrix and flash frozen in methylbutane (Sigma-Aldrich Co. LLC.) that was cooled in liquid nitrogen to -160°C. The outer sections of muscle were frozen in liquid nitrogen for biochemical analysis. At the end of the experiments animals received an overdose of Euthatal and death was confirmed by cervical dislocation.

2.4.2 Group 2: Normoxic- haemodilution (N-HD)

N-HD animals underwent the same protocol as the N-TC animals with the addition of sequential removal of three 1ml aliquots of blood with recovery periods of five minutes between each aliquot before the third, fourth and fifth stimulation. This resulted in a total 9ml HD prior to stimulation 5 (*Figure 6*). Blood was withdrawn from the femoral artery in 1ml aliquots over a 1min period to prevent shocking the animal while simultaneously infusing an equal volume of plasma substitute at a rate of $1ml.hr^{-1}$ (Gelofusine® veterinary, B. Braun) from the 5ml syringe driver.

2.4.3 Group 3: Chronic hypoxia- haemodilution (CH(PaO_2~47)-HD)

The same HD protocol was used for the CH(PaO_2~47)-HD, group 3, animals as was used for the N-HD, group 2, animals. These animals were maintained on 12% oxygen for the duration of the experiment.

2.4.4 Group 4: Chronic hypoxia- haemodilution (CH(PaO_2~33)-HD)

The same protocol was also used for the CH(PaO_2~33)-HD, group 4, animals except that these animals were maintained on 8% oxygen for the duration of the experiment.

The experimental protocol used for groups 2, 3, 4 is summarised in Figure 6.

S1	R1	S2	R2	S3	R3	S4	R4	S5	R5	SNP
	HDx3 Hctx3	BG	HDx3 Hctx3	BG	HDx3 Hctx3	BG	HDx3 Hctx3	BG		

Figure 6. **A diagram of the experimental protocol**
BG=blood gas, Hct=Haematocrit, HD=haemodilution, R=recovery, S=stimulation and SNP=sodium nitroprusside.

2.5 Muscle histology

2.5.1 Lectin histochemistry

Histochemical evaluation of the right EDL muscles of twelve N rats and eight CH rats were used to assess α galactosylated glycan distribution and therefore determine capillary-fibre ratio in three regions across the medial-lateral gradient. Galα1-R carbohydrate localisation was labelled by rhodamine *Griffonia (Bandeiraea) simplicifolia* lectin I (GSL I and BSL I) at a 1:100 dilution (Vector Laboratories, UK). 8μm transverse sections of midbelly EDL were cut at 22°C and thaw mounted onto glass slides (Polysine^{TM}, VWR International). Prior to the 1h lectin incubation at 4°C all sections were fixed in cooled acetone. Sections were washed in phosphate

buffered saline (PBS) three times and coverslipped in aqueous vectashield containing 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, UK) at a 1:8 dilution with PBS. Epifluoresence was observed and images acquired using a Zeiss fluorescence microscope and AxioVision Software.

2.5.2 Capillary quantification

Quantification of capillaries and fibres was carried out in a blinded fashion following an unbiased stereological counting rule using ImageJ analysis software. Analysis was carried out as previously published (Deveci et al., 2001). Briefly, fibres were analysed in three images across the transverse muscle section acquired by a photographer blinded to the experimental condition (Figure 7). Capillaries and fibres, which crossed the right side and bottom of the image boundary, were ignored, the vessels however were included. Only clearly defined capillaries inside the plane of the section were sampled. Biconcave capillary bifurcations within the section plane were counted as one vessel, as were arterioles or longitudinally sectioned vessels.

A **B**

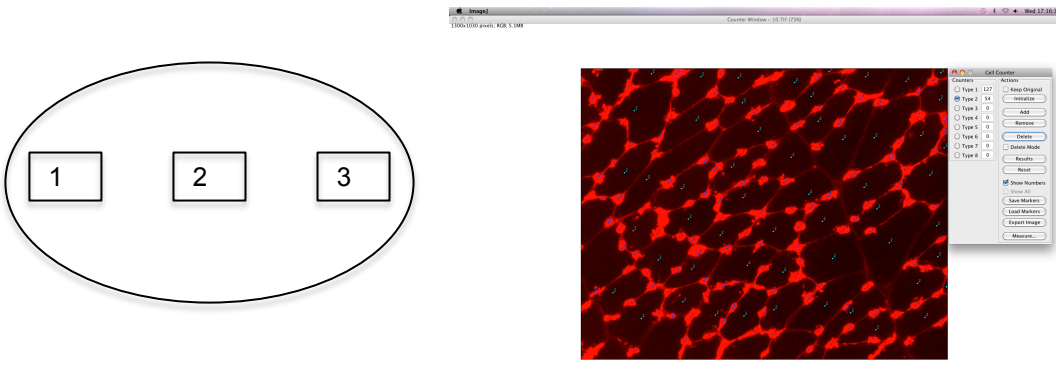

Figure 7. A diagram indicating the location in the EDL of the three regions of interest (A) and a sample image in ImageJ showing analytical cell count (B)

2.5.3 Glycogen protein analysis

The outer sections of EDL muscle were weighed and placed in 0.2ml of KOH (2M) in a preheated water bath at 70ºC for fifteen minutes. Samples were shaken during digestion to ensure adequate tissue breakdown. 0.8ml of ethanol was added to each sample to precipitate the glycogen and remove small proteins after which the Eppendorf tubes were centrifuged at 1000rpm for 5min. The supernatant was decanted and the glycogen pellet resuspended in 0.2ml distilled water (dH_2O) prior to a second wash. The supernatant was decanted and the glycogen pellet air dried for 10min and redissolved in 0.2ml dH_2O. Glycogen was digested into glucose units with 0.8ml 100mM sodium acetate buffer, pH 4.5 and 50µl of amylo-glucosidase enzyme during overnight incubation at room temperature. The enzymatic colorimetric Trinder method (Sentinel CH. Glucose liquid 17630 kit), an end-point assay, was used to quantify glucose concentration. 6µl glycogen sample aliquots, dH_2O (blank) and glucose liquid (standard) were added to 0.5ml of reagent and vortexed. 0.2ml of each sample in duplicate were transferred to a 96 well plate and the absorbance was measured by the Synergy™ HT Microplate Reader (Bio-tek KC4) at a wavelength of 546nm.

2.6 Analysing stimulation responses

Continuous force recordings, FBF, ABP and FVC were averaged across a one-minute baseline, every fifteen seconds during the three minute stimulation (S1-S12) and every minute per ten minute recovery period (R1-10). Δforce and ΔFVC data were calculated for the three minute stimulation period by subtracting the integrated baseline for each parameter respectively. All values were obtained using the

LabChart® datapad export function. ΔFVC and Δforce allowed for comparisons between the total responses of control and CH animals.

2.7 Calculating oxygen delivery

DO_2 to the hindlimb was calculated from the measured Hct level and PaO_2 using the formula below.

Total oxygen content ($mlO_2.min^{-1}.kg^{-1}$)= [Hb x {(Hct/40x15)x1.39}] + [PaO2 x 0.223]

Hb maximum oxygen capacity = 1.39 ($O_2.g^{-1}$ Hb)

Oxygen solubility coefficient = 0.223 $mlO_2.L^{-1}.kPa^{-1}$ for O_2 at 37°C

Hb saturation was taken from an Hb saturation curve derived from published data Appendix 1. $PaCO_2$ was presumed to remain constant at approximately 35 mmHg during the experiment. Total oxygen content was multiplied by blood flow to obtain a value for DO_2.

2.8 Statistical Analysis

The graphics programme GraphPad Prism 5.0 was used to plot graphs and to statistically analyse data. All muscle stimulation response data was plotted as mean±S.E.M. and analysed using repeated measures ANOVA, with Bonferroni post-tests. One way ANOVA, with Bonferroni was used to compare data sets in muscle histology. $P<0.05$ was considered significant.

The significance levels associated with the analysis of the data are represented by symbols in the report according to Table 2.

Table 2. Number of symbols used to represent significance levels

Symbols	Level of significance
*/φ/ϕ	p<0.05
**/φφ/ϕϕ	p<0.01
***/φφφ/ϕϕϕ	p<0.001
****/φφφφ/ϕϕϕϕ	p<0.0001

3. Results

The mean responses to five, three minute periods of continuous, supra-maximal, single twitch isometric contractions for groups 1-4, N-TC, N-HD, 12%O_2 acclimated CH(PaO_2~47)-HD) and 12%O_2 acclimated-acutely hypoxic CH(PaO_2~33)-HD, are noted below.

3.1 Group 1. Normoxic time controls

3.1.1 Group baselines

There was no significant difference between group baselines in HR, FBF, $PaCO_2$ and pH prior to stimulation 1 (Table 3 and Table 4).

***Table 3. Group baselines* for stimulation 1**

	Group 1 N-TC		Group 2 N-HD		Group 3 CH(PaO_2~47)-HD		Group 4 CH(PaO_2~33)-HD	
	Mean	**±SEM**	**Mean**	**±SEM**	**Mean**	**±SEM**	**Mean**	**±SEM**
Body weight (g)	319	4	322	8	346*	8	301$^{\varphi\varphi}$	1
EDL weight (mg)	150	12	160	3	190*	5	150$^{\varphi}$	9
ABP (mmHg)	116	3	114	5	80$^{\varphi}$	4	80$^{\varphi\varphi}$	4
HR (BPM)	408	14	438	11	403	14	411	15
FBF (ml.min^{-1})	3.0	0.7	3.6	0.7	1.7$^{\varphi}$	0.2	2.0	0.6
FVC (ml min^{-1} mmHg^{-1})	0.026	0.006	0.031	0.006	0.019 $^{\varphi}$	0.003	0.024	0.007

**vs. N-TC, $^{\varphi}$ vs. N-HD, n=2-5, one way ANOVA*

Group 3 weighed significantly more than group 1 and group 4 weighed significantly less than group 3. This significance is reflected in the weight of the EDL. Group 3 had significantly lower ABP, FBF and FVC than group 2. Group 4 also had significantly lower ABP than group 2.

Table 4. Gas parameter baselines for stimulation 1

	Group 1 N-TC		**Group 2 N-HD**		**Group 3 CH(PaO_2~47)-HD**		**Group 4 CH(PaO_2~33)-HD**	
	Mean	**±SEM**	**Mean**	**±SEM**	**Mean**	**±SEM**	**Mean**	**±SEM**
PaO_2 (mmHg)	81	6	73	4	$49^{\varphi\varphi}$	3	$30^{\varphi\varphi\varphi}$	1
$PaCO_2$ (mmHg)	42	3	39	1	$28^{\varphi\varphi}$	3	27	12
pH	7.40	0.01	7.43	0.01	7.41	0.02	7.44	0.12

$^{\varphi}$ *vs. N-HD, n=2-5, one way ANOVA*

Groups 3 and 4 had significantly lower PaO_2 than group 2. Group 3 also had a significantly lower $PaCO_2$ than group 2.

3.1.2 Group 1. Response of each parameter to stimulation

Stimulation of the peroneal nerve at 15Hz with a supra-maximal voltage produced twitching of the muscles in the anterior compartment of the hindlimb. A typical pattern of response is shown in Figure 8. The tension generated by the EDL increased from the onset of stimulation and reached a peak during the first fifteen seconds of contraction and then fatigued to a tension that was constant until the end of the stimulation period (Figure 8A). Mean FBF increased at the beginning of stimulation and reached a maximum value during the first ninety seconds, which was then sustained until the first minute of recovery (Figure 8B). A reactive hyperaemia

occurred for ten seconds after stimulation stopped before gradually returning to baseline levels over six minutes (Figure 8B). ABP did not alter during muscle contraction (Figure 8C) and, therefore, the mean FBF response mirrored the FVC (Figure 8D).

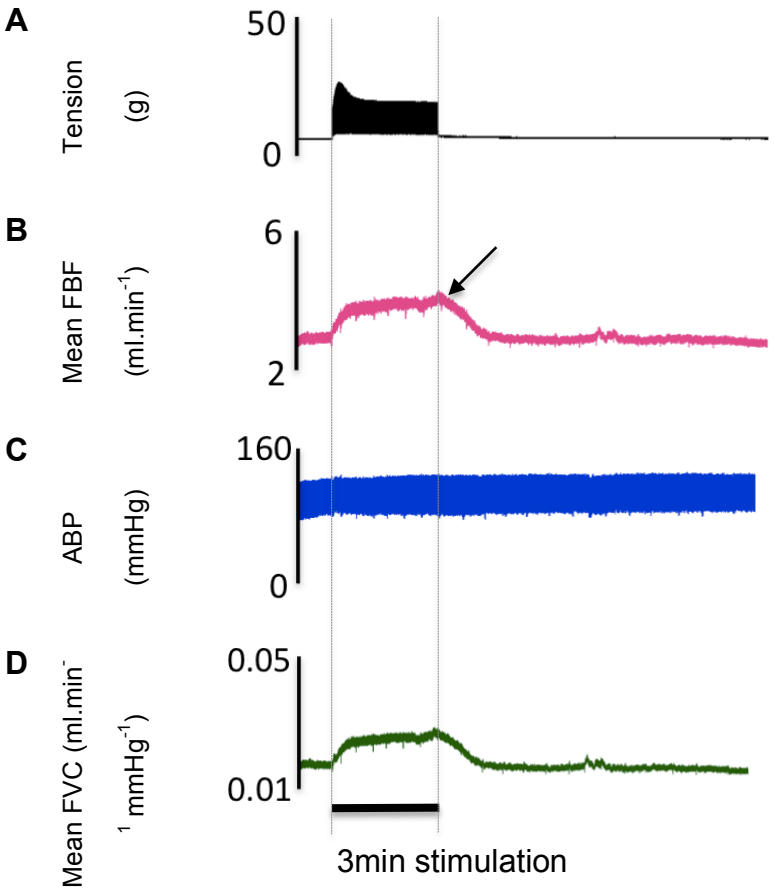

Figure 8. A normoxic isometric twitch contraction.
A representative raw trace showing EDL tension (A), mean FBF (B), ABP (C), and mean FVC (D) during a 1 min baseline, a 3 min stimulation (marked by arrows) and a 10 min recovery period. Reactive hyperaemia is marked by an arrow (B).

During the first fifteen seconds of the three-minute period of high frequency stimulation, the peak tension (P_t) reached by individual twitches increased (Figure

9A). The muscle then performed with maximal efficiency for a short period prior to a progressive reduction in P_t to a plateau.

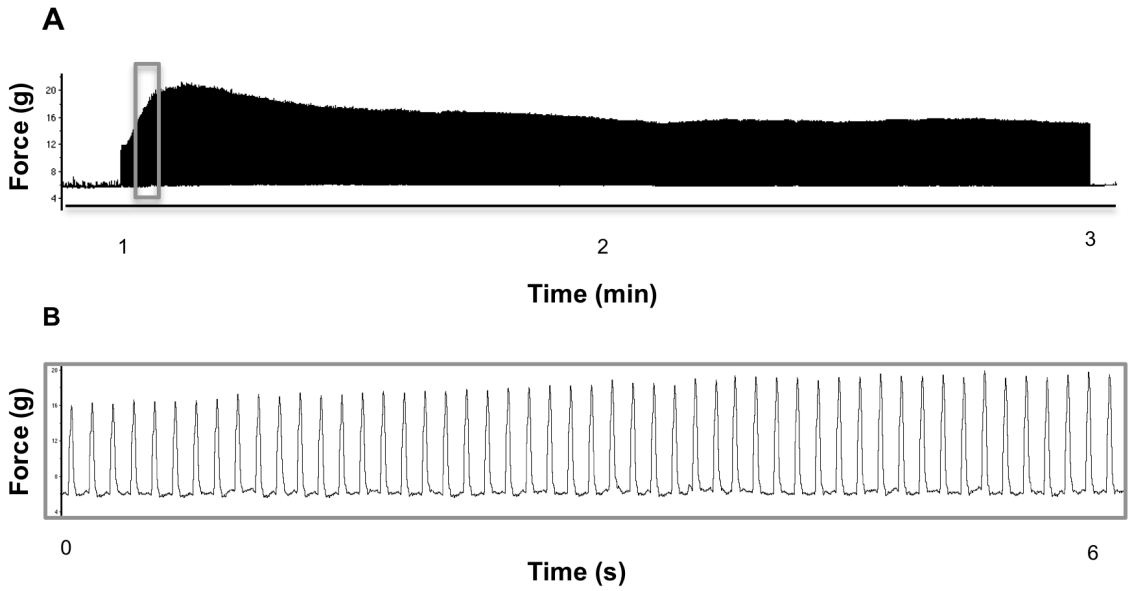

Figure 9. A normoxic fatigue profile.

