MOLECULAR AND PHYSIOLOGICAL BASIS FOR COLD-INDUCED ANGIOGENESIS IN FISHES

by

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Abstract

Angiogenesis- growth of capillaries from a pre-existing network- can be induced in cold-acclimated fishes, where torpor onset and increased oxygen availability, suggests that the primary stimulus for angiogenesis is not metabolic. It was hypothesised that cold-induced angiogenesis was due to increased blood viscosity, therefore endothelial mechanotransduction of high shear stress, and that warm-induced capillary rarefaction was due to reduced shear stress. The reversal of elevated shear stress by vasoconstriction using the nitric oxide synthase inhibitor, L-NNA, and cyclooxygenase inhibitor, indomethacin had different effects. L-NNA administration hinted towards capillary regression at low temperatures but there was a trend towards increased capillarity at intermediate and high temperatures, whereas indomethacin had no effect. Neither warm acclimation nor vasodilatation using the α-adrenoceptor antagonist, prazosin, had an effect on capillarity. Investigation of the effects of NO on heart rate at high temperature showed NO may reduce heart rate at high temperature. However, this does not explain the trend towards an increase in capillarity with L-NNA at high temperature. Evidence is presented for the absence of eNOS in fishes suggesting either nNOS-derived NO or prostanoids are responsible for vascular tone. Microarray analyses were used to identify signalling pathways that would explain the discrepancies, but proved inadequate to reveal significant endothelial responses to cold acclimation.

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Dedication

For Abba, who encouraged me to learn.

1940-2001

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Chapter 1 General Introduction

1.1 Introduction

This thesis describes a series of studies carried out to further our understanding of the molecular and physiological adaptations to low and high environmental temperature, with particular reference to the relationship between cardiac function and the skeletal muscle microvasculature in fishes. The primary role of the microvasculature is widely accepted as being nutrient supply and metabolic waste removal. As such, in skeletal muscle, changes in metabolic demand induce changes in the microvascular supply, to maintain skeletal muscle function. However, as the primarily role of skeletal muscle fibres is to contract and relax to allow movement, and all vessels are exposed to continuous, changing physical forces in the fluid microenvironment, the microvasculature is also heavily influenced by mechanical stressors. The main purpose of this thesis is therefore, to explore the possibility of controlling angiogenesis using mechanical stimuli and to uncouple mechanical and metabolic stimuli for angiogenesis.

1.2 Vertebrate cardiovascular physiology

The primary of function of the vertebrate cardiovascular system (CVS) is convective transport of oxygen, and substrates derived from food (e.g glucose, amino acids, and water), and the distribution of regulatory hormones (Levick, 2003). In addition, it also has the role of

accommodating an adequate removal of waste substances such as carbon dioxide from aerobic metabolism, lactate from anaerobic metabolism, and nitrogenous compounds from protein turnover. In mammals, the CVS is used in temperature regulation; it transports heat from the organs to the skin where it is dissipated. In fishes, however, gill breathing and the single-pass circulation (described below) drives the body temperature to closely follow the environmental temperature.

1.2.1 Structure of the mammalian cardiovascular system

The mammalian heart consists of two ventricles that pump blood out of the heart and two atria that fill the ventricles, within a pericardial sac. The right ventricle pumps deoxygenated blood at low pressure through the pulmonary artery to the lungs and oxygenated blood returns from the lungs through the four pulmonary veins. The left ventricle pumps blood at high pressure to the rest of the body through the aorta, and deoxygenated blood from the body is returned to the heart through the vena cavae. The aorta branches into several arteries including the coronary artery, which provides oxygenated blood to cardiac tissue, in a self-perfusing system (Levick, 2003).

In response to elevated metabolic demand, cardiac output (CO) can be increased without an increase in heart rate (HR), by increasing the stroke volume (SV) (CO = HR X SV) because at rest, SV is only 2/3 of the end diastolic volume. SV depends on a) diastolic stretch (Starling's Law)

concerning myofilament overlap b) contractility which is regulated by sympathetic fibres and hormones (*via* intracellular Ca^{2+} handling) and c) arterial pressure, developed in response to peripheral vascular resistance (which opposes ejection). The distribution of blood to peripheral tissues is generally proportional to the metabolic rate of the tissue, and is altered by dilatation or constriction of the supplying arteries and local arterioles in the tissue in response to a change in metabolic demand, to affect changes in flow through the capillaries, the site of terminal diffusive exchange (Levick, 2003).

1.2.1.1 Blood vessel structure

The mammalian vasculature is composed of serially branching and connecting arteries, arterioles, capillaries, venules and veins. With exception of the capillaries, blood vessels are composed of three layers, the intima (flattened endothelial cells on a layer of connective tissue), the media (smooth muscle cells on a matrix of elastin and collagen fibres), and the adventitia (connective tissue that connects the blood vessel to the surrounding tissue)(Junqueira *et al.*, 2003; Mellander & Johansson, 1968). In large vessels, the adventitia has its own blood supply, the *vaso vasorum* (Levick, 2003). The elastic arteries such as the aorta have high elastin content in the tunica media allowing distension of these arteries on ejection of blood from the heart, smoothing the pulsatile flow of the blood and moderating the pressure waveform. Smaller arteries have high smooth muscle content and they constrict and dilate to alter blood flow to organs. The terminal arteries and pre-capillary arterioles have a small lumen with a low number

of branching vessels and therefore have a high resistance to flow, enabling control of capillary perfusion (Levick, 2003). The capillaries, although they have small lumen, have a low cumulative resistance as the number of capillaries in parallel is high and this offers a large total cross-sectional area. Capillary walls are thin (approx. 0.5µm thick) (Junqueira *et al.*, 2003), which aids diffusive exchange of gases and metabolites. Some exchange also takes place in postcapillary venules (Mellander & Johansson, 1968; Wiederhielm, 1966) and to a lesser extent, pre-capillary arterioles. Veins and venules have thinner walls than arteries and the smooth muscle content of the media is lower (Junqueira *et al.*, 2003). Veins have lower resistance than arteries because of greater compliance, the lumen are larger, and there are more veins in parallel. Blood flow through the veins is aided by muscle contraction and retrograde flow is prevented by venular valves (Vander *et al.*, 1994).

1.2.1.2 Regulation of vascular tone

As resistance to flow is affected by the radius of the vessel, regulation of vascular tone leads to regulation of tissue perfusion. Vascular tone is regulated by sympathetic nerves, circulating hormones, locally produced substances, and the Bayliss myogenic response to altered transmural pressure (a decrease in vessel diameter through arterial contraction (Bayliss, 1902) through stretch-induced depolarisation leading to opening of voltage-dependent calcium channels). However, the extrinsic responses (nervous and hormonal) generally override the intrinsic myogenic responses (Johnson, 1989). Locally produced vasodilators include NO, PGI_2,

bradykinin and adenosine and vasoconstrictors include 5-HT, TXA_2 and leukotrienes (Vanhoutte, 1988). The responses observed may be tissue specific e.g. hypoxia causes vasodilatation in the systemic circulation, but vasoconstriction in pulmonary vessels (Biaggioni *et al.*, 1989; Furchgott & Vanhoutte, 1989; Thomas & Marshall, 1993). Blood flow itself causes arterial dilatation (Khayutin *et al.*, 1986) by stimulating the release of endothelium-derived relaxing factors (Furchgott, 1993); see below.

Nervous control of blood flow is primarily mediated by sympathetic activity as parasympathetic fibres have both sparse and selective distribution. Noradrenaline is released from sympathetic fibres, the terminals of which are usually located in varicosities at the outer media (although they may reach the inner media in the veins) of small arteries but not usually the smallest arterioles (which are controlled by local mediators). Similarly, venules are sparsely innervated whereas veins are more highly innervated although not as highly as arteries, and skeletal muscle veins in particular have little sympathetic innervation (Marshall, 1982).

1.2.1.3 Flow in blood vessels

During laminar flow through a cylindrical tube (like a blood vessel), Newtonian fluids, including plasma, behaves like a set of thin concentric shells (laminae) that slide past each other. The lamina in contact with the vessel wall has zero velocity due to molecular cohesive forces known as the zero slip condition. The velocity of the laminae increases as they reach the centre of the

vessel so the plasma at the centre of the vessel is at the maximum velocity. Therefore in blood vessels, red blood cells have a higher velocity in the centre than the sides. The sliding motion of one lamina on another is the shear and the tug is the shear stress (Levick, 2003). The shear stress is dependent on the shear rate (i.e. rate of sliding) and the viscosity of the fluid (shear stress=viscosity x shear rate). While the development of laminar flow is not possible in small vessels like capillaries, the viscous drag does influence the endothelial phenotype (Takahashi *et al.*, 1997).

1.2.2 Structure of the teleost cardiovascular system

The circulatory systems of different teleost species have similarities and differences to each other, and with mammalian hearts (Satchell, 1991). Therefore, for the purpose of this thesis, comparisons made will primarily be between the rainbow trout, common carp and the human circulatory system. In cases where this information is unavailable for trout and carp, references will be made to similar teleosts.

The teleost heart has four chambers (sinus venosus, atrium, ventricle, bulbus arteriosus), in series, within a pericardial sac (Fig 1.1). Venous blood enters the heart through the sinus venosus, which has a thin wall mainly consisting of connective tissue with an inner endothelial and an outer epithelial lining. The blood passes into the atrium at low pressure, aided by mild

contractions, where the blood is passed into the ventricle through more intensive contractions. The atrium is an irregular shaped chamber with thin trabecular walls (i.e. with supporting connective tissue). The volume of the sinus venosus and the atrium are similar and slightly larger than the ventricle. The bulbus arteriosus, from which the ventral aorta arises, is composed largely of elastic connective tissue and acts to dampen the ventricular pressure wave (Fig 1.1). The wall of the bulbus arteriosus in teleosts also contains smooth muscle and has layers similar to blood vessels (i.e. adventitia, media and intima). The intima consists of squamous, cuboid or elongate cells that are not aligned in the direction of blood flow. The outermost layer, the visceral pericardium, has high collagen content.

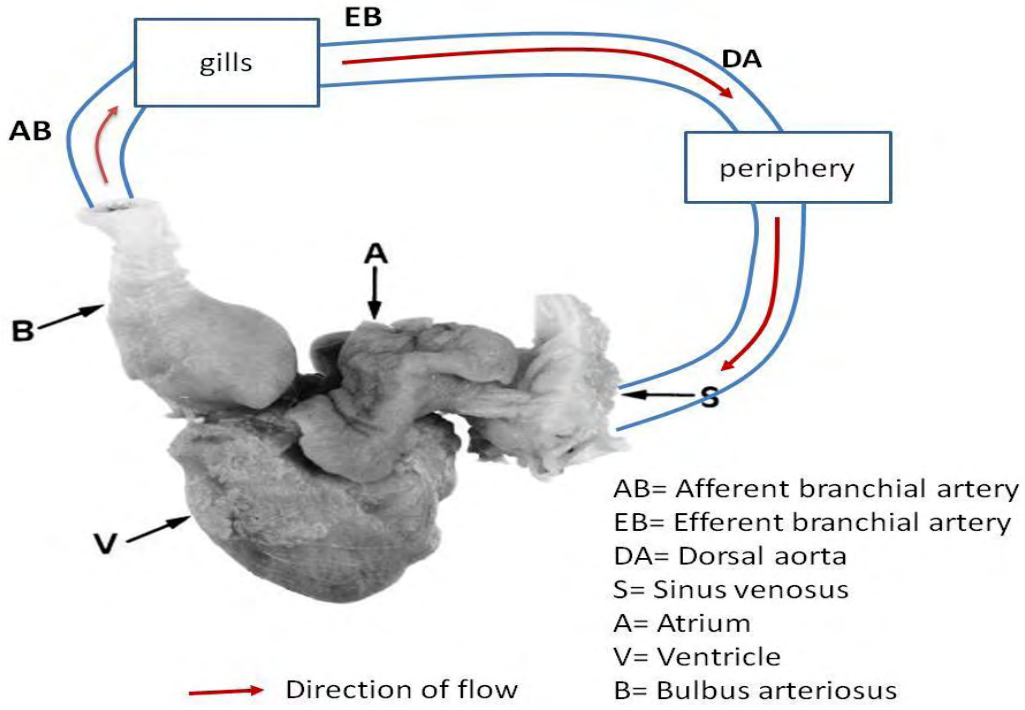


Fig 1.1 Schematic representation of the single pass circulation in teleost species. Adapted from Simões *et al* (2002)

Fish cardiomyocytes are smaller in width but longer than mammalian myocytes (Shiels & White, 2008). Cardiac growth occurs through hyperplasia rather than hypertrophy in rainbow trout, in contrast with mammalian myocardium in which hyperplastic growth occurs only during embryonic development. In addition to the limited SR, the sarcolemma also lacks the T-tubule system found in the skeletal muscle and myocardium of mammals. Instead of having a T-tubule system, the sarcolemma has flask-shaped folds called calveolae. As myoglobin is highly variable, some fish myocytes appear white due to a lack of myoglobin while others are pink. Those species that are highly active and those that are resistant to hypoxia have the highest myoglobin content in their ventricles (Evans & Claiborne, 2006; Hoar *et al.*, 1992).

Luminal O_2 is the major source of oxygen for the spongy inner myocardium (i.e. venous O_2 tension), while the coronary circulation to the compact myocardial layer where it exists reaches the ventricle across the surface of the bulbus arteriosus (supplying arterial O_2 tension). Coronary arteries supply O_2 to the compacta regions of those fish with type II ventricles (e.g. trout) Type I ventricles (like in some Antarctic fishes) contain only spongiosa which usually has no capillary supply, although intermediates exist. This may seem like an inefficient means by which to obtain oxygen supply, but the luminal blood contains a high volume of O_2 compared with the myocardial oxygen demand as the entire cardiac output passes through the heart in the single pass circulation thus fish are usually able to maintain an adequate cardiac output in moderately hypoxic (environmental) conditions (Hoar *et al.*, 1992). If hypoxia is severe, fish hearts become

bradycardic (slowing of AV conduction, with increased cholinergic activity being essential for hypoxic bradycardia in some species (Randall & Shelton, 1963). For example, in hagfish, which do not experience hypoxic bradycardia, there is no autonomic cardiac innervation (Nilsson, 1983), but in shark, hypoxic bradycardia is not dependent on cholinergic activity (Stenslokken *et al.*, 2004). Fishes such as the trout increase their stroke volume during hypoxic bradycardia because bradycardia increases diastolic filling time, thereby maintaining their cardiac output.

1.2.3 Structure of teleost blood vessels

The fish circulatory system is divided into primary and secondary circulation but fish do not have a lymphatic system. The primary circulation consists of branching arterial, capillary and venous networks. The secondary circulation arises from narrow vessels that connect with primary arteries. This secondary circulation is a low-pressure and low-haematocrit system serving a primarily nutritive rather than respiratory function to surface structures that exchange gases directly with water. The secondary circulation is most concentrated at the skin and gills (Olson, 2002b).

The primary circulation in teleosts follows the typical vertebrate pattern. The heart forces blood into the ventral aorta which leads on to the paired afferent branchial vessels, that go upward between gill clefts and rejoin into the efferent branchials to form the dorsal aorta, which exists as an anterior pair of vessels and a single posterior vessel. The dorsal aorta distributes blood to

the peripheral vascular beds where exchange of metabolites, nutrients and waste products occurs (Hoar *et al*., 1992). In the case of the skeletal muscle trunk, this is delivered by a series of bilateral segmental arteries- a pair per myotome.

Fish blood vessels, by and large, structurally resemble those of mammals and the ultrastructure of arterioles in fish is similar to that of mammals (Yasutake & Wales, 1983). However, unlike mammals, in fishes smooth muscle does not appear in venules until those exceeding 300µm in diameter are reached (Soldatov, 2006). Teleost endothelial cells are thinner than their mammalian counterparts, and their cytoplasm is poorly developed, with very few organelles (Boutilier, 1990). Cells lining the capillary are tightly packed but there are fenestrations (~60nm diameter), which are more frequent in the venous end than the arterial end (Soldatov, 2006). The diameter and length of fish capillaries varies greatly and values between 4-73 µm and 200-1300 µm have been reported (Hoar *et al.*, 1992; Soldatov, 2006), probably because of fibre type-dependent and species-dependent differences in capillary structure.

1.2.4 Cardiac control in teleosts

1.2.4.1 Excitation-contraction coupling

Like most vertebrates, in teleosts, cardiac excitation is set by the pacemaker cells which lie in the sinoatrial (SA) node (sinus venosus in lower vertebrates) (Robb, 1965), which spreads rapidly over the atrium and ventricle, with some delay at the SA and atrioventricular (AV) junctions. The spread of excitation is rapid, despite there being little evidence for fast conducting fibres in any species except the zebrafish (Sedmera *et al.*, 2003). The teleost ECG is very similar to the mammalian ECG, it consists of a P wave (atrial depolarisation), QRS complex (ventricular depolarisation), and a T-wave (ventricular repolarisation). The AV delay can be seen in the ECG as a gap between the P wave and QRS complex.

Excitation-contraction coupling is caused by the elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Vornanen *et al.*, 2002). In summary, membrane depolarisation brought about the transient action potential (which increases the membrane potential), causes Ca^{2+} entry into the myocyte through L-type voltage-dependent Ca^{2+} channels and a reversed $Na^{+}Ca^{2+}$- exchanger (NCX). Elevation of $[Ca^{2+}]_i$ causes Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum (SR), through the ryanodine receptor (RyR). In most fish myocytes, except tuna, the extracellular Ca^{2+}-cycling seems to play a more major role in Ca^{2+} influx than Ca^{2+} from the SR, especially in

the ventricle (Vornanen *et al.*, 2002). The Ca^{2+} then activates the myofibrils for contraction. The myocytes are relaxed when $[Ca^{2+}]_i$ is reduced by its efflux through the NCX, and transportation in to the SR through the smooth endoplasmic reticulum calcium transporter (SERCA), and the resting negative membrane potential is restored by the substitution of the Na^+ with K^+ through a Na^+/K^+ ATPase and the efflux of K^+ ions through an inward K rectifier channel (K_{ir}), (Vornanen *et al.*, 2002).