(A) A raw trace showing EDL muscle tension over a 3 minute stimulation period.
(B) A raw trace showing EDL isometric single twitch contractions, 6s insert from A.

3.1.2 The effect of time on baselines

There was no significant difference in any parameter pre-stimulation baseline with time. The Hct level was not altered in N-TC (Figure 11).

Table 5. Normoxic time control baselines for stimulations 2-5.

	Group 1, N-TC							
	S2		S3		S4		S5	
	Mean	±SEM	Mean	±SEM	Mean	±SEM	Mean	±SEM
ABP (mmHg)	110	3	108	3	102	6	102	5
HR (BPM)	403	17	392	19	392	23	372	23
FBF ($ml.min^{-1}$)	1.8	0.5	2.1	0.5	2.0	0.5	2.1	0.6
FVC ($ml\ min^{-1}\ mmHg^{-1}$)	0.016	0.004	0.020	0.005	0.020	0.005	0.020	0.005
PaO_2 (mmHg)	81	6	87	4	83	2	82	4
$PaCO_2$ (mmHg)	42	3	42	4	42	6	44	7
pH	7.40	0.01	7.42	0.02	7.41	0.04	7.40	0.04

No significance, n=2-5

3.1.2 The effect of time on stimulations

There was no significant difference between the tensions generated by the EDL, mean FBF, ABP or mean FVC during consecutive stimulations 2, 3, 4 and 5 (Figure 10A-D). See Appendix 2-5 for trace fatigue profiles. The data corresponding to the second stimulation was used as the control for stimulations 3, 4 and 5. There was no significant difference in the mean responses to S1-S5. However as S1 contractions were not consistent and as Ray and Marshall (2009) found a significant difference in response to S1, S1 was omitted from all the groups in the present study.

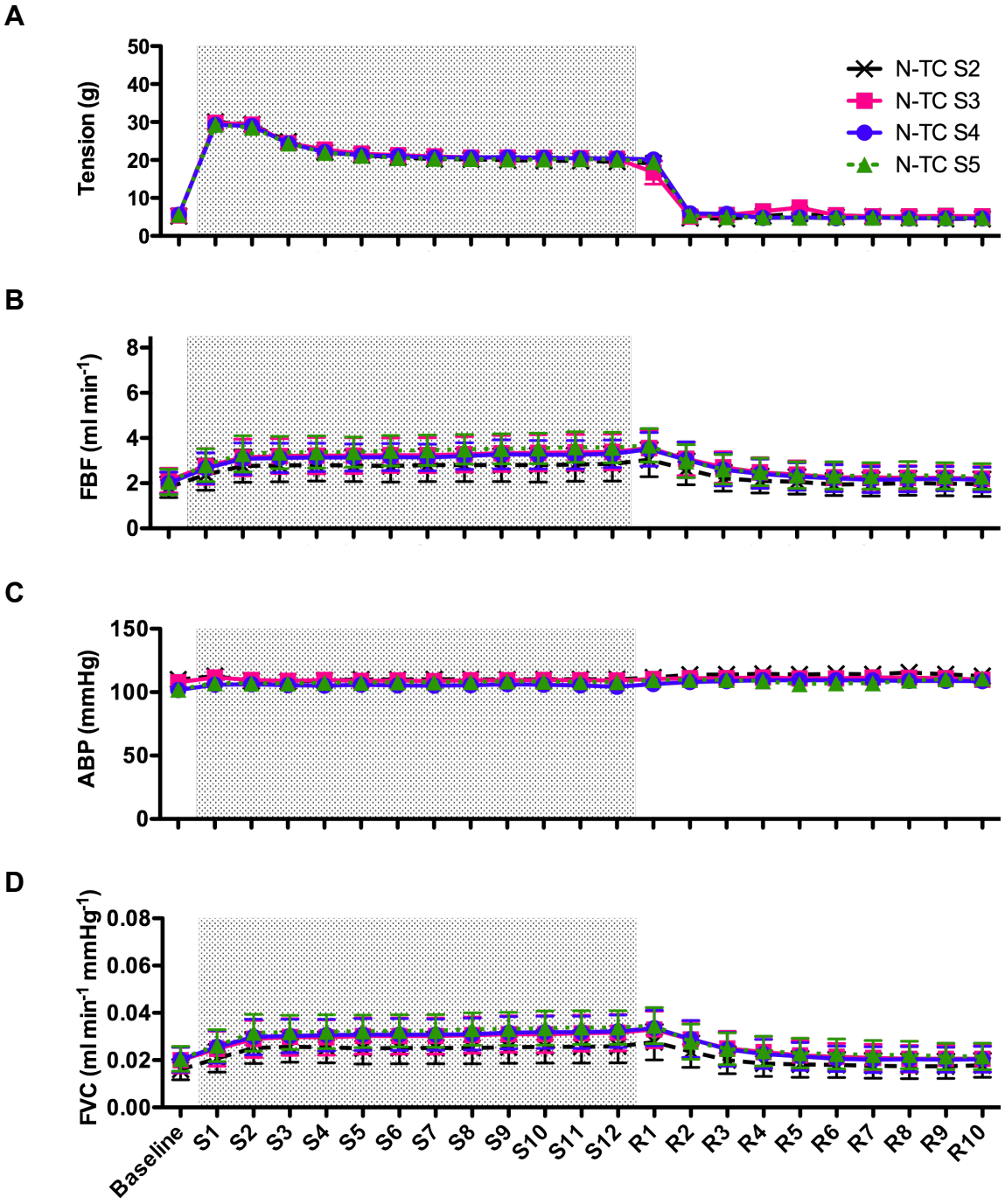

Figure 10. The responses to stimulation in normoxic time controls

Mean data showing EDL tension (A), FBF (B), ABP (C), and FVC (D) for time control stimulation 2 (crosses, black dashed lines), 3 (squares, pink continuous lines), 4 (circles, blue continuous lines) and 5 (triangles, green dotted lines) in the 1 min baseline, 3 min stimulation (grey shading, 15s; S1-12) and 10 min recovery period (R1-10) Error bars show ± S.E.M., n=5.

3.2 Group 2. Normoxic haemodilution

3.2.1 Group 1 vs group 2 stimulation 2 baselines

There was no significant change in ABP and HR between S2 in groups 1 and 2. However, there was a significant difference in FBF and FVC S2 in these groups (Table 5 and Table 6).

3.2.2 The effect of haemodilution on haematocrit

Hct was reduced significantly in N-HD by HD, from 45±2 to 22±1% RBC, representing a 0.5 fold reduction prior to stimulation 5.

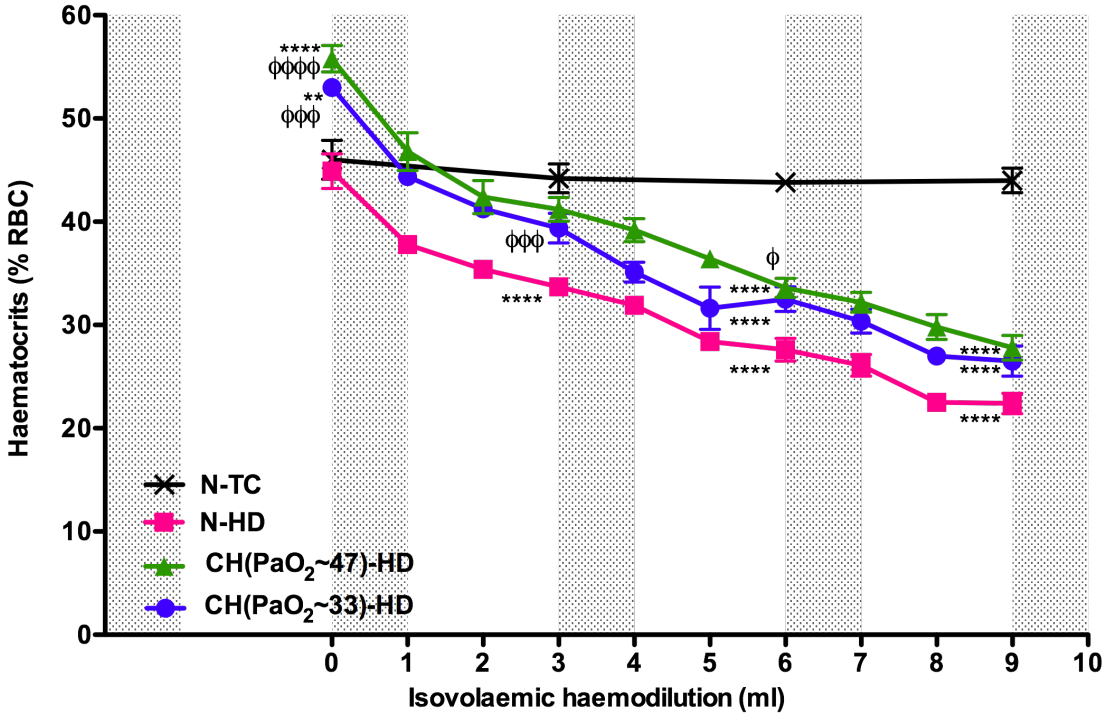

Figure 11. The effect of haemodilution on haematocrit
*Mean data showing N-TC (crosses, black lines), N-HD (squares, pink lines), CH(PaO_2~47)-HD (triangles, green lines) and CH(PaO_2~33)-HD (circles, blue lines) Hct per 1ml HD across stimulations 2 to 5 (grey shading). *vs. N-TC and $^{\phi}$ vs. N-HD, n=5, two way ANOVA.*

3.2.3 The effect of haemodilution on baselines

Throughout N-HD stimulations 2 to 5 there was no significant change in the baselines of any parameter, other than $PaCO_2$, which decreased significantly from S2 and S3 to S4 and S5 (*Table 6*).

Table 6. Normoxic haemodilution baselines

	Group 2, N-HD							
	S2		S3		S4		S5	
	Mean	±SEM	Mean	±SEM	Mean	±SEM	Mean	±SEM
ABP (mmHg)	109	6	105	9	97	8	86	9
HR (BPM)	428	15	443	12	450	11	440	10
FBF ($ml.min^{-1}$)	3.7	0.4	3.4	0.5	2.9	0.4	2.7	0.3
FVC ($ml\ min^{-1}\ mmHg^{-1}$)	0.035	0.005	0.035	0.007	0.031	0.005	0.034	0.007
PaO_2 (mmHg)	73	4	74	1	79	2	80	2
$PaCO_2$ (mmHg)	39	1	37	1	35*$^{\varphi}$	1	34**	1
pH	7.43	0.01	7.45	0.02	7.50	0.03	7.50	0.02

** vs. S2, $^{\varphi}$ vs. S3, n=5, one way ANOVA*

The S4 and S5 baseline $PaCO_2$ decreased significantly from S2 and S3.

3.2.4 The effect of haemodilution on response to stimulations

HD had no significant effect on the tension produced by the EDL, FBF, ABP or FVC across stimulations 2, 3, 4 and 5 (Figure 12). However, there is a trend for ABP to decrease with HD.

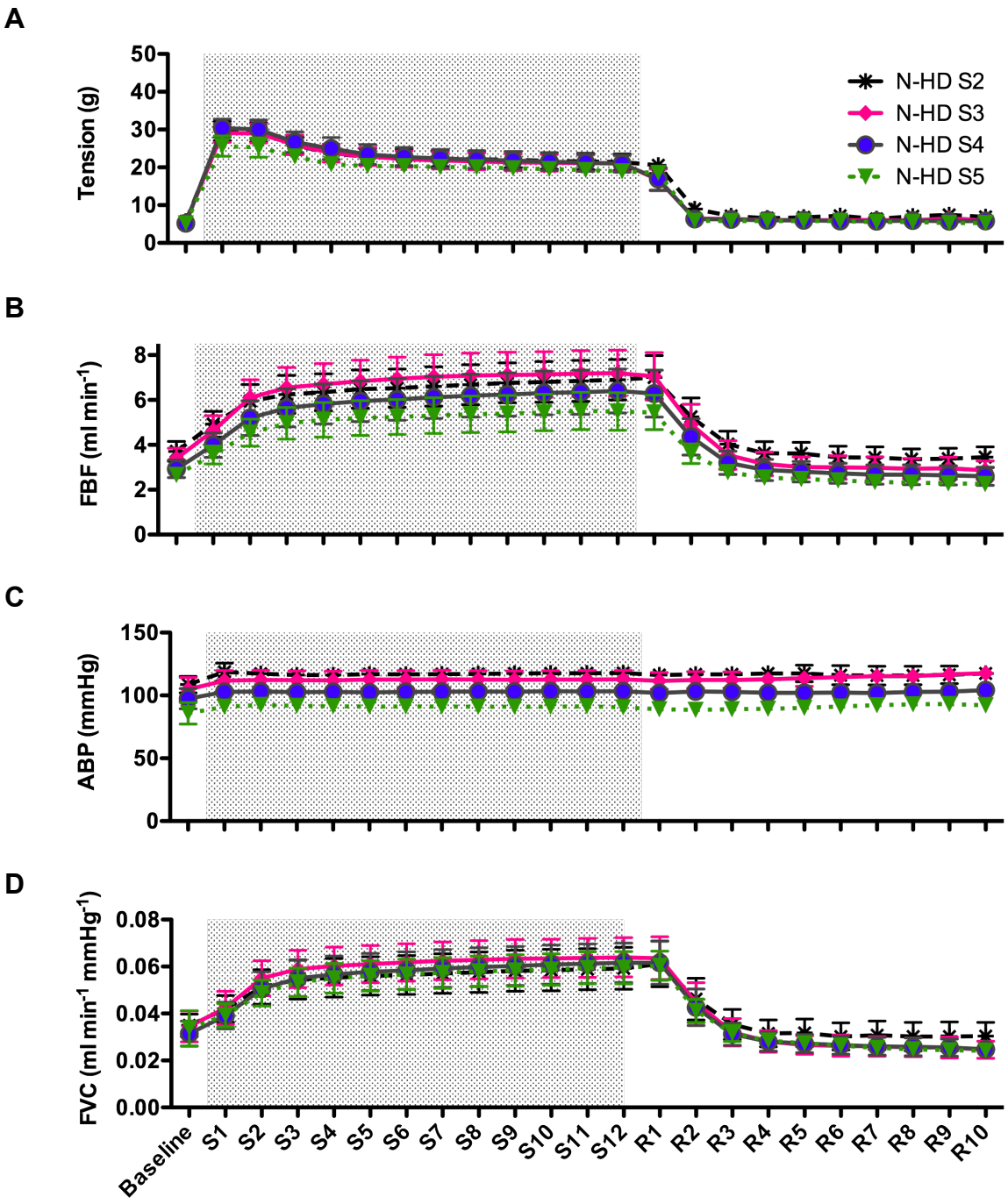

Figure 12. The effect of haemodilution on the responses to stimulation in normoxic animals

EDL tension (A), FBF (B), ABP (C), and FVC (D) for control stimulation 2 (asterisks, black dashed lines), 3 after 3ml HD (solid diamonds, pink continuous lines), 4 after 6ml HD (circles, blue continuous lines) and 5 after 9ml HD (solid inverted triangles, green dotted lines) in the 1 min baseline, 3 min stimulation (grey shading, 15s; S1-12) and 10 min recovery period (R1-10) Error bars show ± S.E.M., n=5.

3.2.5 The effect of haemodilution on oxygen delivery, integrated femoral vascular conductance and tension time index in normoxic animals.

As described, N-TC did not undergo HD and therefore Hct remained unchanged throughout the experiment. In group 1 ΔFVC, a measure of vasodilatation, was significantly increased by a similar amount during each stimulation, which allowed DO_2 to be maintained and muscle performance (ΔTension) to remain constant in every stimulation (Figure 13A-C) (See 2.7 calculating DO_2).

The Hct in group 2 decreased significantly between each stimulation while ΔFVC increased significantly and by the same amount in comparison to baseline values during contractions 2 to 5, resulting in a tendency for DO_2 to decrease. The decrease in DO_2 induced by HD did not affect muscle function as tension time index (TTI) was ~1150g.s^{-1} in both N-TC and N-HD animals (Figure 13D-F).

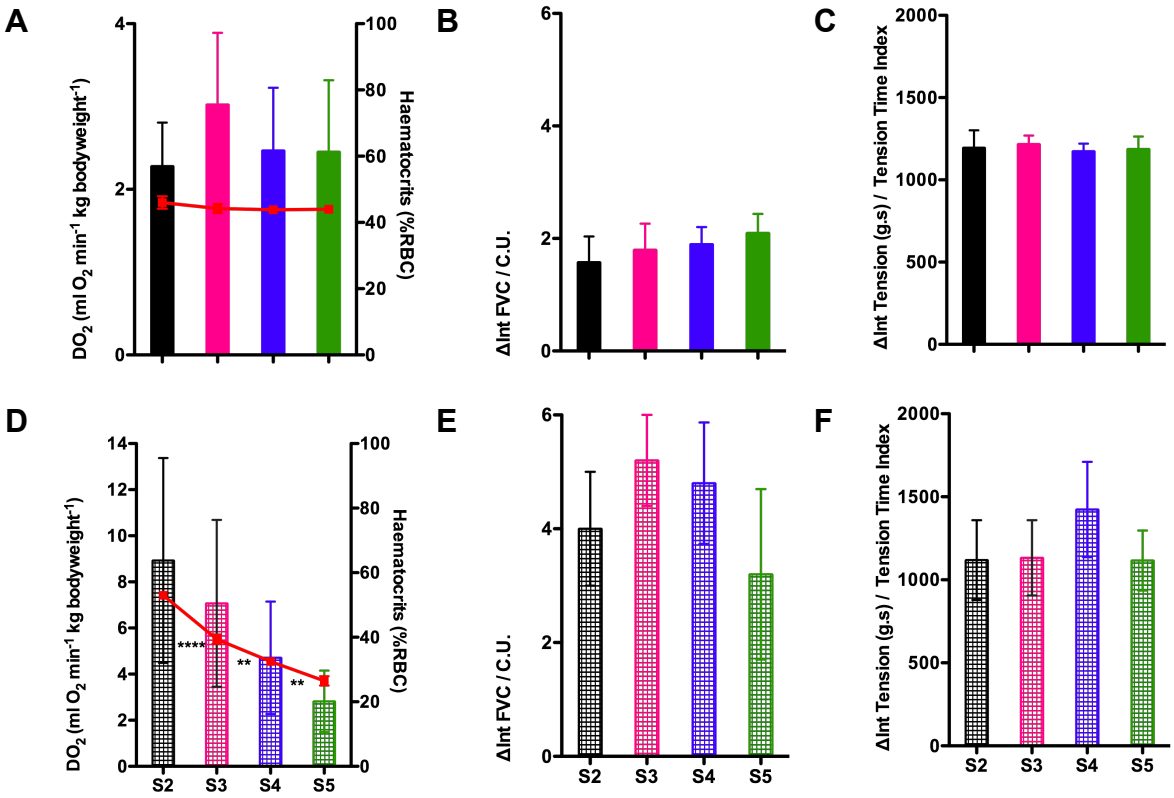

Figure 13. Haematocrit, oxygen delivery, integrated femoral vascular conductance and tension time index for normoxic time control and normoxic haemodilution groups.

*A-C show Hct (red line), DO_2, ΔFVC and ΔTension for group 1 N-TC across stimulation 2 (solid black bar), 3 (solid pink bar), 4 (solid blue bar) and 5 (solid green bar). D-F show identical parameters for group 2 N-HD animals across stimulations 2 (squared black bar), 3 (squared pink bar), 4 (squared blue bar) and 5 (squared green bar). * vs. S2 Hct, n=5, two way ANOVA. Error bars show ± S.E.M., n=5 (n=2-3 DO_2, N-TC).*

3.3 Group 3. Chronically hypoxic (PaO_2~47) haemodilution

3.3.1 Group 2 vs group 3 stimulation 2 baselines

PaO_2 was significantly lower in the CH groups (Table 4). There was no change in either ABP or HR in S2 between groups 2 and group 3. However FBF and FVC both decreased significantly in group 3 in comparison to group 2 (Table 5 and Table 7).

3.3.2 The effect of haemodilution on haematocrit

Hct was significantly higher in CH animals and HD reduced Hct from 56±1 to 28±1 %RBC (Figure 11).

3.3.3 The effect of haemodilution on baselines

There was no change in baseline values between stimulations 2 to 5 in the CH(PaO_2~47)-HD group (Table 7).

Table 7. Chronic hypoxia (PaO_2~47) haemodilution baseline values between stimulations 2-5

	Group 3, CH-HD 12% O_2							
	S2		S3		S4		S5	
	Mean	±SEM	Mean	±SEM	Mean	±SEM	Mean	±SEM
ABP (mmHg)	94	7	89	7	81	3	79	6
HR (BPM)	405	12	412	16	426	16	423	15
FBF (ml.min^{-1})	1.9	0.2	2.2	0.2	2.4	0.3	2.3	0.4
FVC (ml min^{-1} mmHg^{-1})	0.020	0.002	0.025	0.003	0.030	0.004	0.028	0.005
PaO_2 (mmHg)	49	3	47	2	45	2	46	2
$PaCO_2$ (mmHg)	28	3	29	3	30	3	29	3
pH	7.41	0.02	7.46	0.02	7.46	0.02	7.47	0.03

No significance, n=4-5

3.3.4 The effect of haemodilution on response to stimulations

There was no change in the tension generated by the EDL, FBF, ABP or FVC across stimulations 2, 3, 4 and 5 (Figure 14). However, there was a tendency for FVC in stimulations 4 and 5 to increase above levels reached in control stimulation 2 compensating for the reduction in Hct level (Figure 11).

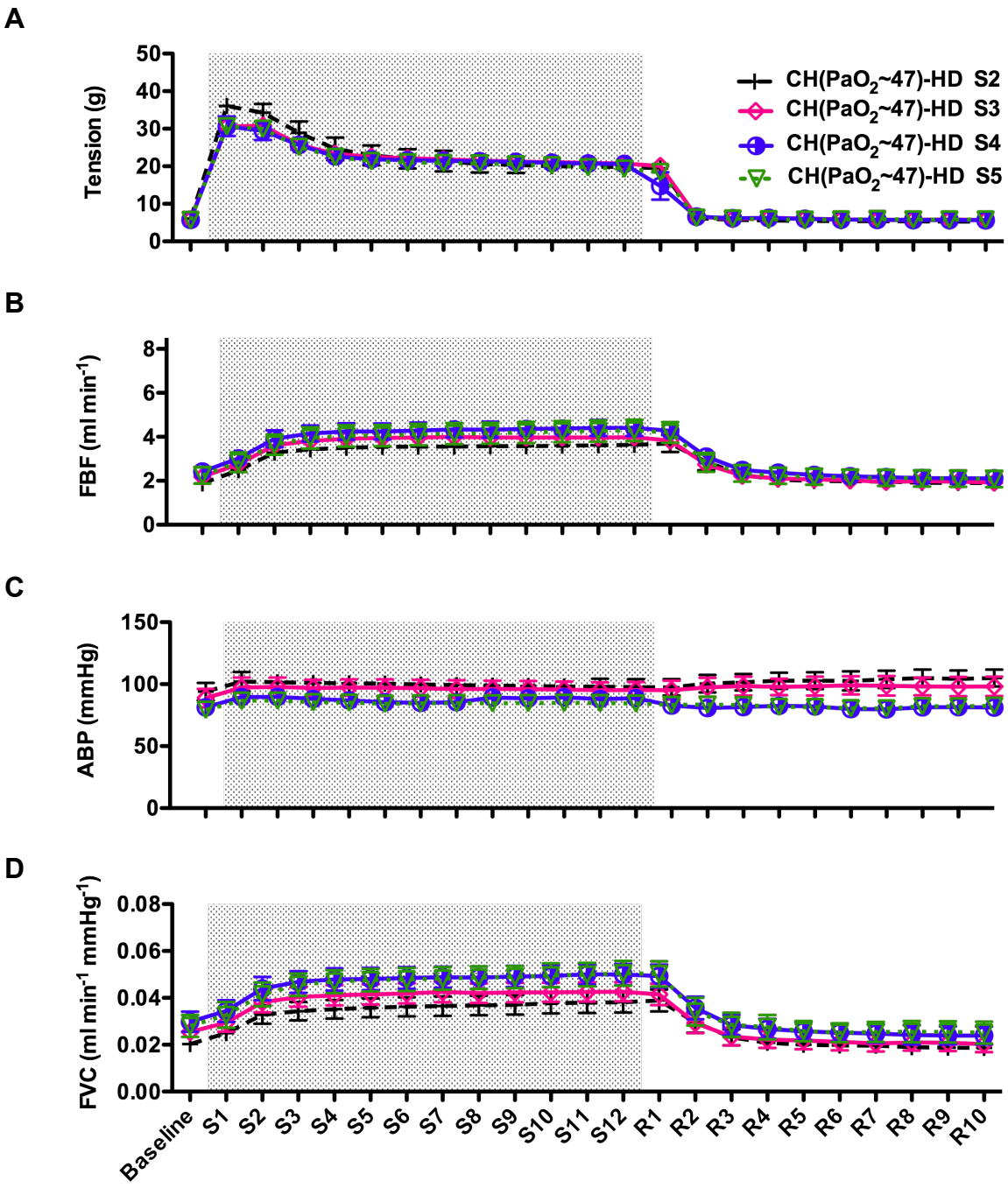

Figure 14. The effect of haemodilution on the responses to stimulation in chronically hypoxic (PaO_2~47)

EDL tension (A), FBF (B), ABP (C) and FVC (D) for control stimulation 2 (pluses, black dashed lines), 3 after 3ml HD (hollow diamonds, pink continuous lines), 4 after 6ml HD (half circles, blue continuous lines) and 5 after 9ml HD (hollow inverted triangles, green dotted lines) in the 1 min baseline, 3 min stimulation (grey shading, 15s; S1-12) and 10 min recovery period (R1-10) Error bars show ± S.E.M., n=5.

3.4 Group 4. Acute systemic hypoxic (PaO_2~33) haemodilution

3.4.1 Group 2 and group 3 vs group 4 stimulation 2 baselines

There was a significant decrease in ABP in group 4 in comparison to group 2 and there was a significant decrease in FVC in group 4 compared to group 3 (Table 5 and Table 8).

3.4.2 The effect of haemodilution on haematocrit

There was no significant difference between Hct in groups 3 and 4. Pre-HD Hct was significantly higher in group 4 than in groups 1 and 2. HD reduced Hct from 53±0.4 to 27±1 (Figure 11) in group 4.

3.4.3 The effect of haemodilution on baselines

Table 8 shows that there were no significant changes in all baseline parameters between stimulations.

Table 8. Acute hypoxia (PaO_2~33) haemodilution baseline values between stimulations 2-5

	Group 4, CH-HD 8% O_2							
	S2		S3		S4		S5	
	Mean	±SEM	Mean	±SEM	Mean	±SEM	Mean	±SEM
ABP (mmHg)	77	7	79	3	71	2	62	3
HR (BPM)	414	16	440	22	423	36	432	23
FBF ($ml.min^{-1}$)	2.2	0.8	2.4	0.9	1.7	0.8	1.7	0.7
FVC ($ml\ min^{-1}\ mmHg^{-1}$)	0.025	0.008	0.031	0.012	0.023	0.010	0.026	0.010
PaO_2 (mmHg)	30	1	33	1	32	4	37	4
$PaCO_2$ (mmHg)	27	12	22	6	22	8	20	8
pH	7.44	0.12	7.54	0.02	7.52	0.04	7.42	0.07

No significance, n=2-4

3.4.4 The effect of haemodilution on response to stimulations

The mean data for group 4 does not indicate that there is a significant change in parameters between stimulations 2 to 5 (Figure 15).

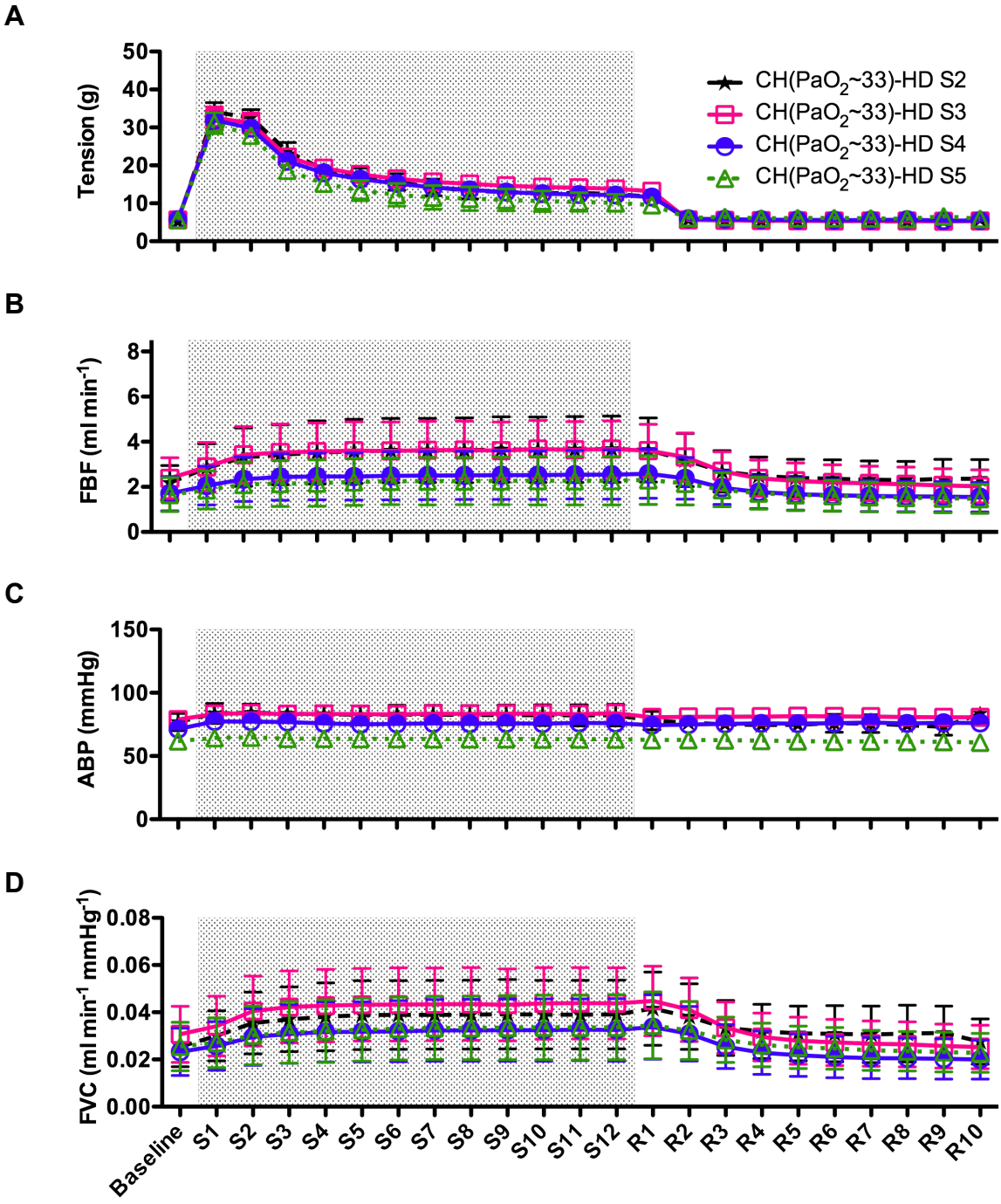

Figure 15. The effect of haemodilution on the responses to stimulation in acutely hypoxic (PaO_2~47) 12%O_2 acclimated animals

EDL tension (A), FBF (B), ABP (C), and FVC (D) for control stimulation 2 (stars, black dashed lines), 3 after 3ml HD (hollow squares, pink continuous lines), 4 after 6ml HD (half circles, blue continuous lines) and 5 after 9ml HD (hollow triangles, green dotted lines) in the 1 min baseline, 3 min stimulation (grey shading, 15s; S1-12) and 10 min recovery period (R1-10). Error bars show ± S.E.M., n=4.

3.4.5 The effect of haemodilution on oxygen delivery, integrated femoral vascular conductance and tension time index in chronic animals.

Figure 16A shows a significant decrease in the level of Hct in CH-(PaO_2~47)-HD animals with HD, accompanied by a trend of increasing IntFVC (Figure 16B) suggesting a vasodilatation to allow a rise in blood flow thereby maintaining DO_2. Muscle performance is sustained (Figure 16C) and is on average 21-26% higher than TTI in N-TC and N-HD animals.