Although heart rate in the fish is set by the intrinsic rhythm of the sinoatrial pacemaker, it can be modulated by at least four extrinsic mechanisms a) the stretch of the pacemaker cells that occurs with increased cardiac filling b) cholinergic (parasympathetic, vagal) fibre activity c) adrenergic (sympathetic) fibre activity and d) systemic or local hormone release (Hoar *et al.*, 1992). Cholinergic and adrenergic inputs have the greatest influence on heart rate, and the influences of these at different temperatures were investigated in the current study. Whereas in mammals an increase in ventricular contractility is primarily due to sympathetic stimulation, in fishes such as tuna, parasympathetic activity is thought have a greater role in the control of heart rate ((Hoar *et al.*, 1992; Keen *et al.*, 1995). In this section, the mechanisms of sympathetic and parasympathetic activity are described; the effects of temperature on these influences are described later (section 1.3.6)

1.2.4.2 Adrenergic control of heart rate

Adrenergic control occurs through the catecholamines adrenaline (AD) and noradrenaline (NAD), which are synthesised and stored in neurons, and AD only is synthesised in chromaffin cells, which is located in the head kidney and is the piscine equivalent to the mammalian adrenal medulla (Nilsson, 1983). The predominant catecholamine in teleosts is AD, which binds to α and β-adrenoceptors (Ahlquist, 1948). In the trout heart, adrenergic activity occurs through β_2-adrenoceptors (Ask *et al.*, 1980), which preferentially binds AD over NAD in mammals (Rang & Dale, 2007). In addition, the existing literature suggests that α and β_1-adrenoceptors do not exist on trout myocytes (Ask *et al.*, 1980; Gamperl *et al.*, 1994), but are present in the coronary circulation (Agnisola *et al.*, 1996). β_2-adrenoceptors, which are G_s-protein coupled receptors, activate adenylyl cyclase, which is involved in the synthesis of cyclic adenosine monophosphate (cAMP), which in turn activates protein kinase (PKA). PKA phosphorylates the L-type Ca^{2+} channels and RyR described above, thereby causing an increase in $[Ca^{2+}]_i$. AD also modifies pacemaker cell activity directly by opening Ca^{2+} channels in pacemaker cells, causing an increase in the decay rate of the pacemaker potential (Hutter & Trautwein, 1956; Levick, 2003).

1.2.4.3 Cholinergic control of heart rate

Parasympathetic innervation exists in all fish hearts, with the sinoatrial region being most highly innervated in teleosts. The muscle fibres of the atrium and the atrioventricular region are

moderately and the ventricles sparsely innervated. Parasympathetic activity causes bradycardia through the neurotransmitter, acetylcholine (ACh). ACh binds to and activates the muscarinic (M_2) receptors, which are G_i-protein coupled receptors (Dhein *et al.*, 2001). G_i proteins inactivate adenylyl cyclase causing a decrease in cAMP and consequently hyperpolarisation of cardiac pacemaker cells in the guinea pig (Leiber *et al.*, 1990), increasing the time taken for threshold potential and therefore an action potential to be reached (Hutter & Trautwein, 1956).

1.2.4.4 Heart rate variability

In a healthy heart, the heart rate itself varies from beat to beat (known as the heart rate variability (HRV)), as a result of the constant but subtle changes in sympathovagal balance and respiration. When all extrinsic mediators of HR are removed, HRV is lost (Devera & Priede, 1991; Kitney & Rompelman, 1980). The R-R interval (the time between the peak of one QRS complex and the next), is used to measure HRV, and is displayed as a tachogram. This time series can be used for geometric analysis of various parameters of the time series such the maximum and minimum HR, the average (mean, median, mode) HR, the standard deviation (SD) of R-R, and SD of successive R-R intervals. With these data, however, the roles of autonomic tonus and respiration under different conditions cannot be isolated.

With power spectral analysis in the frequency domains of R-R intervals, it is possible to isolate the effects of sympathetic and parasympathetic activity and respiratory rate on the heart rate.

In mammals there are three distinct components, high (associated with respiration), mid (peripheral blood pressure) and low (thermoregulation), (Akselrod, 1981). HRV signals have been identified from teleost ECG recordings since the late 1980s (Armstrong *et al.*, 1989) and in the rainbow trout, there are two components with the low frequency component being dominant at low temperature, and the high frequency component at high temperature (De Vera & Priede, 1991). There is also evidence that the low frequency input increases with HR in humans, suggesting that the low frequency signal corresponds with high sympathetic tone, and the high frequency signal corresponds with high vagal tone (Bootsma *et al.*, 1994); this also appears to be the case in rainbow trout (Devera & Priede, 1991).

Fishes including the rainbow trout have a baroreceptor reflex and increases in peripheral resistance (Sandblom & Axelsson, 2005) stimulates the vagus, bringing about reflex bradycardia but fishes respond weakly to hypotension with tachycardia (mainly through angiotensin II-induced vasoconstriction), (Wood & Shelton, 1980). Cholinergic stimulation of the chromaffin cells induces adrenaline release in the eel (Al-Kharrat *et al.*, 1997) and rainbow trout (Fritsche *et al.*, 1993), a possible feedback mechanism to prevent hypotension induced by excessive vagal stimulation.

1.2.4.5 Control of vascular tone

Vascular tone in teleosts is controlled locally by metabolites and paracrine signalling mediators, and remotely by nervous and humoral control (Evans & Claiborne, 2006). In this section, the roles of the local mediators prostaglandins (PGs) and nitric oxide (NO), and the neuronal and humoral mediator, AD, will be discussed. In fish, cholinergic innervation seems not to exist on blood vessels anywhere except the gills and chromaffin cells (Olson, 2002a), which are not the primary subjects of the current study, so will not be discussed in this section. Trout vessels also have non-adrenergic non-cholinergic (NANC) neurotransmitters (neuropeptides such as substance P and serotonin) although little is known about the role of NANCs in fishes (Johnsson *et al.*, 2001).

1.2.4.6 Adrenergic control of vessels

Adrenergic stimulation of vessels occurs through circulating AD originating from chromaffin cells, the concentration of which is usually within the range of 1-10nM but can increase 10-100 times with severe stress (Nilsson, 1983; Reid *et al.*, 1998), and through sympathetic nerve fibres. Control of arteriolar tone occurs primarily through α-adrenoceptors (Nilsson, 1994; Stevens & Randall, 1967), which are G-protein coupled receptors that are located on SMCS lining the arteriole, as well as pre-synaptic sympathetic nerve terminals. Post-synaptic α_1-adrenoceptors and α_2-adrenoceptors are G_q-coupled, so activation of these receptors cause an increase in the enzyme phospholipase C (PLC), which metabolises phosphatidylinositol 4,5-bisphosphate (PIP_2)

into inositol trisphosphate (IP_3) and diacylglycerol (DAG). DAG causes Ca^{2+} influx into the SMC from the extracellular medium and from the SR, leading to SMC contraction, therefore vessel contraction. Pre-synaptic α_2-adrenoceptors are coupled to G_i, an inhibitory G-protein and inhibits further adrenaline release from neurons (Rang & Dale, 2007). Therefore, to avoid the elevation of adrenaline release from sympathetic neurons that would have occurred with non-selective α-adrenoceptor antagonism, in this study, pharmacological blockade of adrenergic constriction was carried out using the selective α_1-adrenoceptor antagonist, prazosin. There is some evidence for β_2-mediated vasodilatation (Wood, 1976; Wood & Shelton, 1980; Wood & Shelton, 1975), but as this is not the predominant method of vasodilatation, this pathway was not pharmacologically modulated in the current study.

1.2.4.7 Prostaglandin-mediated control of vascular tone

Prostaglandins are synthesised by cyclooxygenase enzymes from arachidonic acid (Hamberg & Samuelsson, 1973), and their synthesis is inhibited by the non-steroidal anti-inflammatory aspirin-like drugs (Vane, 1971), such as indomethacin, which is used in this study. The prostaglandins, PGE_2 and PGI_2 are believed to be the primary endothelium-derived relaxing factors (EDRFs) in fishes (Evans & Claiborne, 2006; Evans & Gunderson, 1998). PGE_2 binds to EP_2 and PGI_2 to IP receptors, which are G_s-protein coupled receptors. Activation of these receptors causes vasodilatation by inhibiting contraction by increasing cAMP production through AC activation (Harmar *et al.*, 2009), as described above. However, unlike in the cardiomyocytes, in

SMCs, PKA phosphorylation by cAMP causes relaxation as PKA binds to the myosin light chain kinases (MLCKs) that cause SMC contraction, to inhibit their activity.

1.2.4.8 Nitric oxide-mediated control of vascular tone

NO is synthesised by oxidation of the amino acid, L-arginine (Palmer *et al.*, 1988; Schmidt *et al.*, 1988), by the enzyme nitric oxide synthase (NOS) (Palmer & Moncada, 1989), in the presence of flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (BH_4), reduced nicotine adenine dinucleotide phosphate (NADPH) and oxygen. This results in the formation of L-citrulline and NO (Palmer & Moncada, 1989).

$$\text{L-Arg} + \text{NADPH} + H^+ + 2O_2 \xrightarrow{\text{NOS}} \text{L-citrulline} + NADP^+ + 2H_2O + \mathbf{NO}$$

Nitric oxide is a potent vasodilator in mammals (Hutchinson *et al.*, 1987) that causes vasodilatation by stimulating cyclic GMP production, by activating guanylate cyclase (Arnold *et al.*, 1977). cGMP phosphorylates protein kinase G (PKG), in vascular smooth muscle wall, which inhibits MLCK activity (Gruetter *et al.*, 1981). The source of NO in skeletal muscle may be muscle fibres, endothelium or nerve fibres, although NO acting on SMCs are mostly endothelium-derived in mammals (Furchgott & Vanhoutte, 1989).

There are three NOS isoforms that are encoded by different genes. The Ca^{2+}-dependent endothelial NOS (eNOS) and neuronal NOS (nNOS) are typically thought to be constitutively present. The Ca^{2+}-independent inducible NOS (iNOS) is induced under inflammatory conditions. eNOS, the isoform of NOS thought to be involved in vasodilatation, is primarily located on the caveolae, flask-shaped invaginations of the endothelial plasma membrane. Caveolae are coated with caveolin, proteins that interact with eNOS by terminal NH_2-residues to inhibit NOS activity when unstimulated. eNOS activity is induced by the phosphorylation of one of its serine residues or by mechanical uncoupling from the caveolin to which they are bound (Garcia-Cardena *et al.*, 1996). These processes may be ligand-operated by substances such as bradykinin, acetylcholine and adenosine (Fulton *et al.*, 2008) or induced by mechanical triggers such as elevated shear stress (Ohno *et al.*, 1993), caused by increased blood flow or viscosity (shear stress = viscosity x shear rate). nNOS, the isoform named so because it was originally identified on neurons, also exists on human muscle fibres and endothelial cells (Bachetti *et al.*, 2004) so may exist on the endothelial cells of other species.

NO, the primary EDRF in mammals (Palmer *et al.*, 1987), is clearly a vasodilator in rainbow trout and most other teleosts, as exogenous NO or NO-donors bring about vasodilatation (Kagstrom & Holmgren, 1997; Olson *et al.*, 1997) but there is no direct evidence for the existence of an eNOS gene in trout or carp although the nucleotide sequences for iNOS (Laing *et al.*, 1999) and nNOS (Poon *et al.*, 2003) have been identified in other fishes. Furthermore, there is some

suggestion that vascular NO in fishes (Jennings *et al.*, 2008) and toads (Jennings & Donald, 2010) are nNOS-derived. Indirect evidence for the presence of eNOS include the finding that hypoxia-induced NO production in the cardinal vein (McNeill & Perry, 2005) and L-arginine-induced vasodilatation of the conduit artery (Mustafa & Agnisola, 1998) in rainbow trout are inhibited by endothelial cell removal by saponin and CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), respectively.

In the current study, the irreversible competitive NOS inhibitor, NG-nitro-L-arginine (L-NNA), is used to block NO synthesis, to identify the role of NO in cold-induced angiogenesis. L-NNA is selective for eNOS and nNOS over iNOS, although it cannot distinguish between eNOS and nNOS (Furfine *et al.*, 1993). This is ideal, as at the current time, we are unsure which NOS isoform is responsible for altering vascular tone in trout. As we are investigating physiological and not pathological angiogenesis in this study, the role of iNOS was not investigated.

1.3 Animal life at low temperature

1.3.1 Ectothermy and endothermy

Responses to temperature can be divided into two broad categories, ectothermy and endothermy. Ectotherms are those organisms that conform to their environmental temperature (Cowles, 1962); endotherms maintain a core body temperature and respond to variations in

temperature by their behaviour e.g hibernation or thermogenesis. As fishes breathe through their gills- an effective gas exchange organ which is also a good heat exchanger by way of its large surface area and high blood flow- their body temperature is obliged to follow environmental temperature, and cellular temperatures are very close to water temperature.

1.3.2 Stenothermal versus eurythermal fishes

Stenothermal fish species such as the Antarctic and Arctic (polar) fishes are those fishes that inhabit a relatively thermally stable marine environment and are capable of surviving only a narrow range of temperatures. Antarctic icefishes, for example, are thought to display some features of cold adaptation such as increased mitochondrial and capillary density in skeletal and cardiac muscle to reduce oxygen diffusion distance from capillary to mitochondria (Archer & Johnston, 1991; Sidell & O'Brien, 2006) and loss of genetic information for the production of haemoglobin (Ruud, 1954; Sidell & O'Brien, 2006), a possible mechanism for reducing blood viscosity at chronically low temperatures. Cold adaptation is thought to be that which occurs at the species level due to selection pressures when the species either moves to a cold region or the temperature decreases over longer time periods (years to centuries). Low temperature also induces Metabolic Cold Adaptation, in particular, originally described in the Antarctic fish, *T.Bernacchii,* where it was thought that growth efficiency was higher in these species (Wohlschlag, 1960). However, more recent studies suggest that metabolic cold adaptation most likely does not exist. For example, the validity of the theory that there is an increased standard

metabolic rate in cold-adapted animals has been questioned because oxygen consumption is used as an index of metabolism, and oxygen consumption measurements can vary enormously within one species, depending on the time of day measurements are taken and whether the measurements are taken immediately post-handling of the animals (Steffensen, 2002). Further, it has been demonstrated that Antarctic molluscs do not have an elevated standard metabolic rate compared with temperate species (Peck & Conway, 2000). Additionally, there is some evidence for temperature-independent dormancy and restricted growth of Antarctic fishes in winter (Campbell *et al.*, 2008), suggesting that temperature is not directly the stimulus for winter dormancy and reduced metabolism, and that indirect effects of temperature such as changes in phytoplankton and zooplankton availability (Beaugrand & Reid, 2003) and seasonal cues such as photoperiodicity play a greater role.

Cold acclimatisation is the combined physiological, biochemical, transcriptional and behavioural responses that occur with acute exposure to low environmental temperature, to enable the animal to survive and function. Cold acclimatisation in ectotherms and endotherms differ from one another: the temperature of the organs and tissues of ectotherms change with the environment and physiological adaptations reflect this, whereas cold-acclimatisation in endotherms involves defending and maintaining the core body temperature (Davenport, 1992). Cold-acclimatisation is particularly important in eurythermal species of fish and acclimatory responses to rainbow trout have been studied extensively. Cold acclimation refers to

acclimatisation under laboratory conditions where only one variable (temperature) is altered. Cold acclimatisation and acclimation induce various and profound changes in the skeletal muscle ultrastructure in fishes (see below).

As temperate eurythermal fishes, including common carp and rainbow trout, experience seasonal cues to changes in temperature and successfully acclimatise to their environment, they are ideal organisms for the study of temperature acclimation under laboratory conditions. Cold-adapted fishes such as the Antarctic fishes are better for investigating phylogenetic responses to temperature as the range of temperatures Antarctic fishes are tolerant to is smaller than temperate fishes. In addition, their upper thermal limit is low so reduced blood viscosity at high temperatures cannot be investigated in these species.

1.3.3 Temperature kinetics

It is well known that reduced temperature leads to reduced random movement of molecules owing to lower average kinetic energy of all molecules exposed to that temperature. As such, the rate of activity of these molecules is decreased. This affects processes commonplace in biological systems such as diffusion, enzyme activity, and overall metabolism. The Q_{10} relationship, Van't Hoff (1884) provides an empirical formula describing the temperature dependence of a reaction rate constant, and hence rate of a chemical reaction. In biological

systems this is often ~2. Alternatively, the Arrhenius relationship is used to describe the relationship between reaction rates and temperature.

Van't Hoff rule: $\mathbf{Q_{10} = V_{t+10} / V_t}$, where V_t and V_{t+10} are the rates at temperatures t and $t + 10$.

Arrhenius relationship: $\boldsymbol{v = A.exp(-\mu/RT)}$, where v is reaction rate, R and A are constants, μ is the Arrhenius activation energy, and T is absolute temperature.