As described there is a significant decrease in the level of Hct in the acutely hypoxic animals and the low PaO_2, a key characteristic of group 4, is reflected in the very low DO_2. IntFVC tends to decrease across stimulations 2 to 5 resulting in a reduction in muscle function by approximately a third when compared to group 3 (Figure 16D-F).

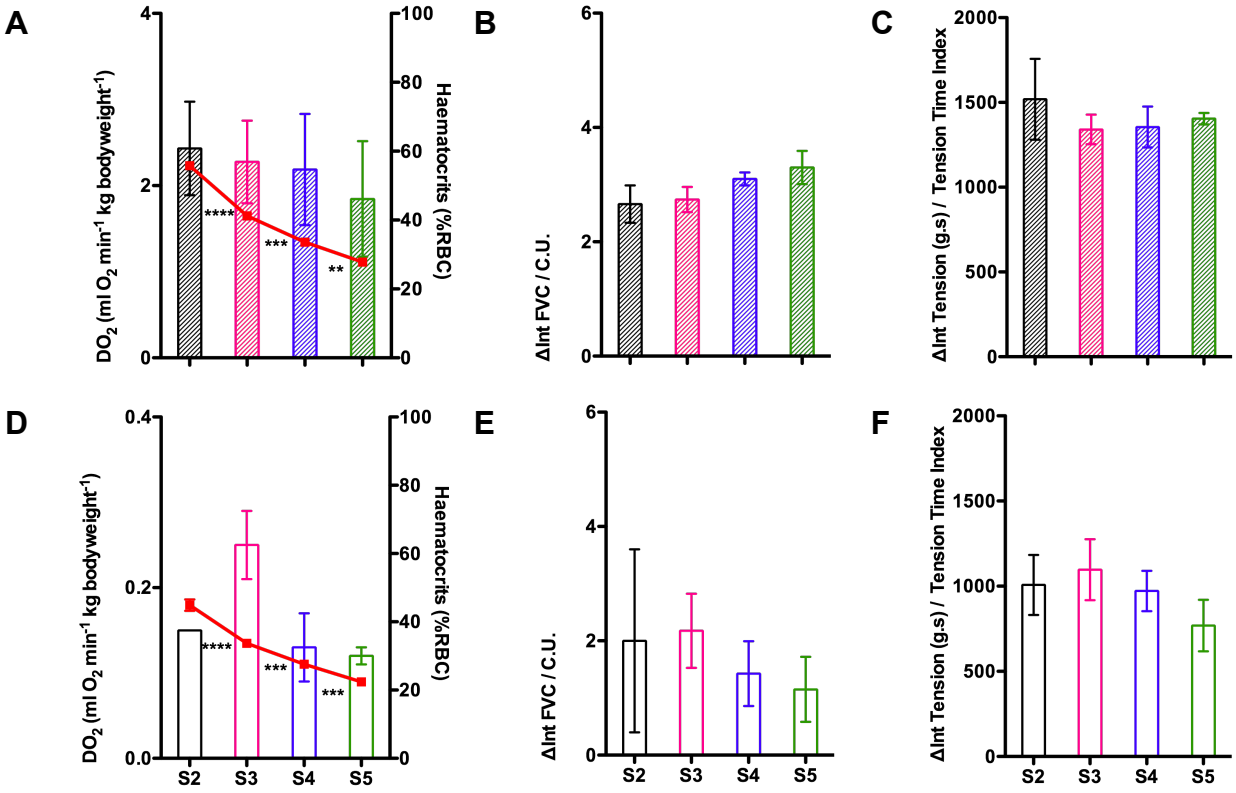

Figure 16. Haematocrit level, oxygen delivery, integrated femoral vascular conductance and tension time index for chronic hypoxic haemodilution groups 3 and 4.

A-C show Hct levels, DO_2, IntFVC and TTI for group 3 CH-(PaO_2~47)-HD *across stimulation 2 (hatched black bar), 3 (hatched pink bar), 4 (hatched blue bar) and 5 (hatched green bar). D-F show identical parameters for group 4 CH-*(PaO_2~33)-HD *animals across stimulations 2 (hollow black bar), 3 (hollow pink bar), 4 (hollow blue bar) and 5 (hollow green bar). * vs. S2 Hct, n=5, two way ANOVA. Error bars show ± S.E.M., n=4-5 (n=1-2 DO_2, CH-*(PaO_2~33)-HD*).*

N.B. different scales in A & D show DO_2 is significantly reduced in CH-(PaO_2~33)-HD*.*

3.5 The effect of haemodilution on oxygen delivery

Figure 17 shows that DO_2 had a tendency to increase between the peak EDL tension and the end of the stimulation, although the rise was not significant. There was no change in the level of DO_2 during stimulations in N-TC, CH-(PaO_2~47)-HD and CH-(PaO_2~33)-HD. However, N-HD data suggested a decrease in DO_2 with HD from ~10.6ml O_2 min^{-1} kg $bodyweight^{-1}$ at the end of stimulation 2 to ~3.4ml O_2 min^{-1} kg $bodyweight^{-1}$ at the end of stimulation 5.

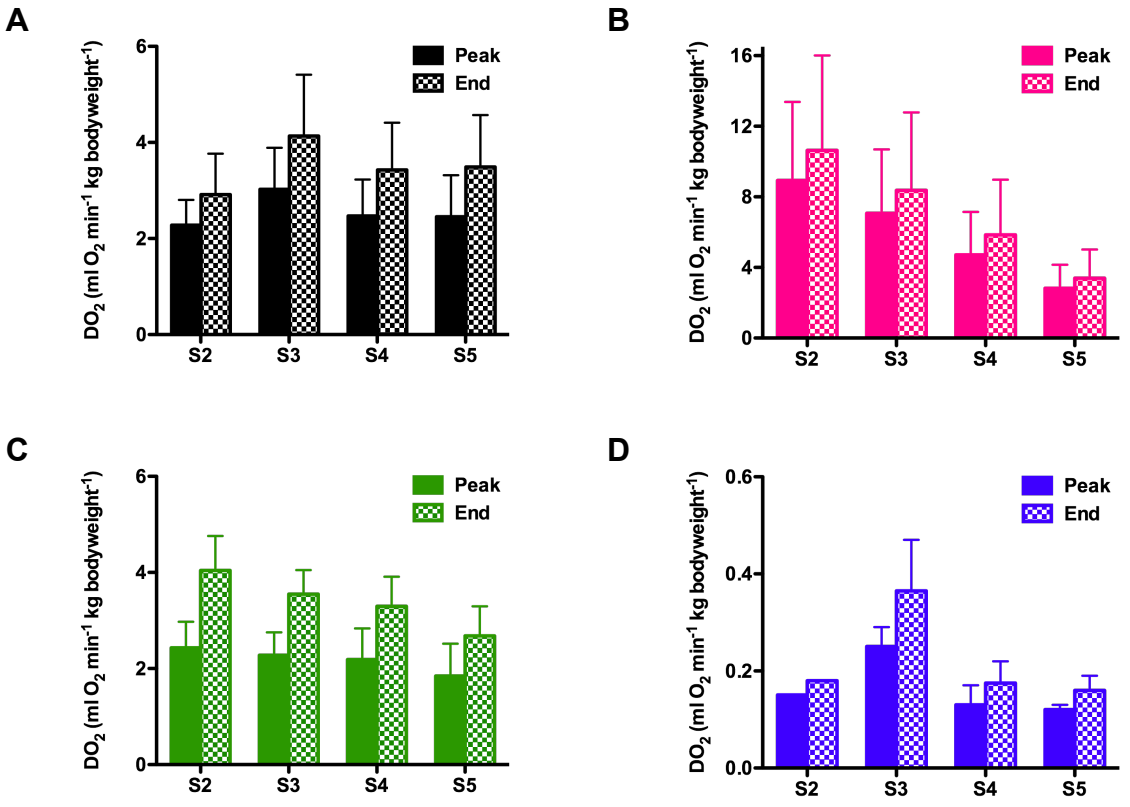

Figure 17. Oxygen delivery during stimulations 2 to 5 in all groups.
A-D show the level of DO_2 corresponding to the first and last fifteen seconds of stimulation matched to the Pt (solid) generated by the EDL and the tension remaining after fatigue (chequered) for N-TC (black bars), N-HD (pink bars), CH-(PaO_2~47)-HD (green bars) and CH-(PaO_2~33)-HD (blue bars). No significant difference. Error bars show ± S.E.M., n=3-5 (n=1-2 DO_2, CH-(PaO_2~33)-HD*).*

3.6 The effect of haemodilution on muscle fatigue

There was no significant increase in fatigue with HD between stimulations across any group, although the acutely hypoxic (group 4) exhibited a significant increase in fatigue in comparison to both N-TC and N-HD. Group 3, showed a tendency towards a higher level of fatigue compared to N-TC and N-HD (Figure 18).

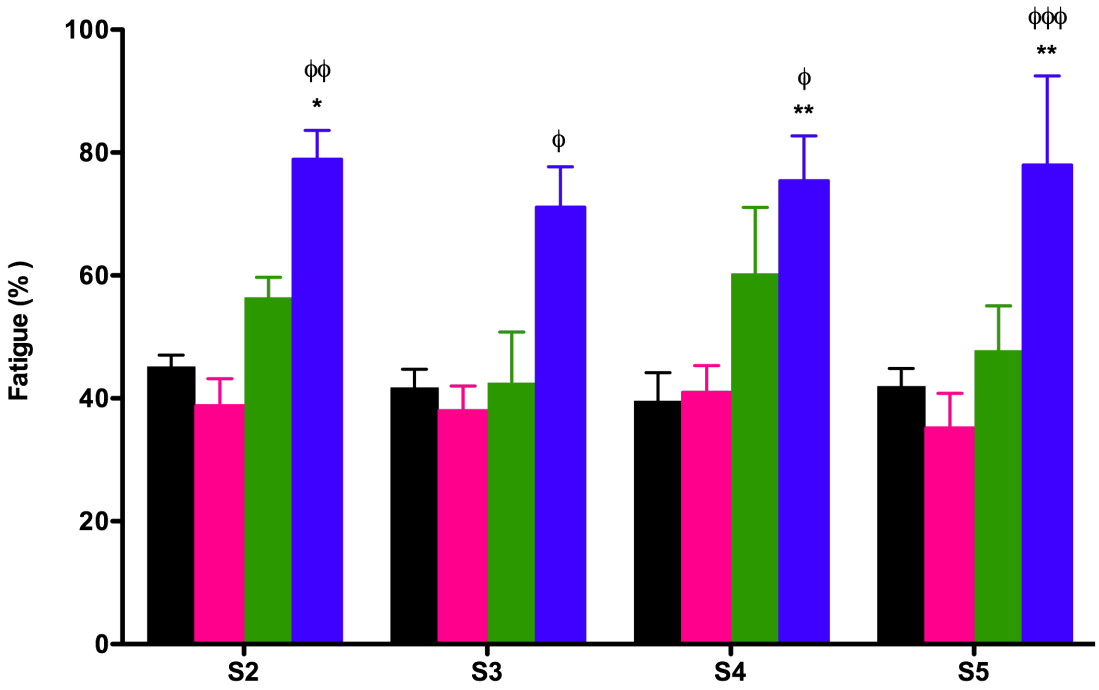

Figure 18. EDL fatigue during stimulations 2 to 5 in all groups.
*Mean data shows the muscle fatiguability for stimulations 2 to 5 in N-TC (black bars), N-HD (pink bars), CH-(PaO_2~47)-HD (green bars) and CH-(PaO_2~33)-HD (blue bars). * vs N-TC and $^{\phi}$ vs N-HD. Error bars show ± S.E.M., n=5, two way ANOVA.*

3.7 The effect of oxygen delivery on muscle fatigue

There was a tendency for DO_2 to decrease with HD in the N-HD group corresponding to the decrease in Hct levels, while the blood flow which was maintained (Fig. 11D-E). N-HD fatiguability is independent of DO_2, remaining constant within the range of 3.4-10.6ml O_2 min^{-1} kg $bodyweight^{-1}$ (Figure 19).

At a matched DO_2 in the range of (3.4±1.6ml O_2 min^{-1} kg $bodyweight^{-1}$) fatigue is significantly increased in all CH-(PaO_2~47)-HD stimulations compared to that of the fatigue in N-HD stimulation 5 (Figure 20). The AH, CH acclimated animals fatigued more significantly than the CH acclimated group corresponding with a decrease in DO_2 (Figure 19). The CH-(PaO_2~33)-HD group is shown on an enlarged scale in Appendix 6.

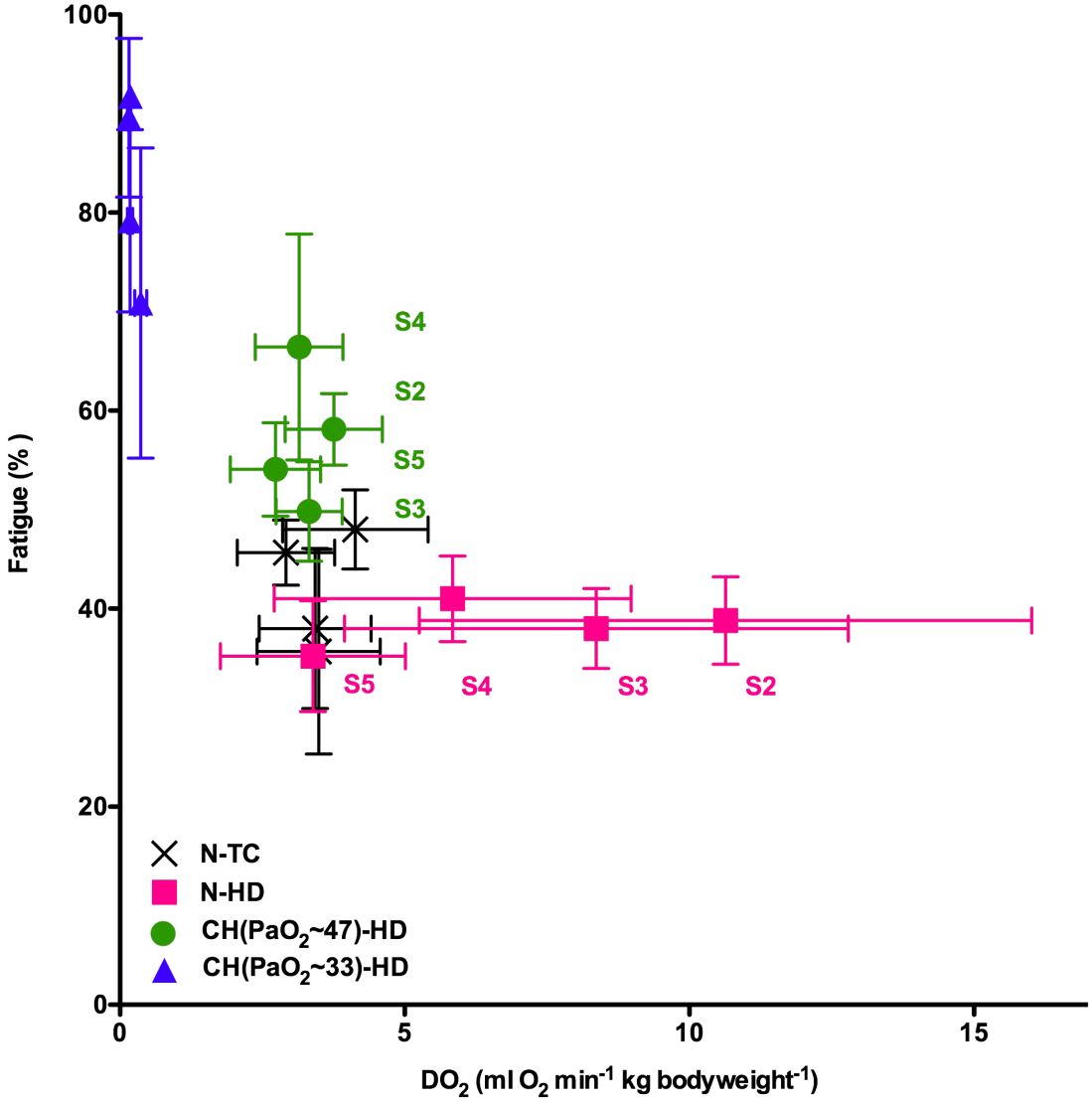

Figure 19. The effect of decreasing oxygen delivery on muscle fatiguability. *Mean fatigue data for individual stimulations is shown against mean DO_2 corresponding to the last fifteen seconds of stimulation for N-TC (crosses, black), N-HD (squares, pink), CH-(PaO_2~47)-HD (circles, green) and CH-(PaO_2~33)-HD (triangles, blue) animals. Stimulation numbers included for N-HD and CH-(PaO_2~47)-HD. Error bars show ± S.E.M., n=4-5.*

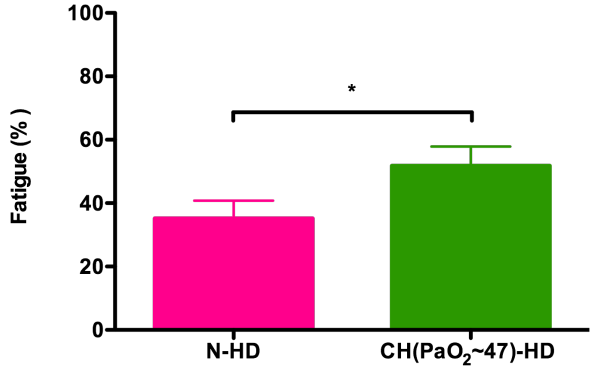

Figure 20. The effect of chronic hypoxia acclimation on muscle fatigue at a DO_2 of 3.4±1.6ml O_2 min^{-1} kg bodyweight^{-1}
*Mean fatigue data for N-HD (pink bar) and CH-(PaO_2~47)-HD (green bar) at a matched DO_2 is shown above. * vs N-HD. Error bars show ± S.E.M., n=4-5, unpaired t-test.*

3.8 Muscle histology

3.8.1 Effect of chronic hypoxia acclimation on angiogenesis

Capillary density (CD), MFCA and capillary to fibre ratio (C:F) were assessed after acclimation to 3 weeks of CH. There was no significant difference in capillary distribution between N-TC and CH and C:F did not show any differentiation between regions 1 to 3. CD showed a significant decrease across muscle regions from 1 to 3 in N-TC and CH rats (Figure 21G-H). MFCA showed a significant increase in size between regions 1 and 3 in control rats (Figure 21H).