Although useful as an estimate, neither relationship provides a complete picture of the responses *in vivo*, because both these idealised mathematical models assume that the only variable is temperature, whereas enzyme activity is also influenced by pH which varies inversely with temperature as described by the imidazole alphastat hypothesis (Burton, 2002), and the physiological outcome of any given system is a cumulative effect of many individual factors that may themselves be influenced by temperature. For example, the rate of oxygen utilisation depends on diffusion rates, cardiac output, and mitochondrial content. Other features of acclimation to low environmental temperature include increased mitochondrial content in some fishes (Egginton & Sidell, 1989; Johnston, 1982), and by changes in enzyme activity, for example the increase in maximal pyruvate oxidation seen in mitochondria from cold-acclimated sculpin muscle (Guderley & Johnston, 1996). Physiological responses to temperature can broadly

manifest in one of three ways: over-compensation, perfect compensation, or no compensation (Precht, 1958).

1.3.4 Temperature and metabolism

Carbohydrates, lipids and proteins are the major sources from which energy is derived by metabolism in animals. Metabolism of these energy-rich molecules ultimately results in acetyl CoA formation which feeds into the citric acid cycle. Glucose metabolism (glycolysis) is the most dominant metabolic pathway. The contributions of fats and proteins for energy provision are dependent on the availability of glucose and vary with factors such as hypoxia and temperature. In order that energy production meets the requirements of the cell or tissue, the metabolism pathways are tightly coordinated. Signals for control include substrate availability, product inhibition, and allosteric inhibitors and activators of individual stages (Champe & Harvey, 1994). Cold acclimation alters the substrate availability in fishes, for example it evokes an increase in glycogen in crucian carp (Johnston & Maitland, 1980) and lipid stores in striped bass (Egginton & Sidell, 1986). Increased lipid content in muscle cells also improves oxygen delivery as oxygen is more soluble in non-polar lipids than in cytoplasm which has a high water content (Hoofd & Egginton, 1997).

On exposure to low environmental temperature, some ectotherms, including fishes, go into torpor, thus reducing metabolic rate (total energy expenditure per unit time). Overall metabolic

rate is attenuated by reducing external work, i.e. skeletal muscle contraction, thereby movement and internal work such as active transport across membranes for example in cardiac contraction (Champe *et al.*, 2005). Oxygen consumption is a commonly used index of metabolic rate and the effect of temperature on basal or standard metabolic rate can be estimated using this measurement. The basal metabolic rate can be measured when food is withdrawn and fish are not reproducing or actively swimming (Schurmann & Steffensen, 1997). Clarke & Johnston (1999) demonstrated a positive correlation between temperature and resting oxygen consumption (Clarke & Johnston, 1999) in various species. Some cold adapted animals, however, have a higher resting metabolic rate at low temperatures, which is indicative of metabolic cold adaptation, although the use of oxygen consumption as measurement of metabolic rate and the methods by which oxygen consumption was measured have been disputed (see above) (Steffensen, 2002). Whether metabolic cold adaptation is a true phenomenon, the animals in this study are eurythermal animals, therefore it is likely that on cold acclimation their resting metabolic rate, and hence oxygen demand decreases.

1.3.5 Temperature and blood (plasma viscosity and rheology)

As the body temperature of teleosts closely follows the environmental temperature, the latter directly affects blood viscosity (Brill & Jones, 1994; Eckmann *et al.*, 2000). As blood is not a Newtonian fluid, blood flow through vessels is affected by those conditions determined by Poiseuille's equation, and blood shear rate, which is proportional to viscosity. Trout blood

comprises of approximately 65% plasma (Fletcher & Haedrich, 1987), which is 92% water therefore its viscosity is expected to be inversely related to temperature. Furthermore, increased rigidity of cell membranes at low temperature increases viscosity of blood further (Cossins & Bowler, 1987). In Arctic fishes such as the char (*Salvelinus alpinus*) (Graham *et al.*, 1985), blood viscosity and its shear rate and temperature-dependency is less than in temperate fishes (Graham & Fletcher, 1983). However, compared with other temperate species such as the winter flounder, the temperature and shear rate-dependency of blood viscosity in rainbow trout is low (Fletcher & Haedrich, 1987). The visocosity of whole blood in Antarctic fishes (Egginton, 1996) and rainbow trout (Fletcher & Haedrich, 1987) is more dependent on temperature than the viscosity of plasma, indicating that factors including erythrocyte deformability play a more important role in temperature-related viscosity.

1.3.6 Temperature and cardiac structure and function

The positive correlation between temperature and heart rate and cardiac output has been demonstrated in a range of vertebrates. Cardiac contractility is depressed with low temperatures in the isolated hearts of the frog (Vovereidt, 1979), turtle (Brown, 1930), cat (Knowlton & Starling, 1912) and is attributed to prolonged action potential durations. Knowlton and Starling (1912) showed that heart rate directly correlates with temperature except at either extreme of temperature, at which points the heart fails. In non-hibernators such as rats, heart rate and cardiac output decreases (Broman *et al*., 1998) with temperature. In rainbow trout,

cardiac output also decreases linearly with decreasing temperature from 62.5 to 19.8 (ml min^{-1} kg^{-1}) at 18°C vs 6°C, respectively; but the proportion of blood flow to red muscle increases at low temperature (Barron *et al.*, 1987). Whether the increase in percentage CO in red muscle reflects the increase in capillarity therefore reduced peripheral resistance, seen in cold-acclimated trout (Egginton & Cordiner, 1997) is unknown at the present time. Cold acclimation increases the heart rate at any given temperature compared with non-acclimated animals, suggesting an adaptive response to cold exposure, although the linear response to temperature is still present (Aho & Vornanen, 2001). Acutely, metabolic depression counters the reduced substrate delivery owing to reduced cardiac output. In mammals, this is temporary, as reduced blood flow to organs during hypothermia (Dudgeon *et al.*, 1980) could eventually bring about tissue necrosis, but in fishes metabolic depression is maintained for long periods suggesting a feedback mechanism that allows their survival.

Despite reduced metabolic rates at low temperature in fishes, increased myoglobin (Lurman *et al*., 2007), and capillary densities in the heart may together serve to protect the cardiac muscle from hypoxia caused by reduced diffusion rates. Ventricular mass and cardiac myocyte diameter is increased in cold-acclimated striped bass (Rodnick & Sidell, 1997) and this is probably a result of increased work carried out to propel more viscous blood. However, contrary to that observed in the skeletal muscle of some teleosts (Egginton *et al.*, 2000), total mitochondrial volume

increases proportionally to ventricular mass, maintaining mitochondrial density of cardiac muscle but not increasing it at low temperature (Rodnick & Sidell, 1997).

HR increases directly with an increase in temperature; $Q_{10} \geq 2.0$. In rainbow trout, the Q_{10} is 2.1 for both cold (6.5˚C) and mid-temperature (15˚C) acclimated fish after bilateral vagotomy, i.e removal of a bradycardic influence. The intrinsic pacemaker rate varies directly with temperature as a direct result of temperature on the permeability of pacemaker cells (Clark, 1920), but this is compensated for in cold-acclimated trout by increasing the intrinsic pacemaker activity by increasing K_{ir} expression (Haverinen & Vornanen, 2007), therefore reducing time taken for repolarisation, hence increasing frequency of depolarisation. Low temperature also attenuates the transient increase in $[Ca^{2+}]_i$ that causes myocyte contraction, although longer-term cold exposure brings about compensation by increasing SR Ca^{2+} stores (Shiels *et al.*, 2010), but does not change the quantity of the RyR receptors through which Ca^{2+} enters the cytoplasm, in trout (Birkedal *et al.*, 2009).

In addition to its direct influences on cardiac myocytes and pacemakers, temperature affects sympathetic and vagal activity on the heart. In cold-acclimated trout, β-adrenoceptor density and adrenaline sensitivity increases (Ask *et al.*, 1981; Keen *et al.*, 1993), which is in contrast to the reduced sympathetic activity seen at low temperatures in man (Jansky *et al.*, 2006). This increase in β-adrenoceptor density in trout is probably compensation for the kinetic influences

of low temperature, and is an example of how that cold acclimation occurs in ectotherms, but not in man. Atrioventricular conduction may be compromised in some fish at low temperatures, including rainbow trout at 5-7°C and can be corrected with adrenergic stimulation. Conversely, at high temperatures, cholinergic activity peaks (Devera & Priede, 1991), probably to counter the high HR caused by increased contractility of the heart. However, this temperature-dependent modulation of autonomic tonus is not encountered in crucian carp (Vornanen *et al.*, 2010) or the Antarctic dragonfish (Axelsson *et al.*, 2000). During hypoxia including at high temperatures, cholinergically stimulated bradycardia occurs in the rainbow trout (Marvin & Burton, 1973).

Overall, acute cold exposure reduces HR, but in cold-acclimated animals, compensatory increases in intrinsic contractility of the heart take place by increasing pacemaker and cardiomyocyte activity (Haverinen & Vornanen, 2007). Acute warm exposure increases HR by increasing pacemaker and cardiomyocyte activity, but beyond the maximum thermal limit of the individual animals, the deleterious effects of heat stress on proteins causes a decrease in HR.

1.3.7 Temperature and skeletal muscle vascularity

There are three distinct regions of muscle fibres in fish skeletal muscle (red-oxidative, white-glycolytic, pink- fast oxidative) (Johnston & Maitland, 1980). In fishes, like mammals, capillary density in oxidative fibres is greater than that of glycolytic muscle. This reflects the greater

demand for oxygen for oxidative phosphorylation in oxidative muscle, although there is some evidence that capillarity is more dependent on fibre size than fibre type (Ahmed *et al.*, 1997), as increased fibre size increases the inter-capillary distance therefore the oxygen diffusion distance.

Vascular growth has been recorded in cold-acclimated or acclimatised fishes including rainbow trout (Egginton & Cordiner, 1997), striped bass (Egginton & Sidell, 1989), brown trout (Day & Butler, 2005), and crucian carp (Johnston, 1982). In cold-acclimated mammals, the relationship is not as straightforward, because whether capillary growth (angiogenesis) occurs depends on the behavioural response of that species to cold exposure. Hibernators such as hamsters which do not defend core body temperature, may have limited capability of increasing capillarity when exposed to low environmental temperature. When exposed to cold, their muscle undergoes disuse atrophy, increasing the capillary density without angiogenesis (Deveci & Egginton, 2002). However, non-hibernators such as rats, which are required to accommodate low temperature, though not to the same extent as fishes, respond to cold exposure with an increase in capillary: fibre ratio, but the accompanying hypertrophy caused by increased muscle activity (shivering) decreases capillary density (Deveci & Egginton, 2002). As with rats, the hypertrophy that accompanies cold-induced angiogenesis in trout, causes a reduction in capillary density (Egginton & Cordiner, 1997).

In cold-acclimated rainbow trout, 10% of the total cardiac output goes to the red muscle and 40% goes to the white muscle (Barron *et al.*, 1987) and at higher temperatures, the cardiac output to the red muscle drops to 2% and the output to white muscle rises (Barron *et al.*, 1987). This may reflect the decreased resistance in red muscle due to angiogenesis at low temperatures and the increased resistance in red muscle owing to capillary rarefaction at higher temperatures. Given that the ratio of red to white muscle is considerably less than 1:4, the relatively higher cardiac output to the red muscle may also be a reflection of the higher basal capillarity and associated conducting vessels in red muscle compared with white muscle.

As low temperature brings about bradycardia and reduced oxygen diffusion rates in most animals, in addition to the increased metabolism required for the maintenance of core body temperature, metabolic cues will likely stimulate angiogenesis in cold-acclimated mammals. However, metabolic rate is lowered whilst oxygen dissolved in water, from which their gills extract oxygen, is higher at low temperature. As such, it seems unlikely that a metabolic demand for angiogenesis exists, so we will explore the possibility that mechanical cues stimulate angiogenesis in cold-acclimated fish.

1.4 Vascular growth and angiogenesis

Capillaries are responsive to mechanical and metabolic stimuli as they are continually exposed to changes in blood flow, and are the site of gas and nutrient exchange. Here, the ways in which

changes in mechanical (primarily blood flow and consequently shear stress) and metabolic factors (primarily oxygen demand) cause the different types of angiogenesis are discussed. It is important to state that the stimuli and the mechanisms often overlap (see Fig. 1.2).

Angiogenesis is the expansion of the microvasculature from a pre-existing capillary network (Ferguson *et al.*, 2005). Angiogenesis is usually a tightly regulated process which is important in tissue repair during wound healing, the menstrual cycle and during exercise to accommodate the increased oxygen requirement, and physical forces exerted on the capillaries by repeated muscle stretch (Hudlicka *et al.*, 1992). Unregulated angiogenesis, however, is implicated in the progression of tumour growth (Folkman, 1971) and retinopathy (Glaser *et al.*, 1980). Conversely, where angiogenesis fails to be stimulated, pathological conditions such as ischaemic heart disease cannot be resolved and permanent tissue damage may occur. The major cells involved in angiogenesis are the endothelial cells that comprise the capillary wall.

Angiogenesis occurs through sprouting, splitting or intussusception (Egginton *et al.*, 2001; Prior *et al.*, 2004). Splitting and intussusceptive angiogenesis are triggered by intraluminal stimuli while sprouting angiogenesis is initiated by extravascular stimuli. Sprouting angiogenesis, the most extensively studied form of capillary growth, requires endothelial cell proliferation and basement membrane degradation, whereas splitting angiogenesis involves little cell proliferation or basement membrane degradation (Egginton *et al.*, 2001). All three forms of

angiogenesis require prior activation of endothelial cells, which are otherwise relatively quiescent cells, requiring significant stimulation to overcome the contact inhibition of neighbouring endothelial cells (Dejana, 2004).

1.4.1 Mechanical angiogenesis

It has long been established that mechanical forces such as increased blood flow velocity and tissue expansion contributes to vascular growth (Ziada *et al.*, 1984), although the mechanotransduction that leads to angiogenesis, and whether mechanically-induced angiogenesis can occur in the absence of a metabolic demand, is not well understood. In skeletal muscle, the triggers for mechanotransduction such as increased blood flow-related shear stress, and skeletal muscle growth, disrupt the endothelium intraluminally and abluminally. Exercise and increased muscle activity also cause angiogenesis, and this may be a combination of influences of mechanical and metabolic stress (Brown *et al.*, 1976). The primary intraluminal mechanical stress of interest here is elevated shear stress on the endothelium; the primary abluminal mechanical influence of interest is stretch due to fibre hypertrophy.

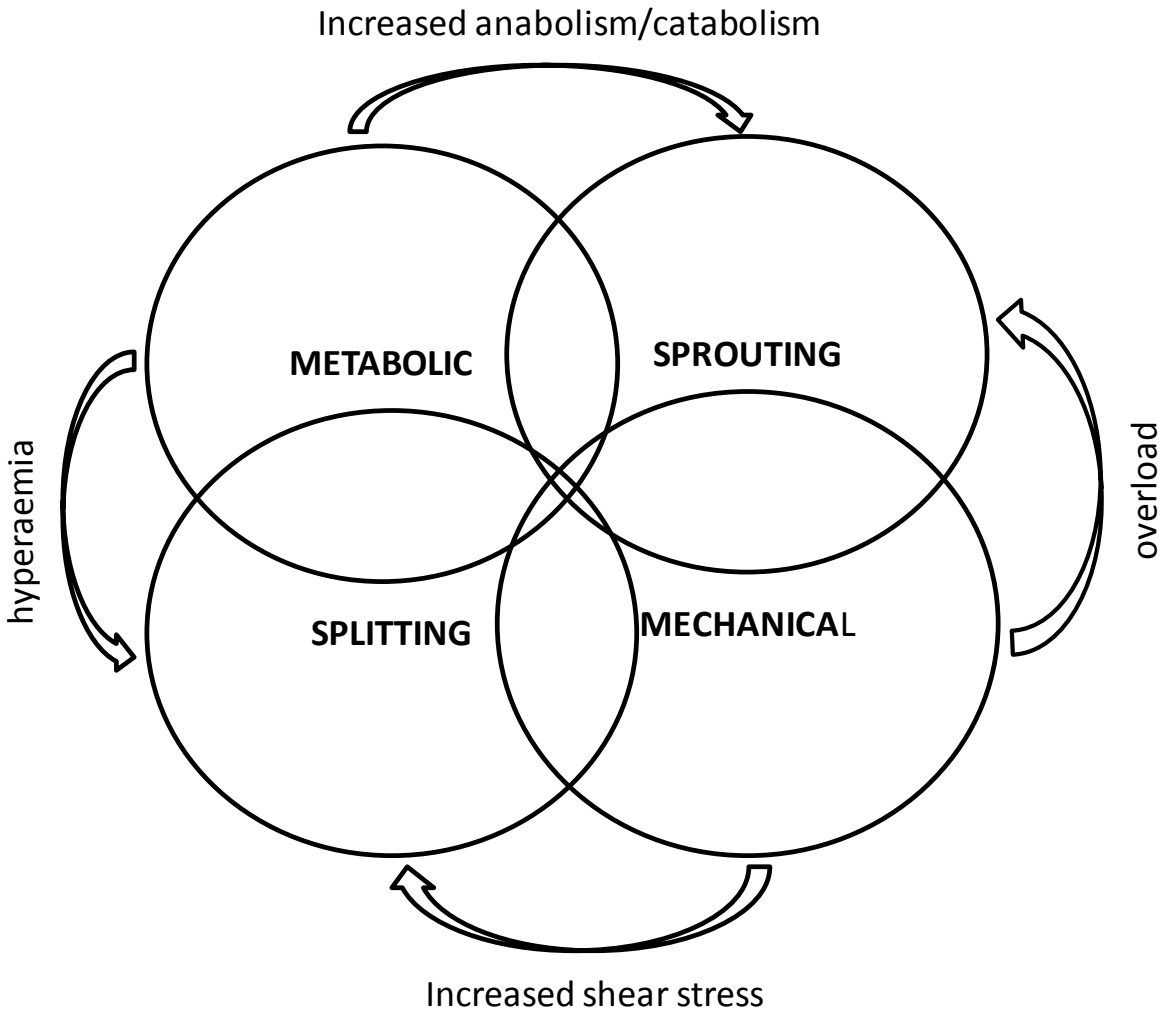


Figure 1.2 Overlapping mechanisms of angiogenesis *in vivo* resulting from metabolic or mechanical stimuli, resulting in different forms of capillary growth.