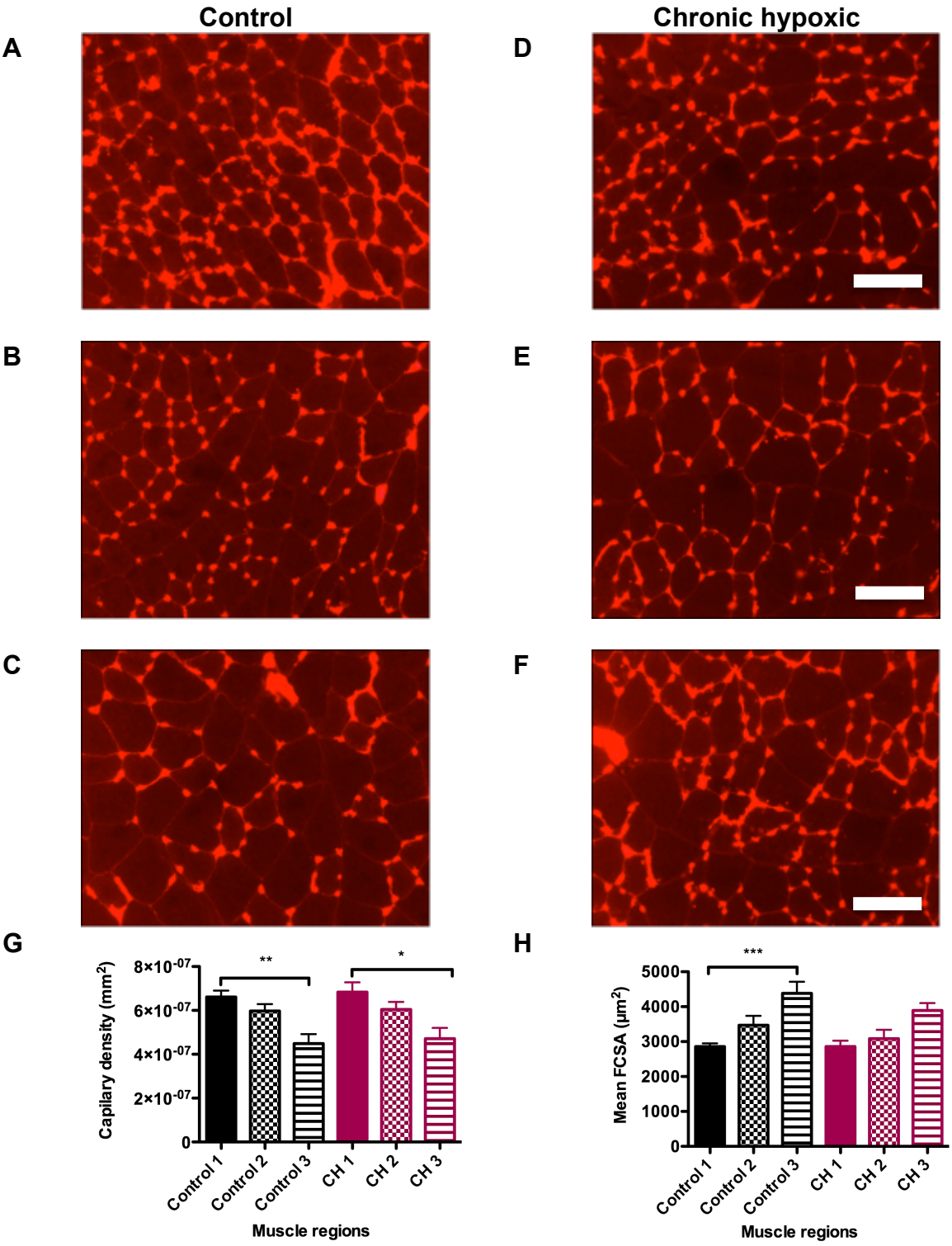

Figure 21. Lectin staining of EDL muscle post 3 week acclimation to 12% O_2. *High contrast photomicrographs representative of transverse mid sections of the medial, central and lateral regions of the EDL, of control region 1 to 3 (A-C) and CH acclimated rats region 1 to 3 (D-F) at 200x magnification, stained with rhodamine-conjugated fluorescent lectin. White scale bar: 100µm (D-F). Panel G and H show quantification of CD and mean FCSA. * vs. region 1. Error bars show ± S.E.M., n=8-12, one way ANOVA.*

3.8.2 Effect of chronic hypoxia and exercise on myofibril glycogen reserves

Glycogen reserves are significantly greater in the EDL muscles of CH non-exercised animals compared to non-exercised controls. There was no significant difference in glycogen content between all exercise groups after five artificially stimulated contractions periods (Figure 22).

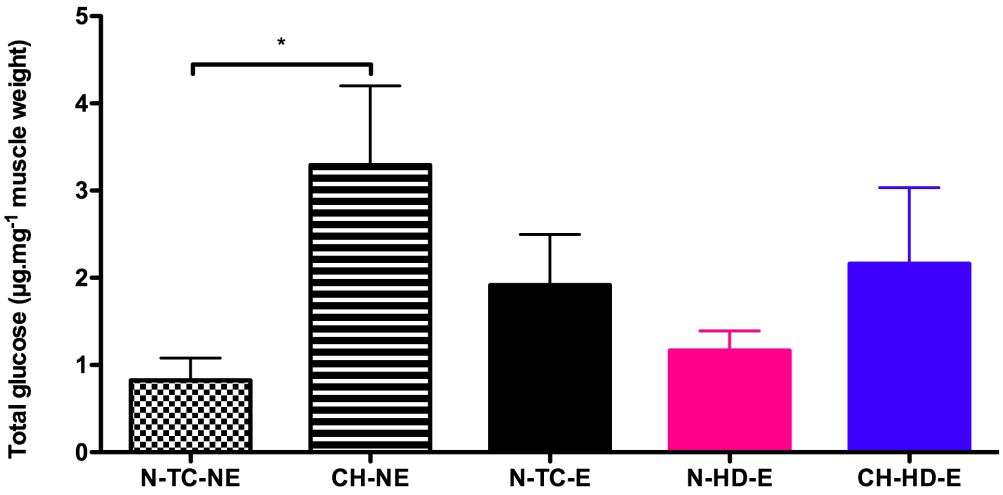

Figure 22. EDL muscle glucose weight in exercised (E) and non-exercised (NE) states 4-6h post anaesthesia.
** vs. N-TC-NE. Error bars show ± S.E.M., n=6, unpaired t-test.*

3.8.3 Effect of chronic hypoxia on the maximum vasodilator capacity

There was no significant difference in the maximum mean FVC recorded in all groups during SNP infusion (Figure 23).

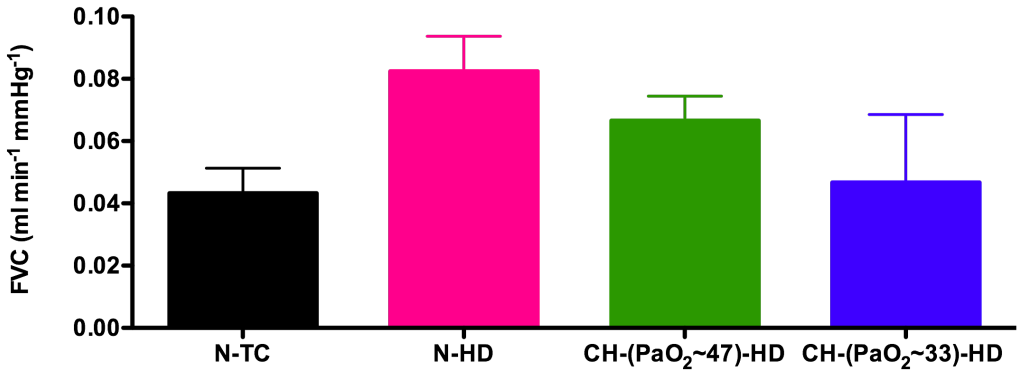

Figure 23. Effect of chronic hypoxia acclimation on the maximum vasodilator capacity of the vasculature in the right hindlimb.
Maximum mean FVC data for N-TC (black bars), N-HD (pink bars), CH-(PaO_2~47)-HD (green bars) and CH-(PaO_2~33)-HD (blue bars) animals. Error bars show ± S.E.M., n=3-5.

4. Discussion

The objective of the present study was to compare the influence of skeletal muscle and vasculature adaptations evoked by CH on the physiological responses induced by isometric twitch contractions by reducing DO_2.

4.1 Group baselines

There was no change in HR at baseline between groups 1-4. FBF, ABP and FVC decreased significantly in group 3 compared to group 2. These findings are inconsistent with Marshall and Davies (1999) who describe comparable cardiovascular parameters between rats acclimatised at 12% and N rats. Group 3 data, however, suggests considerable CH-induced adaptation as parameters vary from those observed by Marshall and Davies (1999) in AH (Table 9). The decrease in ABP in group 3 supports the Smith and Marshall (1999) study and is attributable to an increased peripheral vascular conductance which may be indicative of an increase in structural vascular conductance associated with angiogenesis (Deveci et al., 2001). There was a decrease in FVC in group 3 possibly as a result of a loss of dilator effect, a rise in constriction factors and/or an enhanced myogenic tone in adaptation to CH (Smith and Marshall, 1999). Group 4 showed a decrease in ABP when animals were transferred from breathing 12% O_2 to 8% O_2 with no alteration in FVC, maintaining the FBF. The decreased FVC observed in group 3 was compensated in group 4, potentially by an increase in FVC due to a vascular hypersensitivity to AH (Smith and Marshall, 1999). PaO_2 and $PaCO_2$ decreased significantly in groups 3 and 4 compared to group 2, as expected, due to the nature of the CH treatment. PaO_2 and $PaCO_2$ both for the rats breathing air and for rats

breathing 12% O_2 were, however, in the expected ranges reported in several other studies (Marczak et al., 2004; Marshall and Davies, 1999).

Table 9. Comparison of changes in FBF, ABP and FVC

	Cook, 2012		Marshall & Davies, 1999	
	Group 3 CH 3wk at 12% O_2	**Group 4 AH in CH 3wk at 12% O_2 -> 8% O_2**	**CH 3-4wk at 12% O_2**	**AH 21% O_2-> 8% O_2**
FBF	↓	→	→	→
ABP	↓	↓	→	↓
FVC	↓	→	→	↑

Animal groups above are compared to rats breathing air (21% O_2) reported in Cook, 2012 and Marshall and Davies, 1999. Arrows: ↑increased, ↓decreased, →unchanged; AH=acute hypoxia

4.2 Normoxic time controls

Overall no changes in ABP, HR, PaO_2, $PaCO_2$ and pH baselines between S1-S5 occurred during the N time control protocols and these parameters are therefore unlikely to have affected the responses to stimulation. 15Hz stimulation of the peroneal nerve generated regular twitching of the anterior compartment. The tension peaked in the EDL in the first fifteen seconds of contractions and then fatigued to a steady state for the rest of the stimulation. Similar patterns of contraction were evoked at lower frequencies in the rat EDL muscle: 2Hz, for 15 min and 4Hz, for 5 min in the studies by Lo et al., 2001 and Ray & Marshall, 2009 respectively. The tension may have peaked later in low frequency stimulations as the stimulation parameters may have allowed for increased SERCA activity sequestering calcium between twitches. As a consequence calcium may have accumulated more slowly in EDL undergoing low frequency stimulation.

As anticipated, group 1 N time controls achieved similar mean EDL tensions between S1-S5. However twitches within S1 tended to summate in clonus as the muscle had not fully relaxed before the onset of another stimulus (see Appendices 2-5). All subsequent periods of stimulation did not summate. The summation of individual twitches is generally associated with an increase in P_t. Similar studies (Poucher, 1996; Ray & Marshall, 2009) reported an increased P_t in S1, however, no summation was observed in this study. There are some potential explanations for this noteworthy observation including alterations in the peroneal nerve and neuromuscular junction after the first period of stimulation and changes in the restoration of myofibre energy stores and removal of metabolic by-products from the primary bout of exercise. Reorganisations in contractile filaments and muscle plasticity may also occur to maintain consistency in subsequent contractions (Ray and Marshall, 2009). The author speculates that the lack of crush or sectioning and the patency of the stimulated nerve maintained in this study may have triggered a previously unreported hyper-excitability of the basal ganglia striatum during the first stimulation, via electrically stimulated afferent volley at the peroneal nerve, which may have initiated the release of inhibitory neurotransmitters preventing ganglia excitation in subsequent contractions. The current observation could have critical implications for future investigations and warrants further study.

Overall tension generated by the EDL in N-TC, S2-S5 was fully comparable, so S1 was omitted and S2 was used as the control for S3, S4 and S5.

During stimulation, FBF and FVC, total blood flow and total vascular conductance, to the right hindlimb increased ~1.7-fold, both variables gradually returning to baseline

over a 6 min recovery period. The overall pattern of response agrees with that recorded in rats and cats (Poucher, 1996; Ray and Marshall, 2009), however the increase in blood flow was much lower in the current study. The reduced flow may have been due to the sub-optimally positioned flow probe (due to the inexperienced operator) that mechanically restricted flow. DO_2 remained above the threshold affecting physiological function and therefore muscle performance, represented by the tension time index, described below was unaffected by the mechanical impairment. Probe placement was improved in the remainder of the groups. The increase in FBF and FVC indicated a vasodilatation in the hindlimb muscles during contraction. This supports the established view that vessels vasodilate in metabolically active tissue. ABP remained constant throughout the stimulation, which may have been due to the small muscle mass stimulated and a change in vascular conductance in the peripheral vasculature to counteract the rise in FVC as reported by Calbet et al. (2009).

In summary, the response to stimulations showed no change with the length of experiment signifying that both the experimental setup and the results were reliable.

4.3 Normoxic haemodilution controls

Hct was halved in the N-HD group by S5 through HD, which reduced CaO_2 by approximately 50% but did not change PaO_2. As expected there was no significant change in ABP, HR, PaO_2, $PaCO_2$ and pH baselines between S2 in the control groups N-TC and N-HD.

ABP, HR, PaO_2 and pH baselines between S2-S5 in N-HD, group 2, did not change and these parameters are therefore unlikely to have affected the responses to stimulation. However, $PaCO_2$ decreased significantly in S4 and S5 compared to S2 and S3 which may be attributed to hyperventilation linked with HD described in section 4.4.