1.4.1.1 Shear stress-induced angiogenesis

Vascular wall shear stress can be estimated using the following equation:

$$\tau = 4\mu v/r$$

where τ = shear stress, μ = viscosity of blood, v = blood velocity, r = radius

Although the capillary radius is smaller than arterioles and arteries, shear stress on the capillary endothelium is lower owing to the Fahraeus-Lindqvist effect (velocity-dependent viscosity), plasma skimming and the effect of a small vessel diameter permitting only single-file erythrocyte flow. This leaves a thin layer of cell-free plasma at the periphery of the vessel, reducing the haematocrit and therefore the effective viscosity of the blood, hence shear stress. An increase in blood velocity in pre-capillary arterioles increases shear stress on the endothelium, which induces the relaxation of the underlying smooth muscle through the actions of NO and PGI_2, which increases the vessel diameter and blood velocity. However, as capillaries are not surrounded by smooth muscle or elastic fibres, these vessels do not distend to a great extent, and upstream increase in blood velocity increases shear stress downstream, on the capillary endothelium. Endothelial cells *in vitro,* are very sensitive to shear stress and circumferential stretch, and these induce differential transcriptional responses (Patrick &

McIntire, 1995). An elevation in shear stress (tangential viscous drag) on vessel walls is known, in some cases, to stimulate endothelial cell proliferation and migration by increasing cell surface integrin expression (Ando *et al.*, 1987; Urbich *et al.*, 2002). Shear stress is also necessary for the maintenance of capillary stability as it inhibits apoptotic pathways (Dimmeler *et al.*, 1996). Shear stress activates the intracellular signalling molecule Ras, through G-proteins in cultured human umbilical vein endothelial cells (HUVECs). Ras stimulates the activation of PI3-K, which synthesises PIP_3 from PIP_2. PIP_3 binds to Akt, an anti-apoptotic signalling molecule believed to be involved in angiogenesis and also skeletal muscle hypertrophy partly because it promotes cell survival as Akt activates the IKK/NFkB pathway, which induces the expression of anti-apoptotic genes (Gudi *et al.*, 2003). This increases the survival time of disrupted cells and therefore is permissive of skeletal muscle growth and endothelial cell proliferation.

A major cause of increased arteriolar diameter, therefore shear stress elevation in capillary walls is increased nitric oxide synthesis. Nitric oxide is a potent vasodilator (Hutchinson *et al.*, 1987) and the ability of vasodilators to induce capillary growth has been documented in mice, rats and rabbits (Zhou *et al.*, 1998a; Ziada *et al.*, 1989; Ziada *et al.*, 1984). As a negative feedback mechanism, elevated shear stress itself increases nitric oxide synthesis and this causes dilatation which reduces the stress on arterial walls (Kaiser & Sparks, 1986; Rubanyi *et al.*, 1986; Ziegler *et al.*, 1998). For example with chronic vasodilatation using the α_1-adrenoceptor antagonist,

prazosin (see above), eNOS-dependent angiogenesis occurs (Baum *et al.*, 2004). As nitric oxide activates endothelial cells to stimulate HIF-1α accumulation (Brune & Zhou, 2007) and the synthesis of VEGF (Tuder *et al.*, 1995), an established angiogenic growth factor (Connolly *et al.*, 1989; Leung *et al.*, 1989), increases in HIF and VEGF activity may be a mechanism by which shear stress induces angiogenesis .

Increased blood viscosity either by an increase in haematocrit or low temperature (Eckmann *et al.*, 2000) causes increased shear stress in cold-acclimated ectotherms and in the peripheries of endotherms, and we hypothesised that cold acclimation may elicit similar responses to those brought about by flow-related shear stress. Low temperatures may also increase erythrocyte rigidity (Eckmann *et al.*, 2000) thus increasing deformational strain on endothelial cells.

In vivo, increases in blood flow occur in exercise owing to skeletal muscle contractions and increased cardiac output. The ability of increased blood flow, in the absence of the mechanical demand that occurs with exercise, to stimulate angiogenesis, has been demonstrated using a range of vasodilators (Dawson & Hudlicka, 1989).

On the luminal surface of the endothelium, changes in shear stress are sensed by ion channels, tyrosine receptor kinases, NADPH oxidase, G-proteins and caveolae. These activate intracellular signalling molecules that allow interaction between the sensors, the cytoskeleton and

membrane-bound structures such as integrins that attach the endothelial cell to extracellular components, that induce cell migration and proliferation (Shiu *et al.*, 2005).

Caveolae are flask-shaped invaginations of the plasma membrane that are the primary location of endothelial nitric oxide synthase (eNOS). eNOS proteins normally remain bound within the caveolae where their activity is inhibited (Garcia-Cardena *et al.*, 1996). Disinhibition of eNOS occurs by the binding of Ca^{2+}/calmodulin complexes or when the serine residue of eNOS is phosphorylated by the intracellular signaling molecule Akt (protein kinase B). Akt can be activated by other shear stress sensors such as β_1- integrins (Loufrani *et al.*, 2008) and the VEGF receptor flk-1 (Lee & Koh, 2003). Ca^{2+}/calmodulin complexes form where there is a high level of intracellular Ca^{2+}. Elevation of intracellular Ca^{2+} is brought about by the activation of shear stress responsive Ca^{2+} channels or by the release of Ca^{2+} from intracellular stores. Ca^{2+} may be released from intracellular stores by shear-induced activation of G-proteins that activate phospholipase C to form inositol trisphosphate (IP_3) from PIP_2. IP_3 binds to IP_3R on the SR (Shiu *et al.*, 2005).

The endothelial cell-cell junctional protein, platelet endothelial cell adhesion molecule 1 (PECAM-1) is localised with eNOS. Bagi *et al* (2005) showed that PECAM-$1^{-/-}$ mice have impaired NO-dependent vasodilatation in skeletal muscle arterioles, exemplifying the importance of PECAM-1 mechanotransduction and coupling with eNOS during sudden changes in shear stress. Caveolae mechanotransduction was not as important in this model although it is suggested that

only changes in steady shear stress values are sensed by caveolae (Bagi *et al.*, 2005). PECAM-1 activation leads to Akt phosphorylation (siRNA treatment of endothelial PECAM-1 reduces Akt phosphorylation) which may be a mechanism by which PECAM-1 induces eNOS activation (Fleming *et al.*, 2005).

Integrins link the endothelial cell cytoskeleton to the ECM and are involved in EC migration. On the basal surface, integrin activation induces eNOS activation by intracellular signalling through the PI3-kinase/Akt pathway. Mice lacking the α chain of an integrin show reduced flow mediated vasodilatation, thought to be an effect of reduced Akt/PI3-kinase activity on eNOS activity (Loufrani *et al.*, 2008).

Although haemodynamic stresses induce splitting angiogenesis and there is evidence that matrix metalloproteinases are not involved in shear stress-induced angiogenesis (Rivilis *et al.*, 2002), this does not negate the role of surrounding tissue in this model of angiogenesis. Signals of shear stress are sensed by endothelial cell-surface proteins and the cytoskeleton (Urbich *et al.*, 2002), but the composition of the ECM and how the capillary endothelium is attached to the ECM is likely to mediate and restrict the way endothelial cells respond to changes in flow characteristics.

1.4.1.2 Stretch-induced angiogenesis

Muscle fibre hypertrophy occurs in exercising muscles in mammals (Gonyea & Ericson, 1976; Reitsma, 1969) and cold-acclimation in fishes (Egginton & Cordiner, 1997), but not mammals (Egginton, 2002). Stretch and strain on the abluminal face of the endothelial cells, caused by the growth of muscle fibres is likely to cause sprouting angiogenesis by disrupting the basement membrane and triggering endothelial cell migration (Zhou *et al.*, 1998b), in a matrix metalloproteinase-dependent manner (Rivilis *et al.*, 2002). This may also involve non-endothelial cells e.g. skeletal muscle as a source of VEGF (see below) (Hellsten *et al.*, 2008).

1.4.2 Metabolic angiogenesis

The primary role of blood vessels is to supply substrates to and remove metabolites from tissue. Increased capillary supply where there is high metabolic demand, reduces the maximum diffusion distance from each capillary in order to adequately oxygenate the entire tissue. An important metabolic trigger for angiogenesis is hypoxia.

1.4.2.1 Hypoxia

Hypoxia describes the effect of inadequate oxygen supply, a well studied stimulus for angiogenesis. Hypoxia occurs where there is tissue damage, for example in myocardial infarcts, during intensive exercise, and at high altitudes where low barometric pressure lowers inspired oxygen tension. Hypoxia-driven angiogenesis is seen in the heart and skeletal muscle of some mammals (Hudlicka *et al.*, 1992). However, hypoxia also induces muscle fibre hypertrophy in some muscles such that although the number of capillaries per fibre increases, capillary density is maintained (Deveci *et al.*, 2002). Angiogenesis takes place in human skeletal muscle at high altitude, but there is some dispute as to whether the cause is hypoxia *per se* or the accompanying cold exposure at high altitudes. Hypoxia brings about an increase in number of capillaries surrounding a fibre (CAF) but, in hypoxia-resistant animals such as the crucian carp (*Carassius carassius*), hypoxia has no effect on capillary density (Johnston & Bernard, 1984).

1.4.2.1.1 HIF and VEGF in angiogenesis

Although it is not thought to be the case in fish due to reduced metabolic activity in these vertebrates, at low temperatures, reduced oxygen diffusion rates may render skeletal muscle ischaemic, thus initiating hypoxia-induced angiogenesis. Hypoxia-induced angiogenesis is mediated by hypoxia inducible factors (HIFs), heterodimers formed of α and β subunits (Wang

et al., 1995). Under normoxic conditions, there is an oxygen-dependent inhibition of heterodimer formation. In hypoxia, the oxygen labile HIF-1α subunits can combine with HIF-1β to form functional HIF heterodimers in mammals and teleosts. HIFs are transcription factors for vascular endothelial growth factors (VEGFs) by way of its binding to the hypoxia response elements (HRE) on the VEGF promoter gene in rats and cultured cells (Levy *et al.*, 1995; Semenza *et al.*, 1996). HIFs can be activated in normoxic conditions during cardiac and skeletal muscle fibre stretch and hypertrophy in rats (Kim *et al.*, 2002; Milkiewicz *et al.*, 2007), see below. HIF-1 and HIF-2 homologues that are stable in normoxia have been identified in rainbow trout (Soitamo *et al.*, 2001).

VEGF, a paracrine growth factor for endothelial cells and monocytes/macrophages, increases vascular permeability, and its blockade has been used successfully in the treatment of some diseases associated with angiogenesis, including retinopathy, rheumatoid arthritis and some tumours. VEGF is a key regulator of angiogenesis and vasculogenesis in hypoxia (Shweiki *et al.*, 1992). In humans, there are five VEGF isoforms, 121, 145, 165, 189, and 206, generated from alternative splicing of the VEGF gene. The isoforms perform similar functions but $VEGF_{121}$, $VEGF_{145}$ and $VEGF_{165}$ isoforms are thought to be the most important in angiogenesis. Most cells produce all isoforms but 121 and 165 are the predominant isoforms in most cell types. In zebrafish (Liang *et al.*, 1998) $VEGF_{121}$ is dominant in adults, and $VEGF_{165}$ is dominant in embryos. VEGFs act through the cell surface tyrosine kinase receptors, flk-1 (VEGFR-1) and flt-1 (VEGFR-2),

and are inhibited endogenously by soluble forms of the flt-1 receptor. VEGF can also be produced in the absence of hypoxia, by inflammatory cytokines such as IL-1 and TGF-β, and other molecules such as nitric oxide (Dembinska-Kiec *et al.*, 1997). VEGF, in turn, stimulates nitric oxide synthesis (Dembinska-Kiec *et al.*, 1997; Hood *et al.*, 1998), see below.

Studies have shown that intraluminal stress and extravascular stimuli initiate differential gene expression (Williams *et al.*, 2006b) and protein activity (Rivilis *et al.*, 2002), and different forms of angiogenesis.

1.4.3 Intussusceptive and splitting angiogenesis

Intussusceptive and splitting angiogenesis are triggered by intraluminal stimuli and mainly occur on the venular side of the capillary bed (Burri *et al.*, 2004). During intussusceptive angiogenesis, a pillar core of collagen fibrils surrounded by endothelial extensions is formed in the lumen driven by abluminal surface protrusion into the vessel. The primary source of the collagen is thought to be peri-endothelial cells and to some extent the endothelial cells. The endothelial extensions originate from the endothelial cells of the capillary wall so surrounding the pillar core brings about invagination of the lumen around this region. Additionally, peri-endothelial cell extensions may push into the vascular lumen in between the pillar core and wall extensions,

forming intraluminal folds. Some basement membrane degradation and matrix remodelling is required for endothelial layer rearrangement. Endothelial thinning and transcellular formation of holes form two separate vessels (Burri *et al.*, 2004).

There is persistent plasma flow through splitting angiogenesis, which begins with the thickening of the endothelium followed by protrusion of cytoplasmic processes in the lumen which traverse the lumen and contact and fuse with the opposite end of the capillary to form two lumens. The capillary then splits into two separate vessels. As the entire process occurs intraluminally, unlike intussusceptive angiogenesis, there is little disruption to the basement membrane during splitting angiogenesis (Zhou *et al.*, 1998a).

1.4.4 Sprouting angiogenesis

Sprouting angiogenesis is initiated by an extravascular stimulus and begins with degradation of the basement membrane of the venular end of capillaries close to the site of the angiogenic stimulus. This is followed by migration of endothelial cells towards the stimulus and alignment of endothelial cells in both directions. A lumen is formed either intracellularly by vacuole formation or intercellularly between co-migrating endothelial cells. Blind-ended sprouts connect to each other to form loops through which blood flow or diffusive exchange is increased. Finally, pericytes line the endothelial cells and a new basement membrane is formed. Skeletal muscle

overload and contraction are mechanical extravascular stimuli for sprouting angiogenesis (Hudlicka *et al.*, 1986)

1.4.5 Aims

Metabolically induced angiogenesis has been studied more extensively, but mechanotransduction in endothelial cells for example through shear stress or strain, leading to vessel growth, may be an alternative or independent mechanism by which blood flow is restored. The purpose of this thesis is to explore the mechanical demands, and systemic, proteomic and transcriptional mechanisms for microvascular expansion and rarefaction with temperature acclimation in the temperate fishes, rainbow trout and common carp. This thesis will also challenge some conventional ideas on the regulation of vascular tone, with particular reference to nitric oxide, a molecule that is widely believed to be the primary endothelial-derived relaxing factor in vertebrates. In addition, the potential confounding effects that temperature and /or treatment have on central (i.e. cardiac) responses were explored (see Fig. 1.3).

1.4.6 Hypotheses

1. Cold-induced increases in capillary shear stress will lead to angiogenesis.
2. Warm-induced reduction in shear stress will lead to capillary rarefaction.

3. Shear-induced angiogenesis may be attenuated by blockade of vasodilator pathways.
4. Capillary rarefaction may be attenuated by blockade of vasoconstrictor tone.
5. These effects may be modulated by central cardiovascular effects on blood flow/cardiac output.

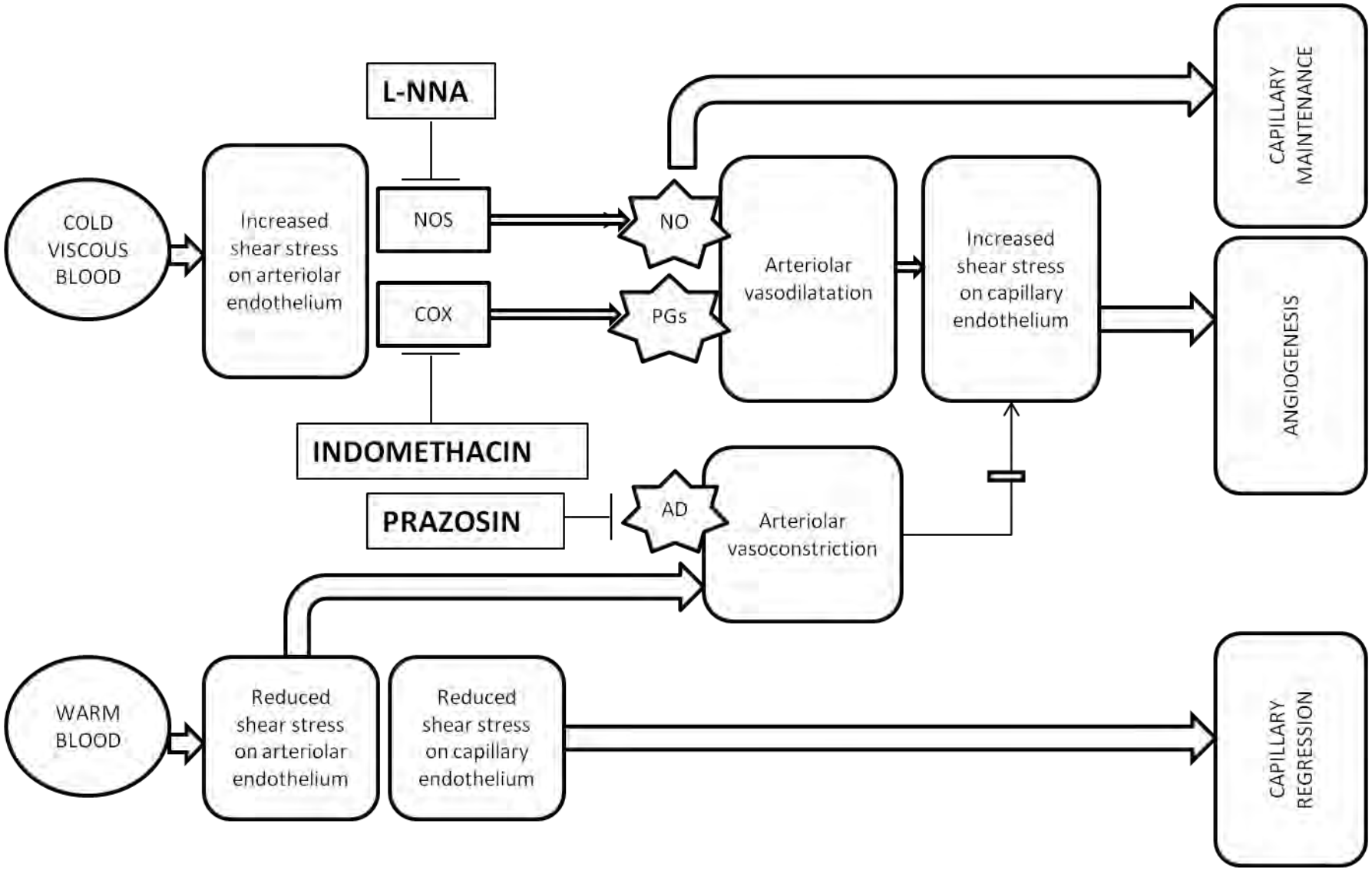


Figure 1.3 Schematic representation of the hypotheses investigated in this thesis. NOS= nitric oxide synthase, NO= nitric oxide, COX= cyclooxygenase, PGs= prostaglandins, AD= adrenaline. Indomethacin is a COX inhibitor, L-NAME is a NOS inhibitor and prazosin is an α_1-adrenoceptor antagonist.

Chapter 2 General methods and method development

2.1 Animal husbandry

Rainbow trout, *Oncorhynchus mykiss*, (190-242g), of unknown age, were purchased from Leadmill Trout Farm, Hathersage, UK, and transported in water containing clove oil to minimise transportation stress and deaths caused by transportation, in tanks of 25 trout per tank. They were held in recirculating, aerated, charcoal-filled Birmingham tap water, and were kept under continuous artificial lighting of 12 hours dark: 12 hours light periods. 36 trout were acclimated to 11°C for 4 weeks before exposure to experimental conditions to minimise the influence of transportation stress. At the end of this initial acclimation period, trout were separated into tanks of 6, 12, or 18 fish depending on acclimation temperature, whether they were to be fed drugs (L-NNA, prazosin, indomethacin), and the duration of their acclimation period. The tank temperatures were raised to 18°C or lowered to 4°C at 7°C per day. During the experimental acclimation periods several fish died in each set of studies, such that in many experiments, there were between 2-6 fishes per condition (Appendix A). All the rainbow trout acclimated to 4 weeks in the indomethacin study, had markers that indicated bacterial infection on their skin.

Common carp, *Cyprinus carpio*, were purchased from Ripples Waterlife, Telford, UK. For the acclimation/gene array studies, 30 carp were initially acclimated to 15°C, after which the fish were separated into 3 tanks of 10 fish. The tank water for cold-acclimated carp was cooled to

5°C and the tank for warm-acclimated carp was raised to 25°C with an immersible heating element. Tank temperatures were monitored with a mercury thermometer with an estimated accuracy of ± 0.2°C. Acute temperature changes were carried out at 1°C/hour to minimise stress to fish without negating the acute effects of temperature change.

All fish in these experiments were fed once every morning by the animal technicians. Many of the fish did not feed properly as was evident from the lack of a feeding frenzy when pellets were thrown into the tanks, and the subsequent settling of pellets at the bottom of the tanks. This introduced two problems to the studies: starvation of trout results in reduced protein synthesis and RNA transcription (Loughna & Goldspink, 1984), and the drugs in the pellets may have leached out when in water for long enough to exchange solutes with its environment. Food was withdrawn from rainbow trout that were to undergo surgical procedures for live physiological recordings, one day prior to surgery. Photoperiod (day length) was controlled by artificial lighting; there were 12 hour light:dark cycles.

2.2 Sampling methods

All animals were killed by stunning with a sharp blow to the head followed by transecting the brain stem, a schedule 1 method, in accordance with the UK Animals (Scientific Procedures) Act, 1986. The animals were weighed and the standard length (measured from the tip of the snout to the end of the caudal peduncle), total length of the antero-posterior axis and maximum

depth of the dorso-ventral axis, were measured (Appendix). Epaxial red and white muscle samples were excised from the lateral line region on both sides of the fish, from the posterior end of the dorsal fin to the caudal fin (Fig. 2.1). White muscle was taken from close to the spine. The ventricle and liver were also excised from each fish. Samples to be used for histochemistry were embedded in an inert mounting medium (Optimum Cutting Temperature compound, BDH, UK) and frozen onto cork discs, in isopentane (2-methylbutane) that was pre-cooled in liquid nitrogen. Samples collected for all other assays were stored in RNase-free, sterile microtubes. All samples were coded before storage at –80°C until use and, where appropriate, further analyses were accomplished blinded to condition and timepoint, but not species.

2.3 Preparation of samples for histochemistry

For histochemical and immunochemical analysis, tissue blocks stored on cork discs were cut at 10µm and mounted onto polysine™-coated glass slides (VWR), in a Bright Clinicut Cryostat at -20°C, and then air-dried for 30 minutes at room temperature. The slides were subsequently stored at -20°C. Prior to use, samples were left to warm to room temperature in a closed box to avoid condensation on the slide. The regions to be stained were marked with a wax pen (Vector laboratories) to produce a hydrophobic barrier. Unless otherwise stated, all staining was carried out in a humid incubation chamber and solutions were pipetted into the region outline with the wax pen. Images were obtained using a Zeiss microscope (Axioskop 2 plus) with an Axiocam MRc digital camera. Images were taken from the same region so that results were not

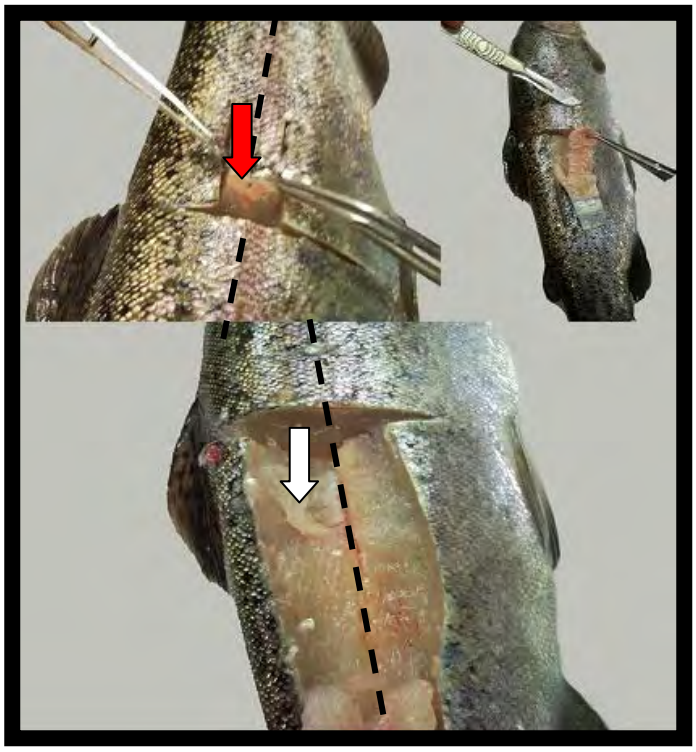


Figure 2.1 Sampling locations on rainbow trout. Red muscle was taken from immediately beneath the skin (red arrow) and white muscle from close to the spine (white arrow) along the lateral line (dotted line).

influenced by fibre size/type (see Fig. 2.2), using Axiovision version 4 software, using a 10X objective lens.

2.3.1 Materials

All materials were purchased from Sigma Aldrich, UK, analytical grade or better, unless otherwise stated.

2.4 Fibre typing

Fibre typing was carried out to examine possible differential effects of cold acclimation on different fibre types. Red muscle is located on either side of the lateral line immediately below the skin, pink muscle lies immediately below the red muscle and white, mosaic fibres are found deeper into the muscle and also ventral and dorsal to the pink fibres.

2.4.1 Succinic dehydrogenase staining

This histochemical assay relies on the activity of succinic dehydrogenase (SDH), an enzyme that is located on the inner mitochondrial membrane. SDH oxidises succinate to produce fumarate and NADH (reduced nicotinamide adenine dinucleotide). NADH cannot be visualised so is complexed with nitro-blue tetrazolium (NBT), forming an insoluble formazan end product that is seen as blue speckles. The intensity and extent of the blue colour is an indication of mitochondrial content, which is used as an index of oxidative capacity**.**

Method

Tissues were incubated in a working buffer solution (60mM NBT, 0.5M NaH_2PO_4, 5M Na_2HPO_4, 3.6M $C_4H_6O_4$; pH 8.0) at 37°C, for 60mins. Although the optimum temperature for enzymatic activity in fishes is expected to be at lower temperatures e.g close to the upper thermal limit of

fishes, preliminary attempts at staining at 5°C and 21°C did result in a visible reaction, so these assays were carried out at 37°C. Slides were then post-fixed in sucrose formol (4% formaldehyde, 120mM $Ca(NO_3)_2$, 330mM sucrose; pH 7.2), for 2 minutes. The slides were wiped with tissue and mounted in aquamount and covered with a glass coverslip. Slides were not fixed prior to staining for SDH as fixation inactivates the SDH enzyme (Bancroft & Stevens, 1996).

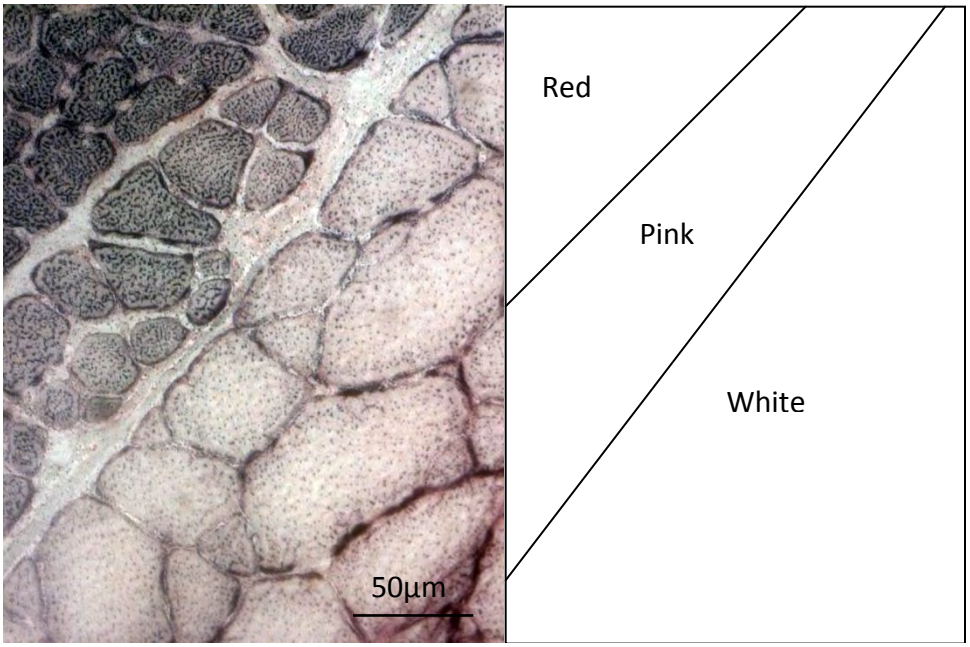


Fig 2.2. Succinic dehydrogenase (SDH) staining in three trout skeletal muscle types, showing the relationship between fibre size and oxidative capacity. Red=slow or type I fibres, pink= fast oxidative glycolytic or type IIa, white= fast glycolytic or type IIb. Note the pattern of formazan deposits, which equates to the mitochondrial distribution observed in electron micrographs from Crucian carp (see Johnston, 1982)

2.5 Capillary visualisation

Several capillary visualisation methods were compared to identify the most robust and practical method for capillarity estimation in fish. These methods included immunohistochemistry for

endothelial proteins, histochemical staining for alkaline phosphatase, glycogen and basement membrane proteins, and using an intraluminal marker. All methods were attempted in both common carp and rainbow trout, except administration of intraluminal markers, which was carried out in rainbow trout alone as a suitable route of administration in common carp was not identified.

2.5.1 Alkaline phosphatase

Alkaline phosphatase localisation was demonstrated by incubating sections in a 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) solution. Alkaline phosphatase activity in the tissue hydrolyses the BCIP to form an indigo dye which then reduces the tetrazolium salt in NBT to form NBT-formazan and produces a stable coloured precipitate that can be visualised under the microscope (Lojda *et al.*, 1979).

Method

300µl of the working buffer (0.5mM 5-bromo-4-chloro-3-indolyl phosphate toluidine salt, 1mM nitroblue tetrazolium, 28mM $NaBO_2.4H_2O$, 7mM $MgSO_4.7H_2O$; pH 9.3) was applied to the slides within a hydrophobic barrier and incubated in a humid tray for an hour, to avoid drying. The working buffer was tipped off the slides and the slides were washed with approximately 300ml of wash buffer (28mM $NaBO_2.4H_2O$, 7mM $MgSO_4.7H_2O$; pH 9.3, adjusted with 0.2M H_3BO_3) for every 10 slides, at room temperature, for 5 minutes. The slides were dried and excess liquid was

shaken off. The tissues were mounted with aquamount (BDH, UK) and covered with a coverslip. Images were saved as jpeg files and analysed using ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health). This method produced clear and reproducible staining of capillaries and terminal arterioles in trout, but in carp muscle staining was sparse and only identified the arterioles (Figs. 2.4a and b).

2.5.2 High molecular weight dextran

The use of intraluminal markers involves the administration of a labelled protein *in vivo* with the assumption that the marker will remain in the vasculature either by binding to components of blood, e.g. erythrocytes or albumin, or because its large size renders the vascular wall impermeable to the protein. Here, fluorescein isothiocyanate (FITC)-labelled high molecular weight (500kDa) dextrans were administered to trout, as it has been shown that it does not escape the vasculature of tuna (Brill *et al*., 1998), which have more permeable vessels than trout or carp.

Rainbow trout were anaesthetised in water containing tricaine methane sulphonate (MS222; 0.1g/L). When the righting reflex was lost, the animal was transferred to a table where it was positioned ventral side upwards. The gills were irrigated with cold, aerated, lightly anaesthetised water (0.03g/L MS222). The dorsal aorta was cannulated by inserting a trocar (sharpened guitar string), slightly thinner than the PP50 catheter into the catheter lumen, to

pierce the roof of the mouth (Soivio & Nynolm, 1975). The heparinised cannula was pushed into the dorsal aorta, and the guitar string withdrawn. The cannula was flushed with heparinised saline, and sutured to the roof of the mouth, passed through the operculum and sutured to the anterior end of the dorsal fin (Fig. 2.3). 500kDa FITC-dextran (Sigma) dissolved in buffered saline (3.5mg/ml) was administered through the cannula. The animal was returned to a bucket of tank water and allowed to recover fully and swim freely for 30 minutes. The animal was then killed with an MS222 overdose and red and white muscle samples were excised, as described in sampling methods (section 2.2). Dextran administration was not carried out in the carp as a suitable method of administration was not found, due to the relatively inaccessible arterial anatomy.

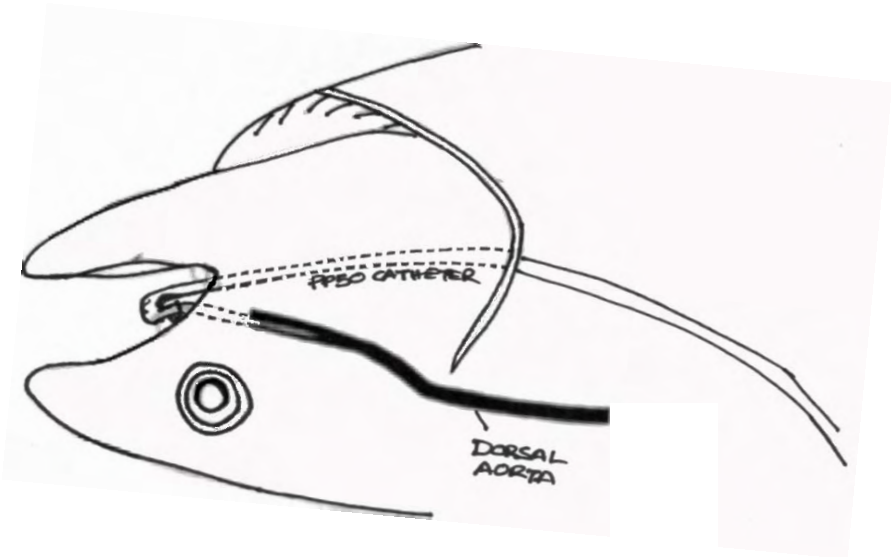


Figure 2.3. Cannulation of the dorsal aorta. The cannula was inserted through the roof of the mouth using a guitar string as a guide (Soivio & Nynolm, 1975), secured to the roof of the mouth by sutures and exited *via* the gill chamber. This allowed unobtrusive injection of substances to the fish.