Hyperventilation may have also been a result of gradual aggregation of mucus in the T-shaped cannula during the protocol, which may have degraded airway patency and lowered pulmonary gas exchange, causing the animals to increase their minute ventilation and instigating the Bohr effect, while preserving PaO_2 but decreasing $PaCO_2$. Calbet et al. (2009) noted that the effects of the Bohr shift were proportional to the size of muscle mass. As the EDL is a relatively small muscle the potential contributions of muscle mass to $PaCO_2$ can be disregarded in this case. The accumulation of carbonic acid released from the exercising EDL and an associated increase in $PaCO_2$ in the blood can also be excluded, as $PaCO_2$ remained unaltered throughout the stimulations in the N-TC group.

Tension produced by the EDL in N-HD, group 2, was comparable to that in N-TC animals. Interestingly, there was no change in tension across S2-S5 in N-HD despite HD. FBF and FVC in the right hindlimb increased ~2-fold, both variables declining to baseline over a 6 min recovery period. These variables are significantly higher than those measured at the end of stimulation in N-TC due to the mechanical restrictions on FBF described in section 4.2. Otherwise the pattern of response is comparable to N-TC, group 1, and with aforementioned studies (Poucher, 1996; Ray and Marshall, 2009). However hyperaemia in the current study remained ~30% lower in amplitude

than that published previously which may be due to the high resting blood flow of ~3ml.min^{-1} rising to ~7ml.min^{-1} compared to ~1ml.min^{-1} increasing to ~3ml.min^{-1} by the end of stimulation as reported by Ray and Marshall (2009). As expected and discussed in section 4.2, there was no significant alteration in ABP during stimulations. There was a slight tendency for ABP to fall with HD, which was probably caused by a decrease in the viscosity of the blood (Figure 12C) as reported by Murray and Escobar (1968).

In summary, the response to stimulations S2-S5 N-HD showed no change with HD. This may be explained by a compensation in DO_2 discussed in 4.4 below.

4.4 The effect of haemodilution on oxygen delivery, integrated femoral vascular conductance and tension time index in normoxic animals.

N-TC ΔFVC, as a measure of dilatation, was maintained across stimulations as was FBF. DO_2 remained constant across S2-S5 as Hct remained unchanged. DO_2 would probably have been similar to that in N-HD S2 had it not been for the mechanical restrictions placed on the flow in N-TC. TTI was maintained in N-TC at similar levels to N-HD S2, despite the restriction in DO_2 suggesting that constrained DO_2 did not influence N-TC TTI and was therefore above the threshold effecting physiological function (DO_{2crit}) (Figure 13C). Consequently, fatiguability remained unchanged across the N-TC group (Figure 18). N-TC TTI, as an index of muscle performance, was maintained at levels of ~1200g.s^{-1} comparable to those found by Ray and Marshall (2009), who reported levels of ~2000g.s^{-1}, for isometric single twitch

contractions at a stimulation frequency of 4Hz for 5 min, as opposed to the stimulation frequency of 15Hz, which was applied for 3 min in this study. N-HD ΔFVC was maintained across S2-S4 but had a tendency to fall in S5, as did FBF. There was a tendency for DO_2 to decrease as HD significantly reduced Hct. Variability between individual DO_2 baselines in the N-HD group can be reduced by baseline normalisation shown in Appendix 7. The present results do not appear to support a role for erythrocyte oxygen sensing as a significant decrease in Hct and decrease in DO_2 does not affect the size of hyperaemia (Ellsworth and Sprague, 2012). The results therefore suggest that there may be a role for a vasodilator mediator such as adenosine, causing hyperpolarisation of vascular myocytes released from the exercising muscle, as reported by Poucher (1996) and Ray and Marshall (2009).

The decrease in DO_2 through S2-S5 may be due to a lack of compensation in ΔFVC and the associated flow. The femoral artery may be close to maximal dilatation and therefore incapable of increasing further in size. This may also provide a possible explanation for the greater decrease in DO_2 than Hct at S4. At S4, 6ml HD had already occurred and Hct had been reduced by 0.34 fold, a decrease from 53±0.4 to 33±1.2% RBC. The corresponding decrease in DO_2 was 0.57 fold from 9±4.4 to 4.7±2.4mlO_2 min^{-1} kg $bodyweight^{-1}$ (Figure 13D) suggesting that flow had not increased to maintain DO_2.

A restricted DO_2 at S4 may explain the decrease in $PaCO_2$ described in section 4.3. Hyperventilation may have occurred as a result of the 6ml HD between N-HD S2 and S4. The reduced DO_2 in S3 resulting from a 3ml HD may have caused a slight AH, decreasing PaO_2, in the active muscle tissue during muscle contraction. As metabolic rate increased in the working muscle, carbonic acid may have been

released triggering a decrease in pH. The reduced pH may have increased oxyhaemoglobin saturation to compensate for the low tissue PaO_2 by increasing O_2 unloading in the tissues consistent with the Bohr shift. The combined decrease in pH and increase in $PaCO_2$ in the blood plasma may have activated the central chemoreceptors potentially stimulating the intercostal muscles and diaphragm, and thereby increasing both minute ventilation and lung volume as reported by Calbet et al. (2009). The potential compensation in hyperventilation may have caused a significant decrease in $PaCO_2$ in the blood, due to increased amounts of CO_2 being expelled from the lungs, noted in the BG prior to S4, causing a slight increase in pH, a respiratory alkalosis (Roach et al., 1999). A hyperventilation may have occurred in N-HD S4 and S5 in contrast to N-TC as the reduction in Hct in N-HD and subsequent decrease in the Hb content of the blood reduced non-bicarbonate buffer (Roach et al., 1999). Hb acts as a non-bicarbonate buffer in the blood to abstract protons reducing carbonic acid concentrations and therefore neutralising pH.

The tendency for FBF to decrease in N-HD S5 is probably due to a severe local tissue hypoxia during muscle contraction, which caused an increase in vasoconstrictor influences on terminal arterioles. Therefore 9ml HD by N-HD S5 decreased DO_2 by 0.65 fold from N-HD S2 and may have initiated vasoconstriction but had no effect on muscle performance, suggesting that DO_2 of 2.8mlO_2 min^{-1} kg $bodyweight^{-1}$ is above the threshold affecting physiological function or that myoglobin stores may act as an alternative source of O_2 for aerobic metabolism when DO_2 is restricted (Calbet et al., 2009). Consequently fatiguability remained unchanged with HD in the N-HD group (Figure 18).

TTIs have been used in other studies to compare force generated by muscle during dynamic exercise (Ray and Marshall, 2009). However it would also be interesting to analyse single twitch dynamics at different time points during fatigue to establish which fibres are likely to be more fatigue resistant. The length of time required for a peak twitch tension to decline to 50% of the generated tension is known as half-relaxation phase ($T_{1/2R}$) and is determined by the types of fibre actively contracting (Close and Hoh, 1968).

To summarise, DO_2 was decreased substantially by HD but did not affect muscle performance or fatiguability.

4.5 Chronic hypoxia haemodilution (12% O_2)

Hct was significantly higher in the CH-HD group in agreement with several studies (Calbet et al., 2009; Gonzalez-Alonso et al., 2001; Lundby et al., 2006). Hct was reduced by 50% in the CH-HD group by S5 through HD, a comparable decrease to that in N-HD which indicates that CH animals may have a similar blood volume to control rats. As expected there was no significant change in HR, $PaCO_2$ and pH baselines between S2 in the CH-HD and N-HD group. There was a significant decrease in ABP in CH-HD compared to N-HD also noted in a study by Smith and Marshall (1999). However PaO_2 decreased significantly in CH-HD animals as was required for adaptations to occur and was expected due to the nature of the CH treatment and which may have resulted in adaptations. FBF and FVC decreased significantly in CH-HD, group 3, in comparison to N-HD, group 2, which is in agreement with Edmunds and Marshall (2001) who observed a decrease in hindlimb flow with the severity of hypoxia. A decrease in flow may suggest an increased

vascular bed (Smith & Marshall, 1999). However DO_2 at CH-HD, group 3 S2 was comparable with DO_2 in N-TC S2 which is consistent with findings of Calbet et al. (2009) who noted that acclimatisation to hypoxia maintained leg DO_2 at levels similar to controls. This may therefore indicate that N-TC FBF was not as restricted as previously thought. This is also reflected in the SNP-evoked maximum mean FVC results in this study that suggested that N-HD animals tended to have larger maximum vasodilator capacity and therefore a higher FBF due to animal variation (Figure 23).

There was no change in baselines between all parameters in the CH-HD S2-S5 and ABP, HR, PaO_2 and pH are therefore unlikely to have affected the responses to stimulation. Tension produced by the EDL in CH-HD, group 3, was comparable to that in N-HD animals. There was no change in tension across S2-S5 in CH-HD despite HD, suggesting that DO_2 had not decreased below the threshold for efficient physiological function. FBF and FVC in the right hindlimb increased ~1.5 fold, both variables declining to baseline over a 4 min recovery period. The rapid return to baseline after contraction may indicate that vasodilator influences and metabolites are removed from the skeletal muscle vasculature more rapidly perhaps due to vascular remodelling (Marshall & Davies, 1999). The pattern of response observed during stimulation was otherwise comparable with that of N-TC and N-HD groups and the observations of Ray and Marshall (2009) mentioned previously.

In summary, the response to stimulations S2-S5 CH-HD showed no change with HD. This may be explained by a compensation in DO_2, enhanced O_2 extraction or an increase in energy reserves in myofibres.

4.6 Chronic to acute hypoxia haemodilution (8% O_2)

The CH-HD, group 4, was transferred to 8% O_2 for the complete duration of the experiment to evaluate the effects of an extremely limited DO_2 on the response to stimulation.

Hct was significantly higher in the CH-HD group in agreement with Gonzalez-Alonso et al. (2001) and other studies mentioned previously. Hct was reduced by 50% in group 4 by S5 through HD, which approximately halved CaO_2 but did not change PaO_2. There was no significant change in HR and pH baselines between S2 in group 4 and the N-HD group, but there was a more significant decrease in ABP in group 4 in relation to group 3 when compared to N-HD, which is consistent with the observations of Marshall and Davies (1999) when switching to AH (Table 9). As in group 3, PaO_2 decreased significantly in group 4 animals as was expected due to the nature of the treatment and may have led to adaptations. However PaO_2 was significantly lower in group 4 during the experimental protocol than it had been in group 3. The present data appears to support an enhanced role of vasoconstrictor activity in severe AH consistent with the findings of Smith and Marshall (1999). FBF and FVC decreased significantly in group 4 in comparison to group 3 which is discussed in section 4.2.

There were no changes in baselines between all parameters in group 4 S2-S5 and therefore these are unlikely to have affected the responses to stimulation. Pt generated by the EDL in group 4 was comparable to that in N-HD and group 3 animals. However tension produced by group 4 animals reached a steady value at a lower tension than all other groups. There was no alteration in tension and therefore

no change in muscle function across S2-S5 in group 4 despite HD. FBF and FVC in the right hindlimb increased ~2.25 fold, both variables declining to baseline over a 4 min recovery period. Group 4 FBF and FVC also returned to baseline rapidly perhaps due to vascular remodelling discussed in section 4.5 (Marshall & Davies, 1999). The patterns of response observed during stimulation for FBF, ABP and FVC were comparable with that of N-TC and N-HD groups and consistent with the findings of Ray and Marshall (2009) mentioned previously. However tension response to stimulation in group 4 declined more quickly than other groups perhaps due to an increased fatiguability in the presence of an extremely low PaO_2.

In summary, the response to stimulations S2-S5 group 4 showed no change with HD. This may be explained as previously by a compensation in DO_2 or perhaps enhanced O_2 extraction in the tissues.

4.7 The effect of haemodilution on oxygen delivery, integrated femoral vascular conductance and tension time index in chronic hypoxia animals.

There was a tendency for ΔFVC in CH-HD, group 3, to increase across stimulations with a decrease in Hct, maintaining DO_2 across S2-S4 (Figure 16). However there was a tendency for DO_2 to decrease in S5. $PaCO_2$ may not have altered in group 3 as the chemoreceptors may not have been activated due to the higher number of erythrocytes and an associated increase in Hb concentration that abstracted more protons neutralising blood pH. The DO_2, which was maintained, also allowed muscle performance to be marginally improved when compared to performance in

controls. The current data supports El-Khoury et al. (2012) as CH was found to significantly increase twitch force. The present result, however, does not support that of Calbet et al. (2009), as performance was found to decline in humans acclimatised for 9 weeks to an altitude of 5260m. Calbet et al. (2009) also suggested that the duration of the acclimatisation period and intensity of the hypoxic stimulus caused the differentiation in performance, as oxygen diffusion and mitochondrial oxidative capacity may be reduced by acclimatisation for 9 weeks which is also consistent with Lundby et al. (2006), who suggest that FVC is a factor limiting exercise tolerance at high altitude. The author speculates that the result of the current study supports the above view as the tendency for DO_2 to fall at S5 may be due to a near maximal conductance. S5 muscle performance, however, is maintained which may therefore be caused by an increased O_2 extraction in the tissues or increased energy reserves in myofibres. It should be noted that fatiguability remained unchanged across group 3 (Figure 18).

ΔFVC for CH-HD, group 4, was maintained in S2 and S3 but had a tendency to decrease in S4 and S5. The present data does not support an increase in hyperaemic response with systemic hypoxia as presented by Donnelly and Green, (2012), however this may be due to the extent of hypoxic stimulus. Donnelly and Green, (2012) used a milder stimulus in their study of humans. The current results may be due to an increase in vasoconstrictor influence induced by severe hypoxia (Smith and Marshall, 1999). Little conclusion can be drawn from the DO_2 data given the small n, except to highlight the severe decrease in DO_2 across the entire protocol due to the lowered PaO_2 and incremental decrease in CaO_2 as a result of HD. Interestingly, muscle performance reflects FVC and is maintained in S2 and S3. TTI

in group 4 S2 ~1050g.s^{-1} was lower than that produced in control groups ~1200g.s^{-1} which may be due to the significant decrease in body weight between group 4 animals and N-HD. There is a tendency for muscle performance to decrease in S4 and S5. Extrapolating from Calbet et al. (2009), a severely decreased DO_2, a result of systemic hypoxia, corresponds to a decrease in peak flow and peak aerobic power as seen in the present data in group 4 S4 and S5. However there was no significant difference in fatiguability across S2-S5.

In summary, muscle performance in group 3 was comparable to that in N-HD animals at S2 and throughout S2 to S5. Therefore 9ml HD had not decreased DO_2 below the threshold required to affect muscle function.

4.8 Fatigue

Group 1 and 2 of the present study fatigued from Pt by ~40% by the end of each 3 min stimulation S2-S5. Fatigue in group 3 was more variable between S2-S5 but remained at ~50% of initial Pt. Group 4 fatigued significantly more than the other groups by ~75% of initial Pt. The present results support El-Khoury et al. (2012) as they found CH significantly decreased EDL endurance *in vitro.*

The current study suggests that at a matched DO_2 where O_2 diffusion does not limit muscle function in N-HD animals, CH-HD, group 3, muscle fatiguability is significantly increased. This appears to implicate skeletal muscle adaptations, influenced by CH, as the more critical limiting factor in exercise tolerance as opposed to vasculature remodelling. As far as the author is aware this is a new finding.

One possible adaptation that may be responsible is a transition towards fast glycolytic fibre types that could have contributed to the observed tendency for group 3 animals to have an enhanced muscle performance while significantly increasing fatiguability between group 2 and group 3 at matched DO_2 levels .

4.9 Capillary to fibre ratio

The transition towards a glycolytic fibre-type or switch in fibre type remains controversial, however it has been deliberated in a number of reviews (Allen et al., 1995; Spangenburg and Booth, 2003; Westerblad et al., 2010). The current study supports the view of a transition to a fast fibre type as significantly increased glycogen reserves were observed in CH muscle (Figure 22). CH muscle also appeared in relation to FCSA to become more homogenous in nature which may be indicative of a switch to intermediate fibres or perhaps myofibre atrophy (Figure 21H). Faucher et al. (2005) supports this modest change in histology. There was no change in C:F which may suggest there is vascular remodelling in the terminal arterioles in accord with Smith and Marshall (1999).

4.10 Potential improvements

This study would have been improved with the addition of two further experimental groups, firstly a CH-TC group to determine the effects of the length of the experiment on CH animals and allow the effects of HD within the CH animals to be assessed against a CH “control”, and secondly, an N group, i.e. acclimatised to 21% O_2, that was transferred to 8% O_2 for the duration of the experimental protocol to determine the effects of systemic AH. Furthermore, additional experiments could have been

carried out to obtain interpretable BG results for group 4, had the appropriate equipment been available.

There were limitations in the interpretation of FBF in relation to the tension produced as the tension was solely generated by the EDL, although the entire anterior compartment was stimulated and therefore the blood flow was representative of flow to the whole hindlimb. It is important to recognise therefore that any increase in flow is attributable to the metabolic demands of all of the muscles in the anterior compartment and not just the EDL (Clifford and Hellsten, 2004).

4.11 Recommendations for further work

An investigation to determine the relationship between muscle performance and DO_2 and whether the effects of CaO_2 or PaO_2 are more important in regulating exercise tolerance would be beneficial to this field of research. The study should ideally contain control groups that reduced CaO_2 further than levels in the current study (i.e. by HD greater than 9ml) as 9ml HD in N-HD S5 animals only just matched DO_2 to CH-HD animals, group 3. The new investigation should also include several N and CH-HD groups with step-wise decreases in PaO_2. It would also be beneficial to measure VO_2 via venous blood samples prior to, and after, stimulation to confirm the extent of O_2 extraction in the active muscle, thereby enabling changes in skeletal muscle to be identified.

TTI and absolute fatigue provide a superficial analysis of muscle performance and endurance. A more detailed picture of muscle performance could be developed through analysis of the single twitch relaxation phase and length of twitch, thereby enabling twitch dynamics in relation to muscle fibre energetics and fatigue resistance

to be evaluated. Further histological assessment would allow muscle fibre typing and mitochondrial number to be evaluated. See Appendix 8 for sample micrographs following staining with a succinic dehydrogenase and alkaline phosphatase stain. The current model of adaptations evoked by CH is not immediately translatable to clinical conditions of COPD and CHF. To establish a more robust link to the clinical condition, disease models should be evaluated. For example, either with an aged model, which is relevant as adult muscle is more oxidative in composition and may therefore experience a greater level of CH-evoked adaptation, or a model of CHF.

4.12 Conclusions

EDL muscle performance and fatiguability in N and CH rats remained unchanged when the Hct was reduced by 50%. Phenotypic adaptations in response to CH allowed the muscle to perform and fatigue at levels comparable to N rats. However, adaptations to CH did not allow CH animals to maintain a fatigue profile comparable to N rats at a matched DO_2 as absolute fatigue was significantly increased in comparison to their N counterparts. The latter suggests that muscular adaptations are more critical than vascular changes in limiting exercise tolerance in rats, a finding that may be applicable to COPD or CHF patients.

It can be concluded from the results of this study that the original hypotheses 1 and 3 can be refuted whereas hypothesis 2 is supported.

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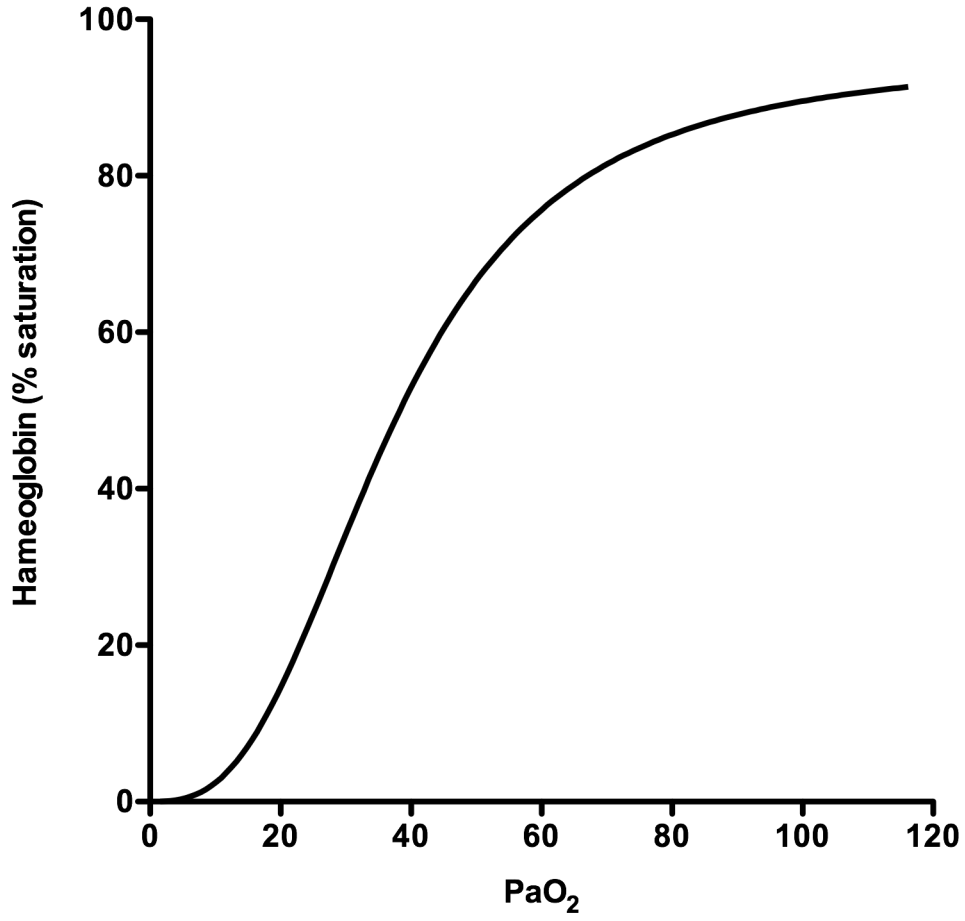
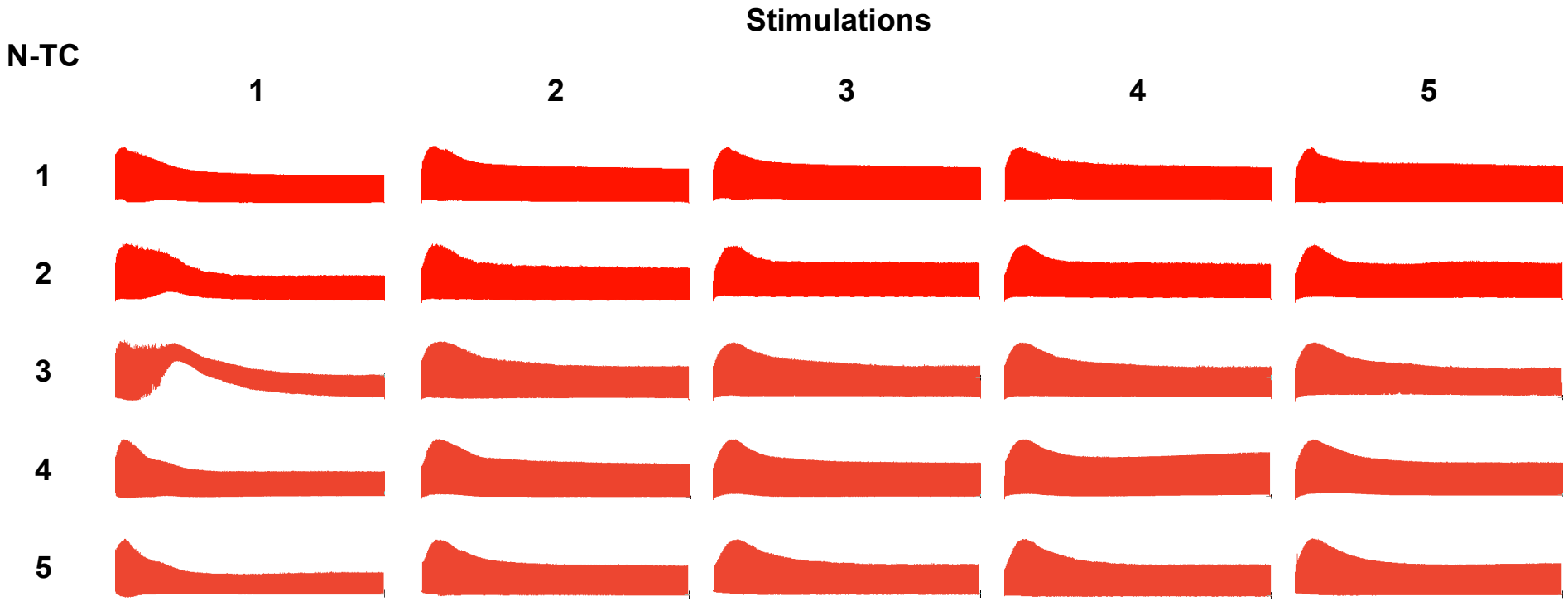
Appendix 1: Oxyhaemoglobin saturation curve

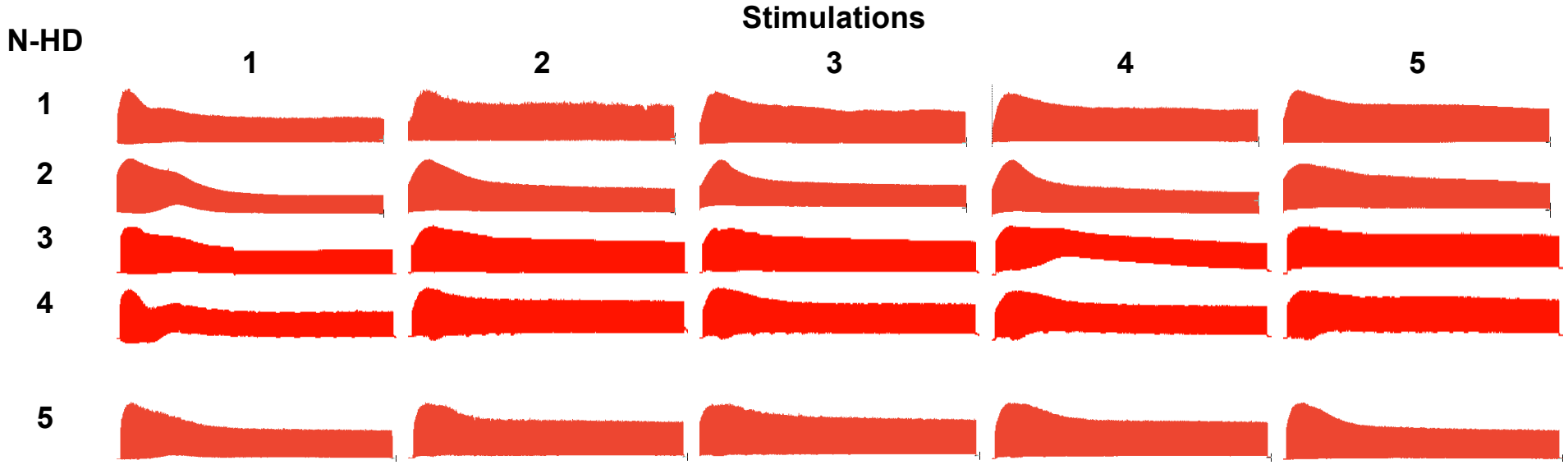


Oxyhaemoglobin dissociation of rat blood.
The solid line combines previously published observations(Cartheuser, 1993).

Appendix 2: Normoxic time control fatigue profiles

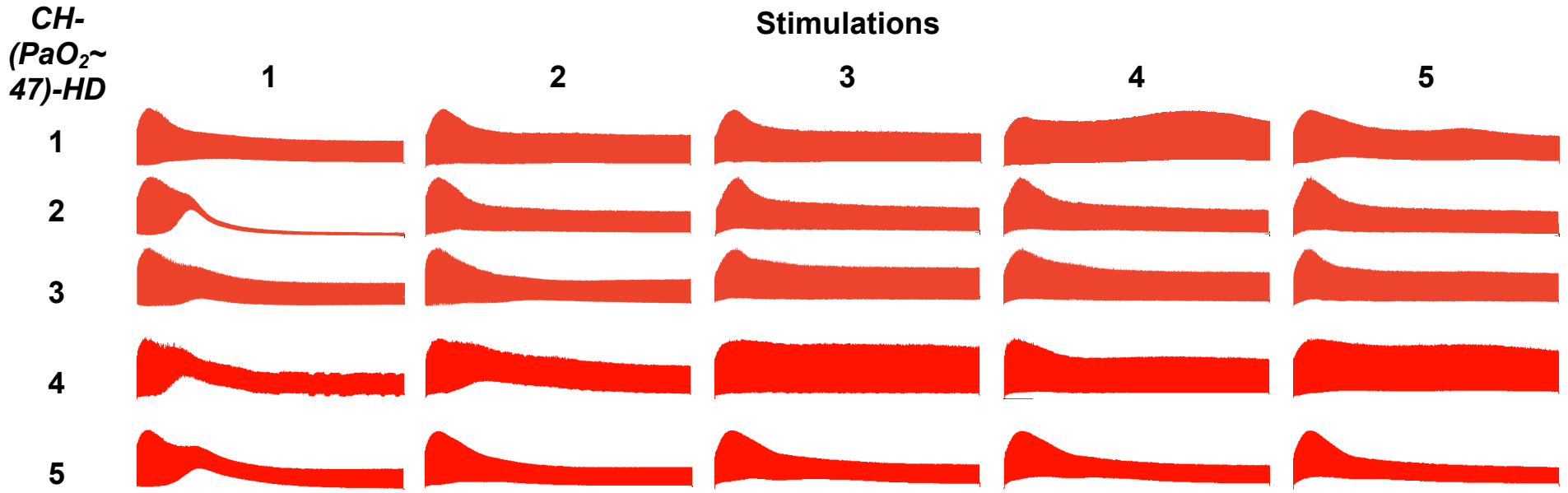


Appendix 3: Normoxic haemodilution fatigue profiles



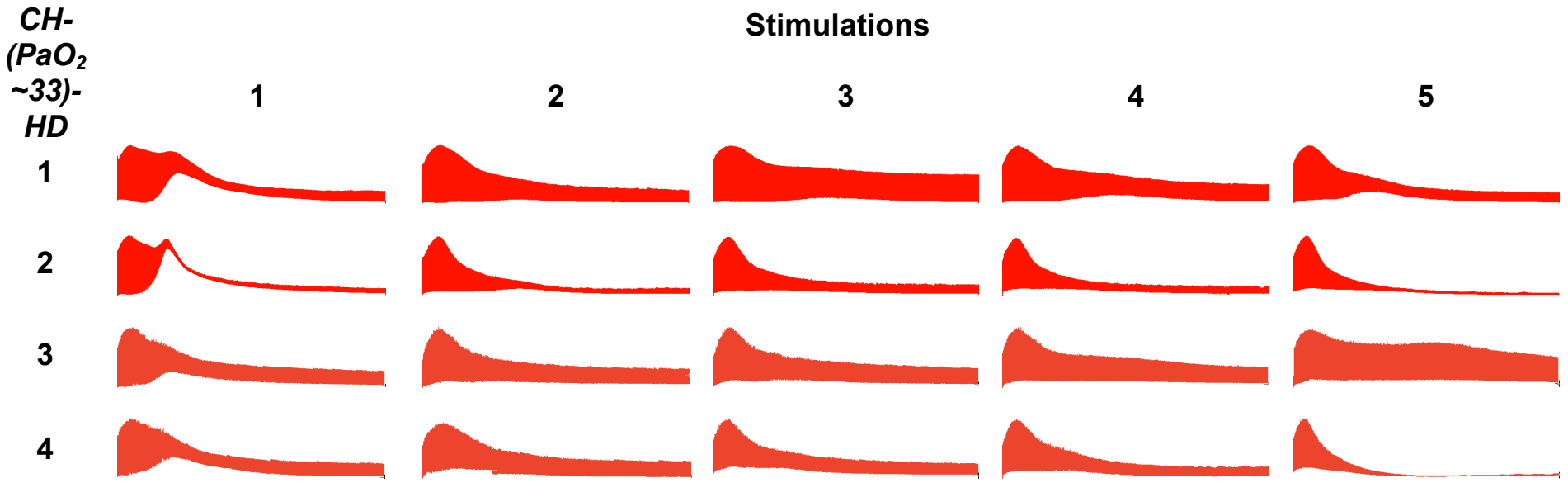
N-HD tension fatigue profiles in the EDL muscle at a supra-maximal stimulation of 15Hz, 0.15m