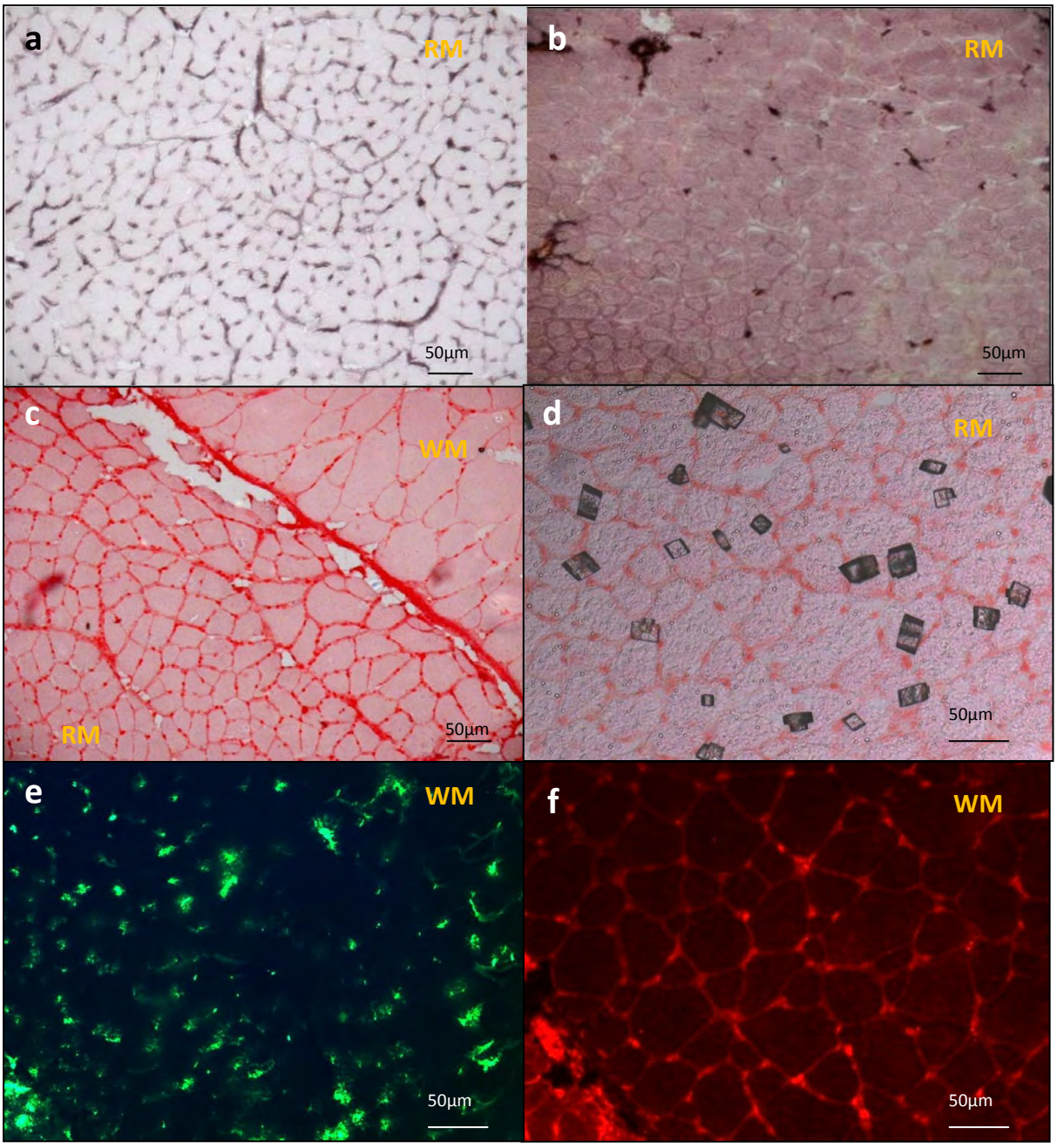


Figure 2.4. Alkaline phosphatase staining in the red muscles of a) rainbow trout and b) common carp. Magnification X10. Note the sparse staining of vessels in carp muscle which renders the ALP stain unsuitable for marking capillaries in this species. PAS amylase staining in c) red and white muscle of trout (X10) and d) red muscle of common carp (X20). Although this method of staining produced clear identification of when staining was successful, the method was not always successful, thus unreliable. Parallel e) FITC-dextran (green) and f) Tie-2 (red) stains in trout white muscle. Lack of clarity of fibre outlines and distinction between adjacent capillaries made FITC-dextran labelling unsuitable. Tie-2 staining was clear and consistent in both red and white muscle. RM=red muscle; WM= white muscle

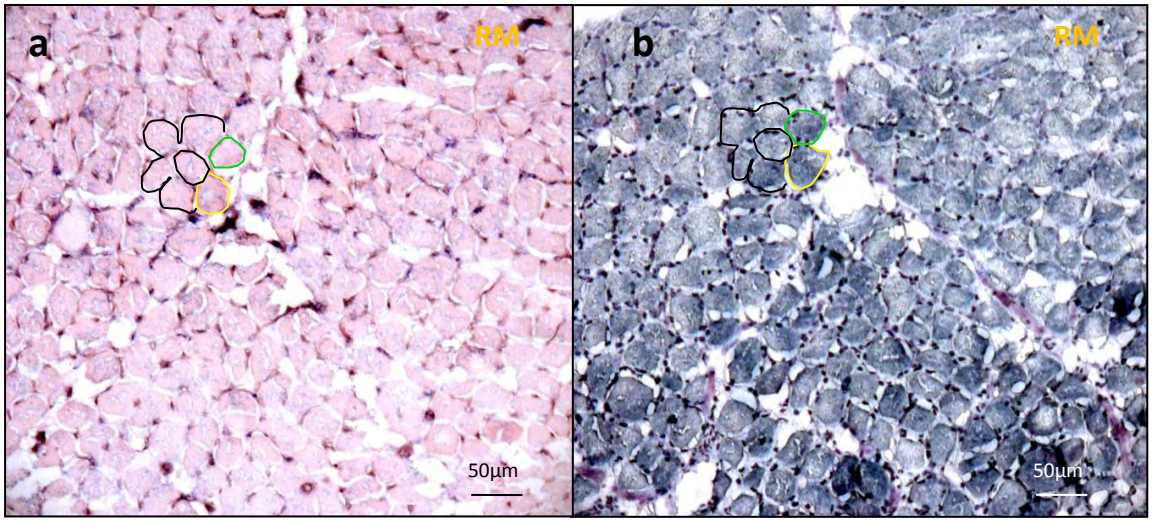


Fig 2.5. Serial sections of trout red muscle to compare a) alkaline phosphatase staining with b) silver impregnation as a method for measuring capillarity. The latter was inconsistent in staining, and nuclei could not be distinguished from capillaries which would result in overestimation of capillarity.

2.5.3 Alpha-amylase/Periodic Acid Schiff staining

This method (adapted from Andersen, 1975) stains for carbohydrates, which are oxidised by periodic acid, to aldehydes that bind to Schiff's reagent and stain pink (Andersen, 1975).

Method

Period Acid Schiff- Amylase staining was carried out at room temperature. Slides were fixed in Carnoy's fixative (6 parts ethanol: 3 parts chloroform: 1 part acetic acid) for 10 minutes. The fixative was washed in distilled water once for 3 minutes. The slide was shaken dry and was incubated with 10% (m/v) alpha-amylase (from *Bacillus subtilis*) for 30 minutes. This was

washed with distilled water for 3 minutes. The slide was then incubated with 1% (v/v) periodic acid for 10 minutes, which was followed by staining with Schiff's reagent for 5 minutes. This was washed with three changes of distilled water for 5 minutes each. The sections were photographed immediately as mounting caused crystal formation (see Fig. 2.4d), possibly due to limited washing needed to preserve section integrity.

2.5.4 Silver impregnation

This silver impregnation technique modified from Gomori (1937), stains for reticulum in the basement membrane so can be used to locate blood vessels surrounded by a basement membrane.

Method

All solutions used for sliver impregnation were diluted in distilled water and the protocol was carried out at room temperature. Slides were incubated in 0.1% (m/v) potassium permanganate for 20 seconds, 1% (m/v) sodium metabisulphite for 1 minute, 10% (m/v) silver nitrate solution for 2 minutes and then ammoniacal silver for 1 minute. Ammoniacal silver was prepared by making a 1:6 10% (m/v) KOH:10% (m/v) silver nitrate solution to which 30% (m/v) ammonium hydroxide was added slowly with a Pasteur pipette whilst continuously stirring until the precipitate was completely dissolved. Silver nitrate solution was added to this solution drop by drop until the precipitate easily disappeared when shaken. Finally, the volume of the solution

was doubled by adding an equal amount of distilled water. Slides were washed in distilled water for 3 minutes between each incubation period. After ammoniacal silver incubation, samples were rinsed quickly in distilled water for 30 seconds and fixed in 4% (m/v) formaldehyde for 3 minutes. After fixing, samples were incubated in 0.2% (m/v) gold chloride solution for 10 minutes and washed again with distilled water. Tissues were incubated in 1% (m/v) sodium metabisulphite once more for 1 minutes; this was followed by incubation in 1% (m/v) sodium thiosulphate for 1 minute and washing in distilled water for 3 minutes, after which samples were shaken dry and mounted in aquamount and covered with a glass coverslip. Pictures were taken immediately.

2.5.5

2.5.6 Tie-2

The endothelial receptor tyrosine kinase receptor, Tie-2 is the receptor for angiopoietins (Davis *et al*, 1995), substances that mediate VEGF activity during angiogenesis. Although angiopoietins are involved in angiogenesis, Tie-2 was considered a suitable endothelial marker due to its constitutive expression on endothelial cells. Tie-2 homologues have been cloned in zebrafish (Lyons *et al.*, 1998) therefore are likely to be present in other fishes. Tie-2-positive endothelial cells were identified using fluorescent immunohistochemistry (IHC) in carp and trout, first described by (Coons, 1951). The Tie-2 antibody used was raised in rabbit against a human

antigen (Santa Cruz biotechnology). Buffered solutions were either prepared in phosphate buffered saline (PBS) or Tris-buffered saline (TBS). A fluorescent dye-coupled secondary antibody was used to visualise the locations of the primary antibody-binding. Comparison between fixing with acetone or formaldehyde showed that acetone fixation produced clearer staining. When TBS was used, binding could not be seen in either rainbow trout or common carp. However, binding could be seen in 5°C-acclimated rainbow trout but not common carp acclimated to any temperature, when PBS was used. It was also noted that increased washing at each stage reduced background staining but Triton X (0.3% (v/v), 5 minutes) blocked all binding. The final method used is described below.

Method

All stages of IHC were carried out at room temperature unless stated otherwise. Samples were fixed in pre-cooled acetone, on ice, for 5 minutes and washed with four volumes of 300ml phosphate buffered saline, PBS (for 6-10 slides), for 5 mins each. Each slide was incubated in 300µl of blocking solution (1% (m/v) BSA, 10% (v/v) normal goat serum, 0.5% (v/v) PBS-Tween 20), applied within a hydrophobic barrier created with a wax pen. Slides were then incubated in 300µl rabbit polyclonal Tie-2 antibody (Santa Cruz, sc-1026, 1:100) overnight at 4°C, in a refrigerator. Negative control slides to which only the primary antibody vehicle (1% (m/v) BSA, 0.5% (v/v) PBS-Tween 20) was applied were also used. After 16 hours, samples were washed in 4 volumes of 300ml 0.5% (v/v) PBS-Tween 20. Samples were then incubated with 300µl of

Alexafluor-594 goat anti-rabbit secondary antibody (Invitrogen, 1:500), in the dark, for 60 minutes. Slides were washed in the dark with 3 volumes of 300ml (for 6-10 slides) of 0.5% (v/v) PBS-tween, and 3 volumes of 300ml PBS for 5 minutes each. Slides were mounted with 1-2 drops of Vectashield mounting medium (Vector Laboratories) that contained 4',6 '-diamidino-2-phenylindole (DAPI), for visualising nuclei, diluted 1:9 in PBS, and covered with a glass coverslip.

2.5.7 Acid ATPase

Acid-stable ATPase can also be found in red skeletal muscle and acid myofibrillar ATPase (mATPase) is used to identify this fibre type. The acid ATPases used for capillary visualisation are optimally active at a lower pH than those of mATPases and it has been shown in rats that ATPases produce an estimate of capillarity similar to that produced by alkaline phosphatase staining (Sillau & Banchero, 1977). The sections were preincubated in a 0.2M veronal-acetate buffer adjusted to a range of pH (3.6-4.2) to establish the optimal pH for endothelial cell ATPases. All sections were then incubated at room temperature for 20 minutes in an incubation medium (glycine, $CaCl_2$ and ATP, adjusted to pH 9.4), followed by rinsing with 2% $CaCl_2$, incubation in 1% (m/v) $CoCl_2$ for 3 minutes and incubation with 1% (m/v) ammonium sulphide for 30 seconds. In this assay, ATPase is the enzyme, ATP is the applied substrate and phosphate is the reaction product. As phosphate cannot be seen on its own, it is reacted with calcium to

form calcium phosphate ($CaPO_4$), incubated with $CoCl_2$ and then ammonium sulphide to produce cobalt sulphide (CoS_2), which is brown-black and can be seen easily.

2.6 Angiogenesis and cell proliferation

Angiogenesis is modulated by several substances including VEGF, so VEGF expression was used as an index of angiogenesis. As sprouting, but not splitting angiogenesis involves cell proliferation (Chapter 1), estimates of cell proliferation were made in order to identify the primary mode of capillary growth in cold-induced angiogenesis.

2.6.1 VEGF

Changes in VEGF expression were measured by IHC. The method used for VEGF IHC is identical to that used for Tie-2 IHC (2.5.5). The primary antibody was substituted for rabbit polyclonal VEGF antibody (Santa Cruz A-20).

2.6.2 PCNA

PCNA (proliferating cell nuclear antigen), is a DNA polymerase co-factor expressed by cells undergoing mitosis, and so can act as a measure of cell proliferation. Co-localisation of PCNA with capillaries can identify proliferating cells associated with capillaries (for example endothelial cells or pericytes) and those associated with interstitial cells. PCNA stains were

counterstained with DAPI, a nuclear stain, in order to discriminate between proliferating and non-proliferating nuclei.

Method

Slides were fixed in 4% (m/v) formaldehyde (diluted in PBS) for 5 minutes, and washed 3X 300ml PBS for 5 minutes each. Slides were shaken dry and incubated in blocking solution (1.3% (m/v) BSA, 0.6% (v/v) Triton X-100 in PBS). Blocking solution was tipped off the slides and replaced with rabbit polyclonal PCNA antibody (Autogen Bioclear; ABP2908) and incubated in a 4°C refrigerator overnight. A negative control slide in which the antibody vehicle (1.3% (m/v) BSA, 0.6% (v/v) Triton-X in PBS), was used. After 16 hours, slides were washed in 3X 300ml PBS, for 7 minutes each. This was followed by 60 minutes incubation with Alexafluor goat anti-rabbit secondary antibody (diluted in antibody vehicle) in the dark, followed by three washes in PBS for 7 minutes, in the dark. Slides were mounted with Vectashield with DAPI.

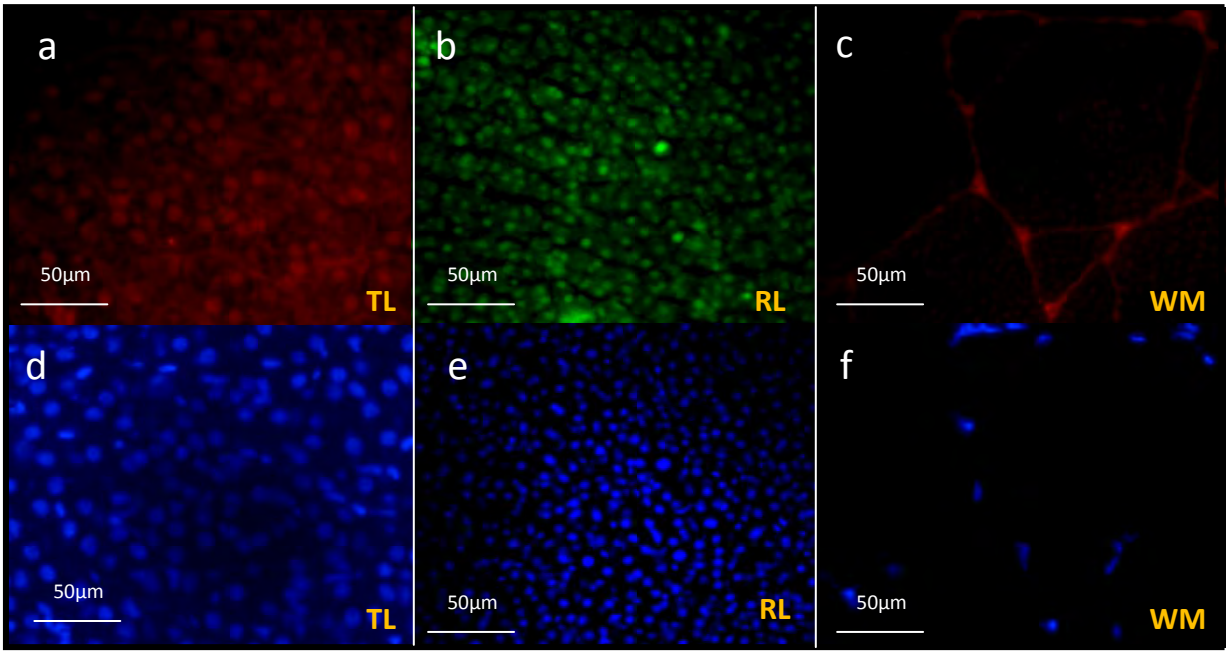


Fig 2.6. Determination of cross-reactivity of a mammalian PCNA antibody in trout muscle. Rat liver (b) was used as a positive control for staining shown in trout liver samples (a), which in turn was used as a positive control for trout white muscle staining (c). DAPI staining on the same slide (bottom panel; d,e,f) showed that the PCNA co-localised with the nucleus. TL= trout liver; RL= rat liver; WM= trout white muscle

2.7 Discussion- Immunohistochemical techniques

2.7.1 Capillary visualisation

The use of dextrans as an intraluminal marker was found to be unsuitable as the proteins did not gain entry into all capillaries, as demonstrated by parallel staining with the endothelial marker Tie-2 (Figs. 2.4e&f). Moreover, the overlap of dye from adjacent capillaries, together with the halo effect of the fluorescent dye and perhaps some protein leakage from vessels, smeared the appearance of the vessels. This was more problematic in the red muscle as the

inter-capillary distances were shorter. The likelihood of underestimating capillarity and the lack of clarity of the fibre outlines meant this method was not used for capillary estimation.