Appendix 4: CH-HD 12% O_2 haemodilution fatigue profiles



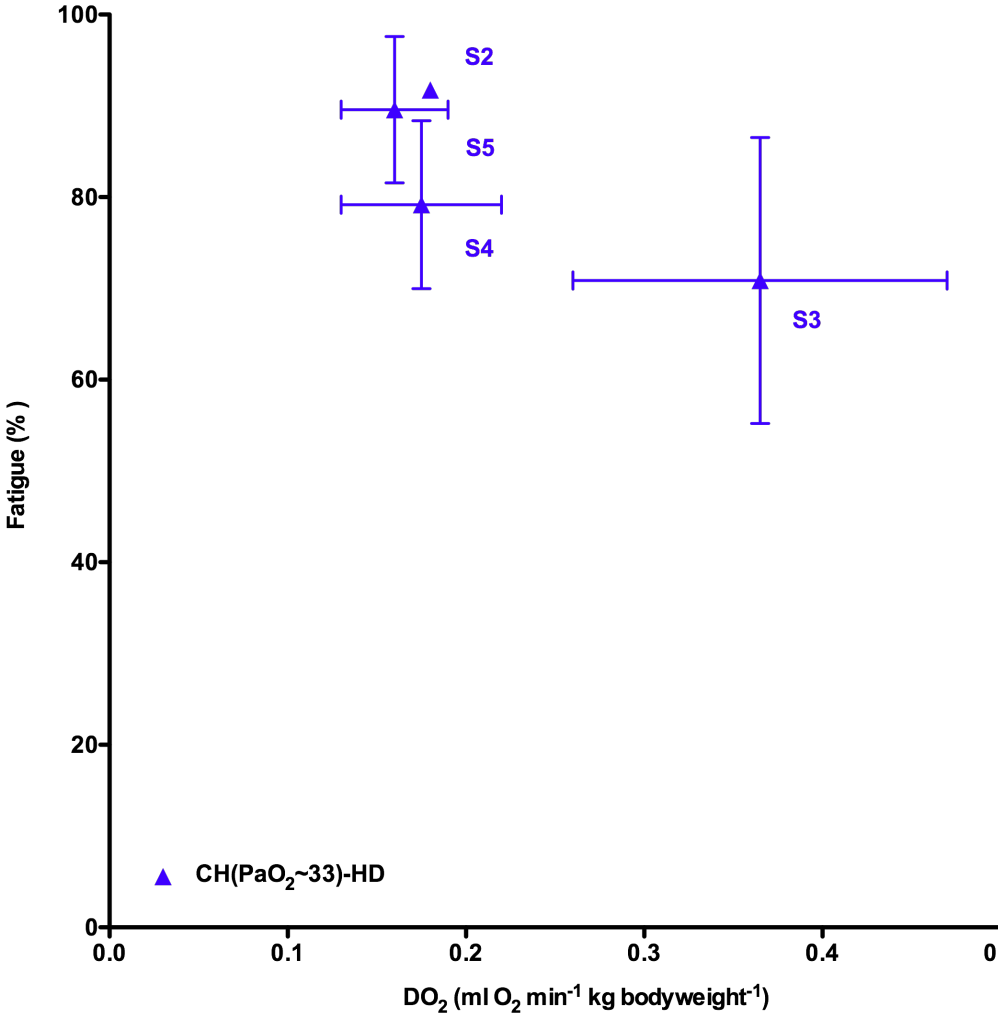
CH-(PaO_2~47)-HD tension fatigue profiles in the EDL muscle at a supra-maximal stimulation of 15Hz, 0.15ms.

Appendix 5: CH-HD 8% O_2 haemodilution fatigue profiles



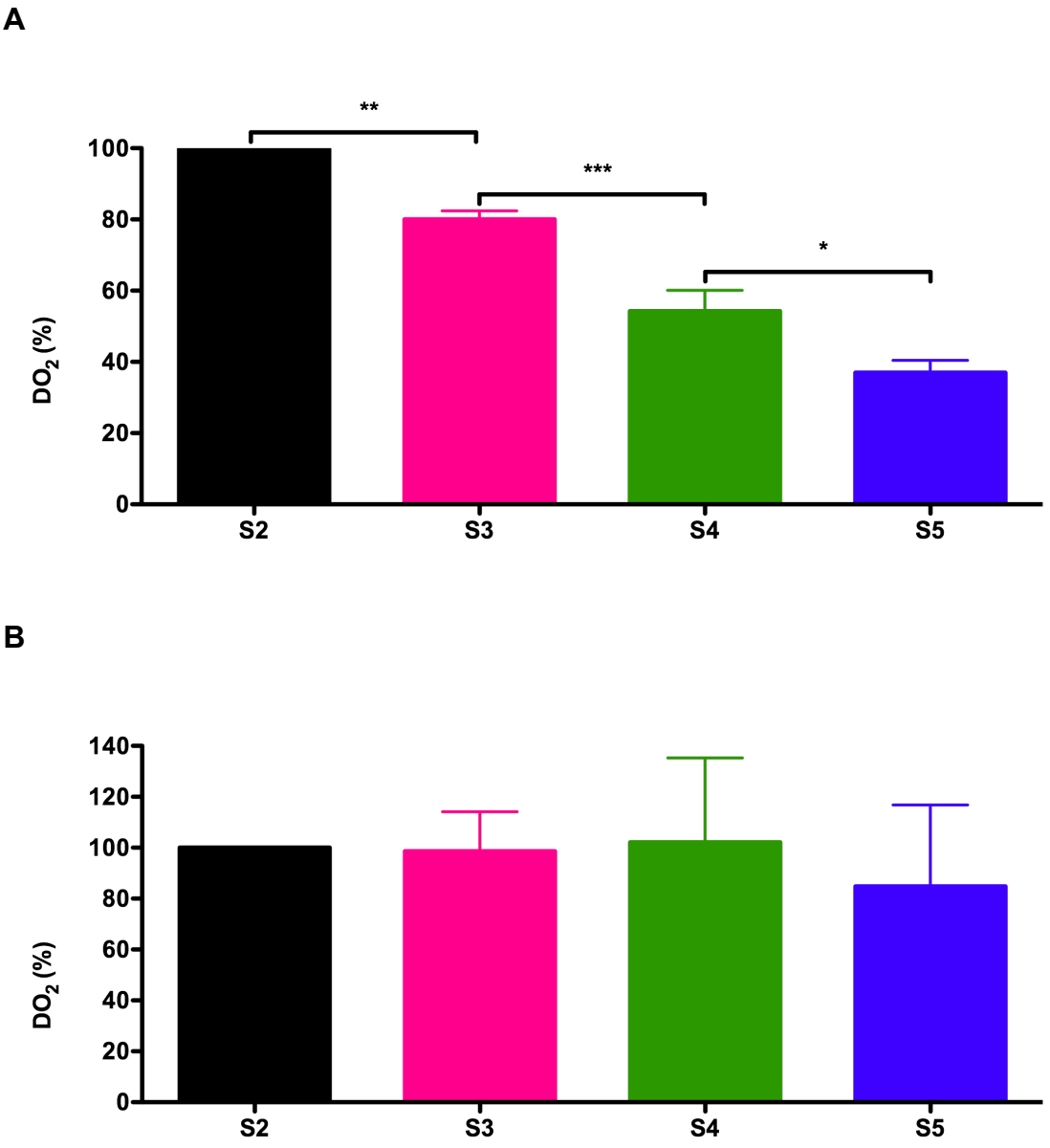
CH-(PaO_2~33)-HD tension fatigue profiles in the EDL muscle at a supra-maximal stimulation of 15Hz, 0.15ms.

Appendix 6: CH-(PaO_2~33)-HD group, the effect of oxygen delivery on muscle fatigue



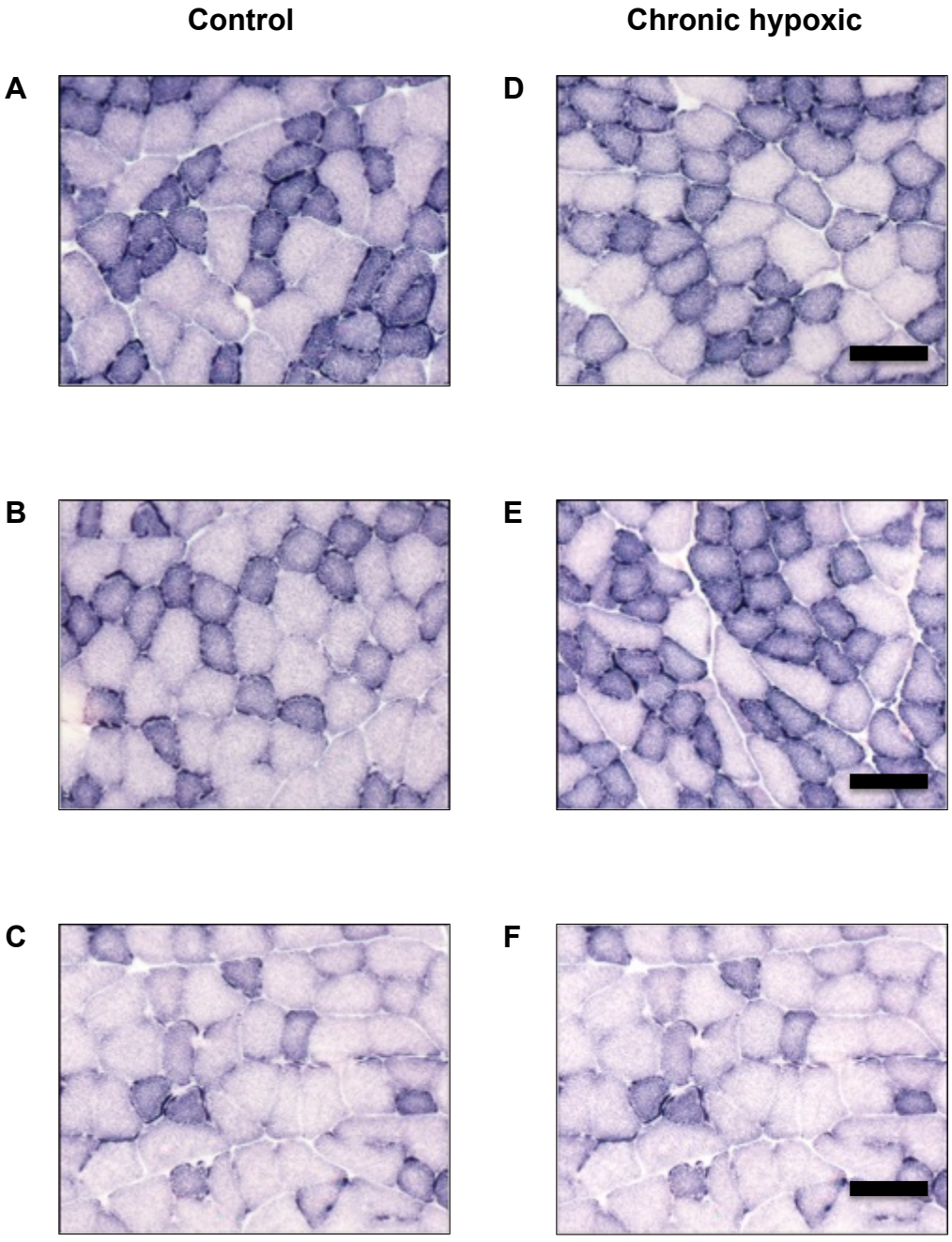
The effect of decreasing oxygen delivery on muscle fatiguability. *Mean fatigue data for individual stimulations is shown against mean D corresponding to the last fifteen seconds of stimulation for CH-(PaO_2~3 animals (triangles, blue). Error bars show ± S.E.M., n=4.*

Appendix 7: Oxygen delivery normalised by baseline

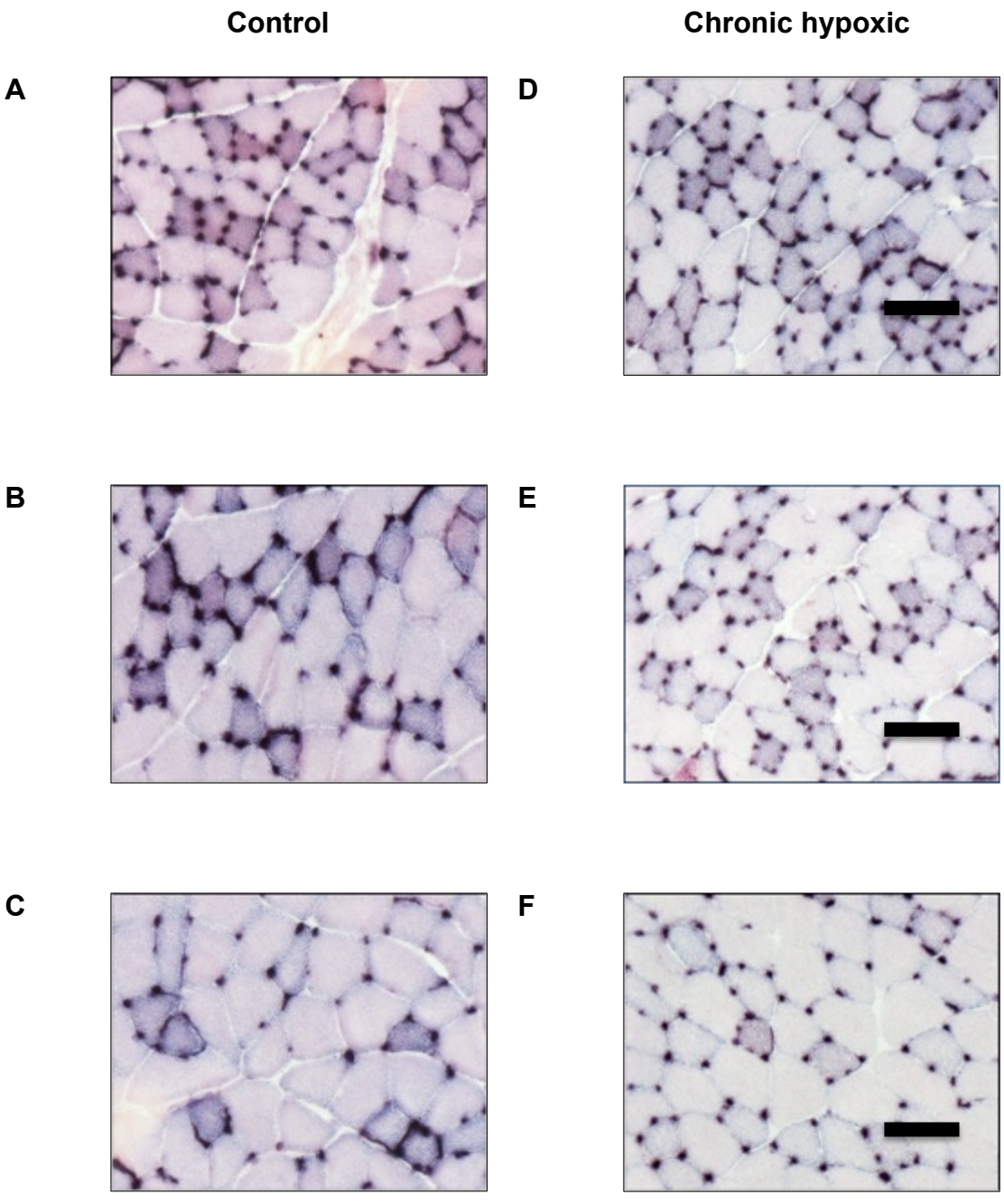


The effect of haemodilution on DO_2
Mean data normalised by baseline DO_2 for N-HD (A) and CH-(PaO_2~47)-HD (B). Error bars show ± S.E.M., n=4-5, repeated measures ANOVA.

Appendix 8: Succinic dehydrogenase and alkaline phosphatase staining



Mitochondria distribution indicative of relative oxidative capacity in the EDL muscle post 3 week acclimation to 12% O_2.
Bright field photomicrographs representative of transverse mid sections of the medial, central and lateral regions of the EDL, of control (A-C) and CH acclimated rats (D-F) at 200x magnification, stained with succinic dehydrogenase (SDH). Scale bar: 100μm D-F).



Capillary identification in the EDL muscle post 3 week acclimation to 12% O_2.

Bright field photomicrographs representative of transverse mid sections of the medial, central and lateral regions of the EDL, of control (A-C) and CH acclimated rats (D-F) at 200x magnification, stained with alkaline phosphatase. Scale bar: 100μm (D-F).