The PAS-amylase method demonstrated vessels clearly in trout and carp muscle (Fig. 2.4c&d) but the staining was not successful in all samples, so could not be used for capillary estimation. Further, compared to other stains, there seemed to be an overestimation of capillarity using this method, suggesting that there was an incomplete digestion of glycogen by the α-amylase, or confusion with other anatomical structures that were PAS-positive. The ATPase staining method demonstrated only the arterioles in tissue from common carp and was not tried in rainbow trout.

The silver impregnation, when successful, did not distinguish between reticulum, collagen fibres and nuclei, which with successful toning with gold should have appeared black, brick red and rusty brown, respectively (Gomori, 1937) so this technique was unsuitable for the current purpose. Comparison of serial sections from the same muscle, stained for alkaline phosphatase and silver, shows the extent of over-staining with silver impregnation (Fig. 2.5). Further, oxidisation with potassium permanganate often lifted the tissue off the slide, so loss of sections was an additional risk associated with this method, although this was lowered by reducing the potassium permanganate concentration to that described above.

Tie-2 was a successful marker for endothelial cells in trout red muscle and showed reproducible staining (see Fig. 2.4). However, as Tie-2 IHC was unsuccessful in carp and is more time-consuming and expensive than alkaline phosphatase staining, this method was not used routinely for either species. Platelet endothelial cell adhesion molecule 1 (PECAM1, CD31) IHC was also attempted, as this is also constitutively expressed on mammalian endothelium, but there was no cross-reactivity with mammalian antibodies in carp or trout muscle. The method for PECAM1 IHC was identical to that for Tie-2 staining, but the primary and secondary antibodies were substituted as appropriate.

A systematic review of the existing literature yielded no information on methods for immunohistochemical staining of the capillary endothelium in carp. The method previously used to identify capillaries in carp was electron microscopy (Johnston, 1982). However, as this is both time-consuming and expensive, an alternative method was sought. Extensive investigation into alternative methods for capillary visualisation was unsuccessful, generating results that were inconsistent, non-specific, or none at all. In some cases, the stains were damaging to the muscle fibre integrity, particularly in red muscle, so could not be used to generate reliable data on capillary to fibre ratios or capillary density in carp muscle.

2.7.2 Angiogenesis and cell proliferation markers

PCNA IHC in trout liver sections (Fig. 2.6a) was comparable with rat liver sections (Fig. 2.6b), so this antibody was suitably cross-reactive for trout tissue. DAPI stains (Figs. 2.6d & e) show that PCNA staining co-localises with a reliable nuclear marker and the stain is not non-specific. VEGF IHC was also successful and staining was present local to capillaries and within muscle fibres, as expected. Negative controls for the Alexafluor-488 (rat) and Alexafluor 594 (trout) in which the secondary antibody was used in the absence of the primary antibody showed no stain so there was no non-specific interaction of either of the secondary antibodies with the tissues.

2.7.3 Capillary and fibre counting

Images, 1300X1030 pixels, were taken at X10 magnification and analysed using Image J software. A counting frame consisting of 6000pixels^2 squares, were overlaid on the images (<plugins> <analyze> <grid>). Fields of 6X6 squares were selected for analysis and the area outside the selected region of interest (ROI) was made transparent (Fig. 2.7). Two sides of the grid were selected as exclusion zones (Fig 2.7. -solid black lines) such that any capillaries or fibres touching the line were not counted; the other two sides of the square (black dotted lines) were considered inclusion zones so that any capillaries or fibres that touched these sides exclusively, were included. ROI of the same size were selected from all images and capillaries and fibres were counted. Capillaries/fibres were marked using the 'point selection' tool, and

counted automatically (<Analyze> <measure>). Capillary to fibre ratio (C:F) was calculated by dividing the number of capillaries by the number of fibres in the same field. Capillary density (CD) is the number of capillaries per mm^2 fibre area. Mean fibre size was calculated by dividing the area of the selected field by the number of fibres in that field, subtracting any non-muscle area of an image if necessary. One tissue block from each trout or carp was used, and 7-8 each fields were analysed from each block. Using 7-8 fields was found to be suitable because at this point onwards the running mean of fibres and capillaries from the same block was constant. There were approximately 100 fibres per field with some variation depending on the size of the fish.

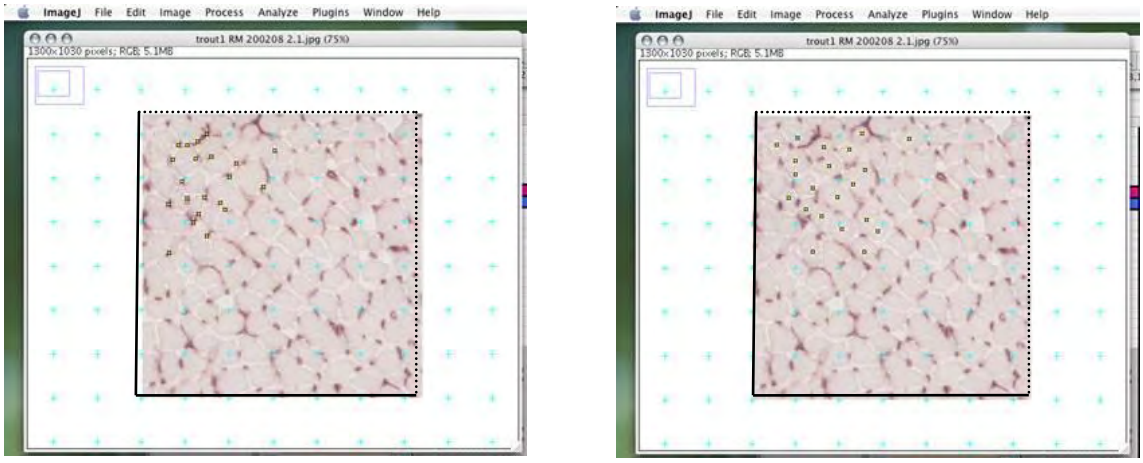


Fig.2.7 Representative snapshot demonstrating the method used to estimate capillary:fibre, capillary density, and mean fibre size. The ROI is highlighted and the unbiased counting frame is superimposed

2.8 Assaying nitric oxide synthase activity in tissue homogenates

Nitric oxide (NO) is a gaseous signalling molecule that is produced by many cells in the body, but of particular interest here, is its production in the endothelium and its effects on vascular tone. NO, also known as an endothelium-derived relaxing factor (Palmer *et al.*, 1987), brings about vasodilatation by diffusing into smooth muscle cells (SMC) and binding to the haem moiety of cytosolic guanylyl cyclase (GC). This activates GC to convert guanidine triphosphate (GTP) to cyclic guanidine monophosphate (cGMP). cGMP interrupts the Ca^{2+}-dependent contractile filaments of smooth muscle cells by inhibiting Ca^{2+} entry. cGMP also activates protein kinase G, which inactivates myosin light chain kinase by phosphorylation. Thirdly, cGMP activates K^{+} channels therefore hyperpolarising the cells, which causes SMC relaxation. Vasodilatation in pre-capillary arterioles gives rise to elevated shear stress in capillaries, which is a trigger for angiogenesis (Egginton, 2002).

NO is synthesised by oxidation of the amino acid, L-arginine by the enzyme nitric oxide synthase (NOS), in the presence of the co-factors flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (BH_4), reduced nicotine adenine dinucleotide phosphate (NADPH) and oxygen. This results in the formation of L-citrulline and NO.

$$\text{L-Arg} \xrightarrow[\text{nitric oxide synthase}]{} \text{L-citrulline + NO}$$

There are three NOS isoforms, each encoded by different genes. The Ca^{2+}-dependent endothelial NOS (eNOS, NOS-III) (Mayer *et al.*, 1989) and neuronal NOS (nNOS, NOS-I) are typically thought to be constitutively present. The Ca^{2+}-independent inducible NOS (iNOS, NOS-II) is induced under inflammatory conditions. The level and activities of eNOS and nNOS can also be regulated by stimuli for enzyme activation and gene transcription.

2.8.1 Radiolabelled citrulline assay

NO is highly reactive and is converted into nitrites and nitrates within 6 to 10 seconds, therefore estimates of nitric oxide synthase activity are often obtained based on the stoichiometric formation of the more stable by-product L-citrulline (Bredt & Snyder, 1994). This forms the basis of a commercial kit (Cayman Scientific), supposedly optimised for human NOS. However, reports from other labs suggested it may not be a robust assay, and extensive trial were conducted to establish a) its robustness and b) optimal conditions for fish tissue.

A common method used for this assay is by incubating tissue samples in a Tris-HCl buffer (pH 7.4) containing 1µl 14C-Arginine, 50µM $CaCl_2$ and co-factors (BH_4, 3µM; FAD, 1µM; FMN 1µM, NADPH 0.8µM), required for citrulline/NO synthesis from L-arginine, for 60 minutes. NOS activity is inhibited using a solution containing ion-chelating agents, at pH 5.5. This inhibits all NOS activity as Ca^{2+} chelation inhibits the Ca^{2+}-dependent eNOS and nNOS, and iNOS is inactive

at pH5.5. ^{14}C-citrulline is quantified by liquid scintillation counting after separation from ^{14}C-arginine by cation exchange. Citrulline is ionically neutral at pH 5.5, so it passes through cation exchange resin and any unconverted arginine, which is positively charged, remains bound to the resin.

Materials

All reagents were obtained from Sigma Aldrich, UK unless otherwise stated. 10X homogenisation buffer (250mM Tris-HCl, 10mM EDTA, 10mM EGTA, pH 7.4) and 1X stop buffer (50mM HEPES, 5mM EDTA, pH 5.5) were stored at room temperature until use. 20X reaction buffer (1M Tris-HCl, 120µM tetrahydrobiopterin, 40uM flavin adenine dinucleotide, 40uM flavin adenine mononucleotide, pH 7.4) was stored at -20°C till use. The homogenisation and reaction buffers were cooled and thawed on ice, respectively. A cation exchange resin was prepared by soaking 10g resin (Dowex 50WX8) in 3X 100ml 1M NaOH resin for 3 hours, each. The excess NaOH was removed by washing the resin repeatedly with distilled H_2O, until it reached pH≤6. Excess water was then poured out and replaced with Stop buffer. Some preliminary assays were carried out in the absence of BH_4, FAD and FMN as it was thought probable that endogenous co-factors would suffice. In these preliminary assays, the reaction volume was 400 µl.

Methods

Samples that were previously snap frozen in liquid nitrogen and stored at -80°C, were homogenized in ice-cold 1X homogenisation buffer in a Potter-Elvehejm Tissue Grinder to make a 2.5%-5% m/v solution. Samples were centrifuged at 10,000g for 15 minutes at 4°C (Hettrich Zentrifugen, Germany). For the preliminary assays, the working reaction mixture, per reaction was prepared on ice as follows:

100µl	tissue
50µl	[^{14}C]-arginine (Amersham Biosciences)
50µl	2% $CaCl_2$
50µl	NADPH
100µl	homogenisation buffer
50µl	L-NAME

Where L-NAME was not used, it was replaced by 50µl homogenisation buffer.

For later assays, the working reaction mixture, per reaction was prepared on ice, as follows:

25µl	2X Reaction buffer
5µl	10mM NADPH (made fresh in 10mM Tris-HCl, pH 7.4)
1µl	[^{14}C]-arginine (Amersham Biosciences)
5µl	6mM $CaCl_2$
4µl	dH_2O

The reaction mixture was added to a 1.5ml microcentrifuge tube, containing the desired volume of tissue with or without 5µl of 80mM L-NAME, and the final volume was made up to 60µl, on ice. The final reaction mixture was mixed well with a whirlimixer and centrifuged for 20 seconds at 6,500rpm. This was left at room temperature (22°C), unless otherwise stated, for 60 minutes to allow the reaction to take place. Assays at 4°C, were carried out in a refrigerator, for 5 hours. The reaction was terminated with 200µl Stop Buffer, at room temperature, mixed well, and centrifuged at 6,500 rpm. The solution was left to stand for 5 minutes at room temperature, after which mixing and centrifugation was repeated. 100µl ion exchanger resin (200µl for the preliminary assays) was applied to the microtube, and mixed with the whirlimixer. This was left to stand at room temperature, for 10 minutes, and was mixed again. Samples, *including all resin*, were then transferred to a 1.5ml spin column (Cayman chemicals) and centrifuged for 30 seconds at 6,500rpm, or until all the liquid passed into the collection tube. The eluent was transferred to a 5ml scintillation vial, 4.5ml scintillation cocktail (Packard SafeFluor) was added and radioactivity was measured using a scintillation counter (Packard MS 2500). Radioactivity was used as an index of citrulline synthesis and was expressed as pmol radioactive product synthesised/min

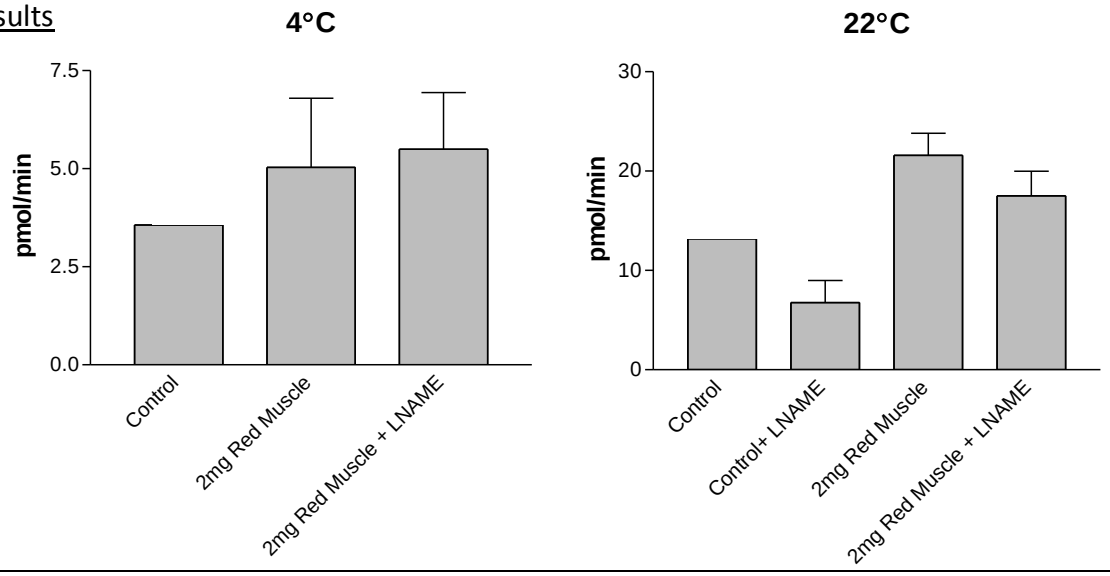


Figure 2.8. Effects of temperature on the citrulline assay, in the absence of exogenous co-factors, in common carp red muscle (RM). Citrulline assay with carp RM , 5 hours at 4°C, n=5 and 1 hour at 22°C (room temperature), n=8, except 0µg controls, for which n=2. Values expressed as mean ± SEM. Muscle samples were from carp acclimated to 5°C, tissue mass= 0-2mg. This assay showed no significant effect of NOS inhibition. ANOVA showed no difference between means.

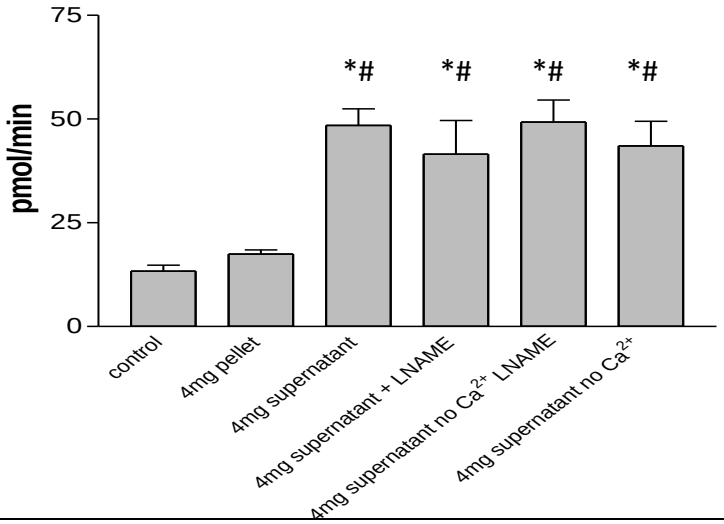


Figure 2.9.Citrulline assay, assessing the requirements of the pellet fraction and Ca^{2+}, carried out in the absence of exogenous NOS co-factors. The supernatant and pellets from carp RM homogenate (centrifuged at 10,000rpm), were used. Values are expressed as mean ± SEM, n=3-4, tissue mass= 0-4mg. This assay was carried out to identify whether the pellet fraction carried any nitric oxide synthase, to determine whether whole homogenate should be used for further assays to amplify the signal; and to assess the effect of Ca^{2+} inhibition. The assay showed no effect of NOS inhibition or Ca^{2+} omission from the assay, and that the pellet fraction contained a negligible amount of, if any, NOS. *P<0.01 compared with control, #P<0.01 compared with pellet fraction, ANOVA followed by Bonferroni's post-test.

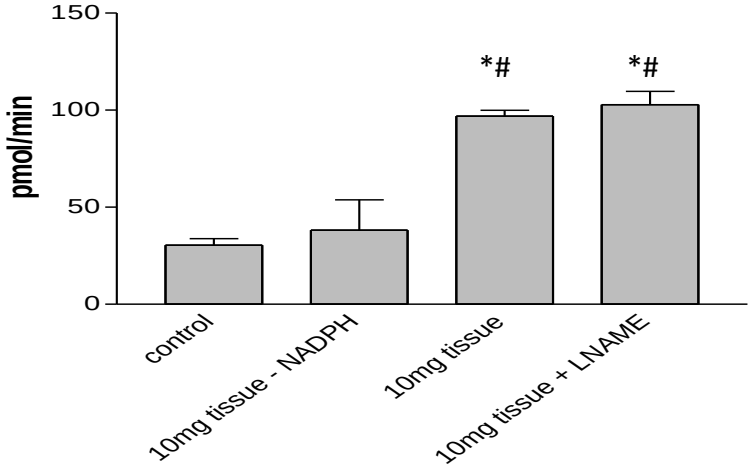


Figure 2.10. Citrulline assay carried out, in the absence of exogenous NOS co-factors, in mouse brain, tissue known to have high NOS activity, to assess the NADPH-dependence of this assay. Values are expressed as mean ± SEM, n=3, tissue mass= 0-10mg. This assay showed that the assay was NADPH-dependent but NOS-independent, in mouse brain. *P<0.01 compared with control, #P<0.01 compared with tissue without NADPH, ANOVA followed by Bonferroni's post-test.

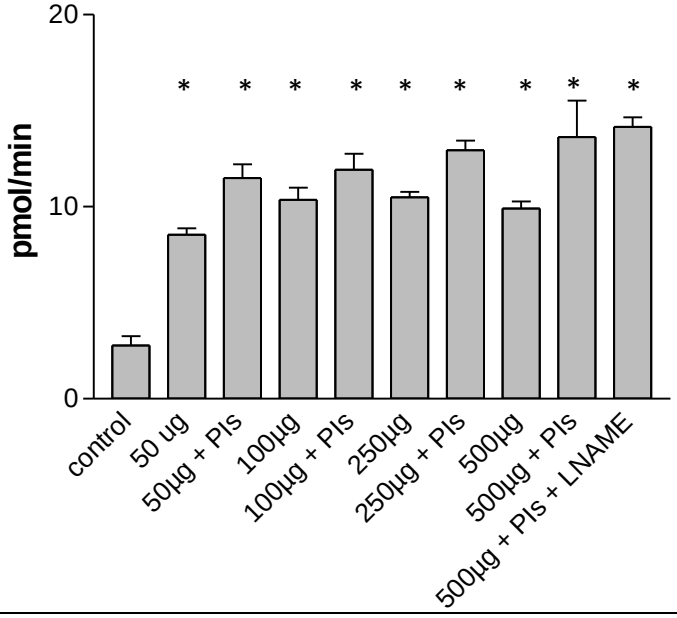


Figure 2.11.Citrulline assay carried out, in the presence of NOS co-factors, in rat liver tissue. Total reaction volumes in this assay are 40ul and mass of tissue used range from 0ug-500ug. Values are expressed as mean ± SEM, n=3. Red bars represent tissues homogenised in the presence of PMSF, and a protease inhibitor cocktail. *P<0.01 conpared with, ANOVA followed by Bonferroni's post-test. This assay showed that the use of protease inhibitors enhanced the activity slightly at each tissue concentration, though not statistically significantly. Overall, the reaction was not enzyme concentration-dependent, and was not attenuated by NOS inhibition, as with the previous assays.

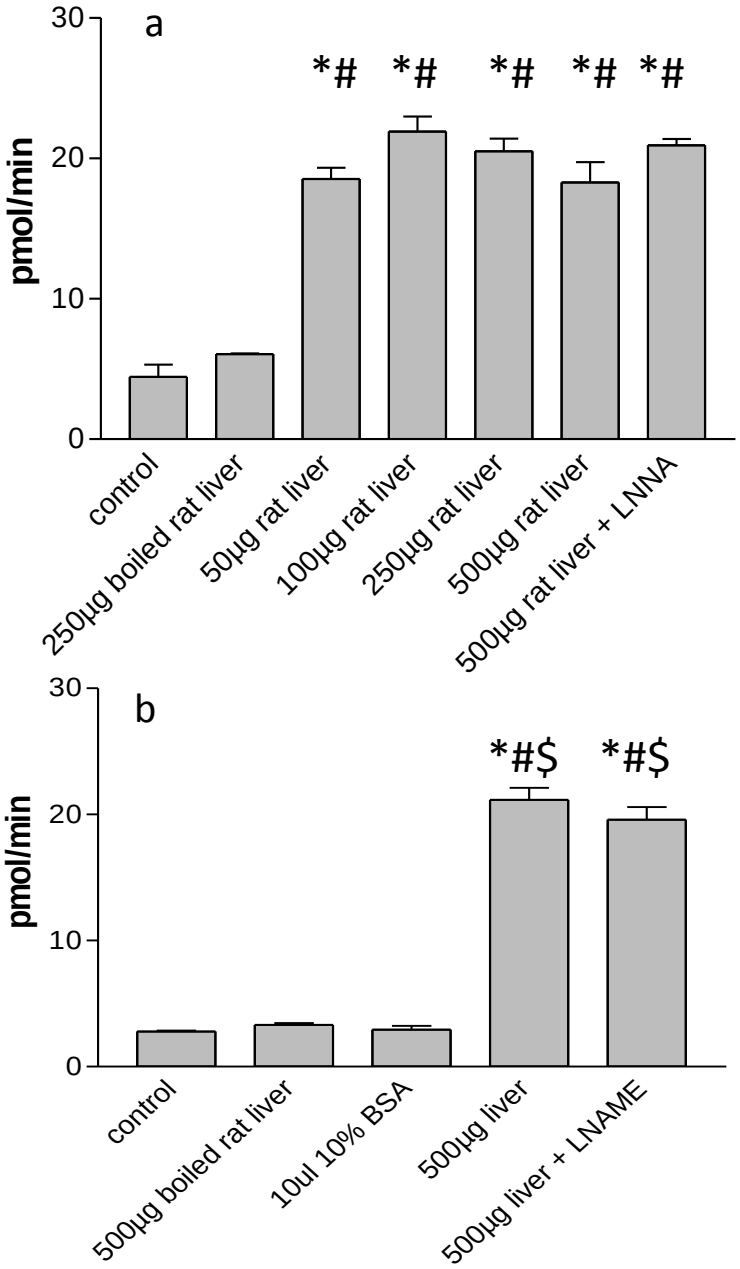


Figure 2.12**.** Citrulline assay carried out, in the presence of exogenous NOS co-factors, with a) boiled rat liver and b) bovine serum albumin. Values are expressed as mean ± SEM, n=3, tissue mass= 0-500µg**.** This assay was carried out to determine whether the response seen in previous assays was a non-specific protein response. Here, the response seen with BSA or boiled tissue was the same as background. Again, the response with intact tissue was not attenuated with NOS inhibition. Values are recorded as disintegrations per minute (dpm). *P<0.001 compared with control #P<0.001 compared with boiled liver, $P<0.001 compared with BSA, ANOVA followed by Bonferroni's post-test

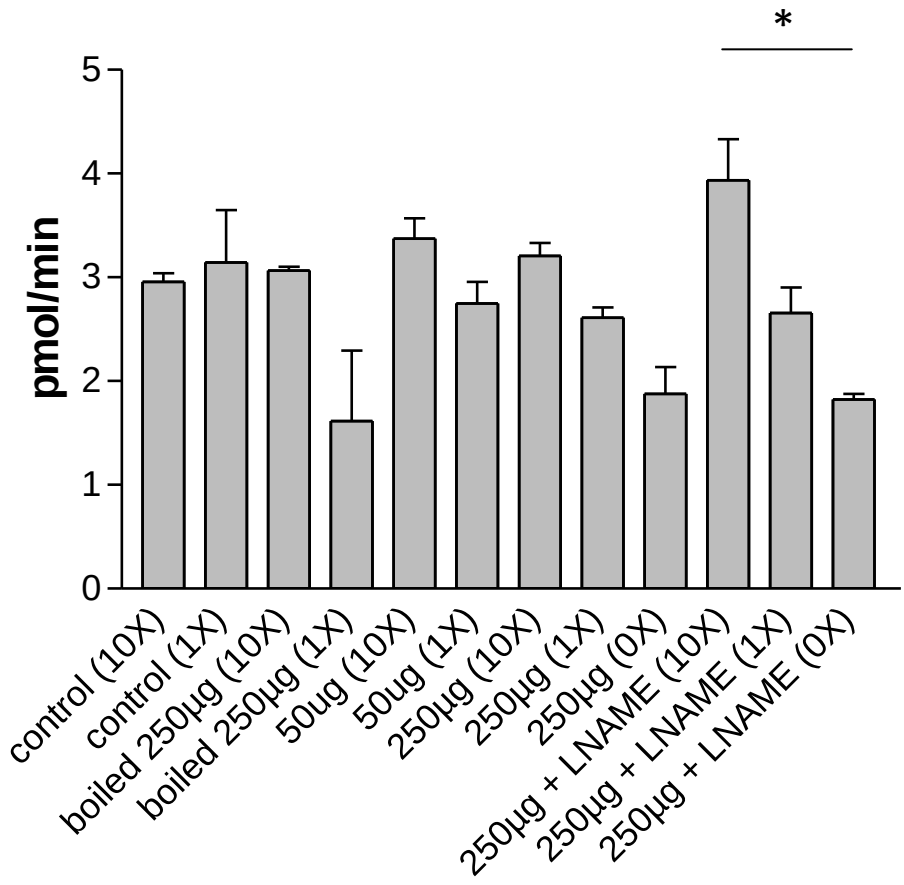


Figure 2.13.Citrulline assay carried out using rat brain with two concentrations of exogenous NOS co-factors, in the presence of two concentrations of tissue, and boiled tissue. Values are expressed as mean ± SEM, n=3. This assay was carried out to ascertain whether the response observed using the low tissue levels of rat liver, where the urea cycle is present, could be repeated in the rat brain, where the urea cycle is absent. With rat brain, there was no significant increase in activity, compared to control. However, in the presence of tissue, there was a NOS co-factor concentration-dependent response (though not statistically significant): in the absence of co-factors, activity levels were below 0ug control. *P<0.01, ANOVA followed by Bonferroni's post test

This investigaton showed that the radiolabelled citrulline assay that is commonly used to quantify NO synthesis is unreliable, and an alternative is required.

Initial assays (Figs. 2.8, 2.9, 2.10) carried out, in the absence of exogenous NOS co-factors, were used with relatively large volumes of tissue, ranging from 2-20mg of tissue, compared with later assays. Although some of these assays showed a response above control (no tissue), this response was not seen in all assays conducted (data not shown). Fig. 2.8 suggests that the response observed using carp red muscle was higher at 4°C than 22°C. However, the assays carried out at 4°C were for 5 hours, whereas the assays at room temperature were carried out for 1 hour. A previous observation (data not shown) was made that there was no response occurred at 4°C within one hour. To eliminate the possibility that any NOS was being discarded with the pellet after homogenisation and centrifugation, the pellet was also assayed (2.10) and no response above background was observed. The absence of an observable response may have been because the co-factors had degraded/been depleted in samples used in those assays that had not shown a response. However, co-factors are functional at 37°C, and BH_4, for example is recycled *in vivo*. Alternatively, the observed responses were not NOS-dependent, therefore the presence of NOS co-factors was unnecessary. That neither L-NAME, nor L-NNA was able to inhibit or attenuate the observed response in all assays (Fig. 2.8- 2.13) suggests that the citrulline assay did not demonstrate a NOS-dependent response.

Assays carried out to ascertain the optimum mass of tissue for this assay (Fig. 2.11 & 2.12a), showed with 50µg liver tissue, a 2-fold increase in radiolabelled citrulline and a 3-fold increase in the presence of protease inhibitors, but no further significant increase in activity with further increases in tissue (100, 250, and 500µg). In the preliminary assays, 1/3 of the citrulline was unlabelled. The lack of an enzyme concentration-dependent activity was unlikely to be due to substrate limitation as the relative conversion of arginine to citrulline was, at its highest, 28.9%. However, to be sure that labelled substrate limitation was not the cause, in all subsequent assays, the unlabelled arginine was replaced with labeled arginine so 100% of the arginine in the reaction was labelled, and therefore all citrulline synthesis could be measured. In these assays, no more than a 20% conversion rate was seen, and no more conversion was seen compared to the equivalent conditions where 1/3 of the citrulline was unlabelled. This further supports the conclusion that substrate limitation is not the cause of a lack of an enzyme concentration-dependent response. Other possibilities including co-factor limitation and further assays were carried out to investigate this.

Incubation of the tissue with the irreversible, non-selective NOS inhibitor, L-NAME did not attenuate the response observed, indeed it seemed to consistently augment the response, although this was not statistically significant. This suggested that the response observed was mainly a NOS-independent conversion of arginine to an ionically neutral substance that may or may not be citrulline, and NOS blockade, by reducing competition for arginine, promoted the

observed response. To ensure the response observed was not a non-specific response to protein, the assay was carried out with both boiled enzyme and 10% BSA (Fig. 2.12), and the response was the same as control (homogenisation buffer only).

As the rat liver has a urea cycle it was thought that the product measured may have been arginase-mediated breakdown of arginine. In order to investigate this possibility, the assay was carried out with rat brain, where there is high NOS activity, but no reported arginase activity (Fig. 2.13). This demonstrated no activity above control with 50µg or 250µg tissue. Further, it revealed a co-factor concentration-dependent response, in the presence of tissue. It seemed that with higher co-factor concentrations, the activity levels were the same as control, but in the absence of co-factors, the activity level was below control. This suggests that the co-factors themselves interact with arginine directly, inducing conversion of arginine into a neutral product. The findings also suggest that this assay is not sensitive enough to detect nitric oxide synthesis at such low levels.

A scaled-up citrulline assay with rat liver, mouse brain and white and red skeletal muscle from rainbow trout, where 2.5mg tissue was used per assay, was tested. Briefly, tissue was homogenised in and samples were tested with and without co-factors using 500µl tissue homogenate. In these assays, the charged resin was allowed to settle at the bottom of the test tube, and a portion of the solution was pipetted off the top for measurement. This method also

showed a lack of sensitivity, and neither L-NAME nor LNNA were able to inhibit the reactions. There was no significant increase in radioactivity when tissue was added, compared to control.

2.8.2 Measurement of nitric oxide synthesis using an amperometric sensor

NO synthesis can be monitored continuously using an NO-sensitive electrode that measures real-time NO synthesis *in vivo* and *in vitro*. The Apollo 4000 NO-sensing system (World Precision Instruments), like other amperometric NO detection systems utilises a gas-permeable, hydrophobic membrane that allows diffusion of NO to the electrode surface while limiting access of hydrogen peroxide, NO_2^- and other ionic species. A potential is applied to the probe relative to a reference electrode. The electrode is applied to the sample and as NO is produced; the current due to the electrochemical oxidation of NO is monitored according to this reaction:

$$NO + 4OH- \; NO_3 + 2H_2O + 4e-$$

The slope of NO detection is the maximal NOS activity in the sample.

Methods

Samples were homogenised (50mg/ml), on the day of use, in ice-cold phosphate buffer (100mM K_2HPO_4; 100mM KH_2PO_4; 0.1mM EDTA; pH 7.4), using a Potter-Elvehejm tissue grinder and stored on ice until use. The NO-meter was calibrated as per the manufacturer's instructions, by

the chemical generation of known amounts of NO, according to the reaction: $2NaNO_2 + 2KI + 2H_2SO_4 \rightarrow 2NO + I_2 + 2H_2O + K_2SO_4 + Na_2SO_4$.

All reactions were carried out in a 5ml beaker sufficiently deep to allow immersion of the electrode without touching the base of the beaker, which was filled with 5ml phosphate buffer and a magnetic flea was placed at the bottom to stir the contents at all times so all added substances were evenly distributed. The responses to rat liver (a NOS-abundant tissue) alone and in the presence of 5µM bradykinin (a stimulator of eNOS), a cocktail of NOS co-factors (3µM BH_4; 4mM NADPH; 1µM FAD, 1µM FMN) and the antioxidant glutathione (25mM) was tested (Fig. 2.14).

Although this method of NO detection can be used to measure NO production in isolated blood vessels from rats (Ray *et al.*, 2002), it was not sensitive enough for use in homogenised rat liver. This may have been because in the preparations described by Ray *et al* (2002) the tip of the electrode was placed apposed to the exposed endothelium, whereas in the liver homogenate, cells other than endothelial cells were present and are likely to have come in contact with the electrode. The volume-dependency of the change in current (Fig. 2.14iii) also suggests that the electrode is highly sensitive to movement and any responses seen were a direct result of change in volume and not the actions of the mediators on the tissue.

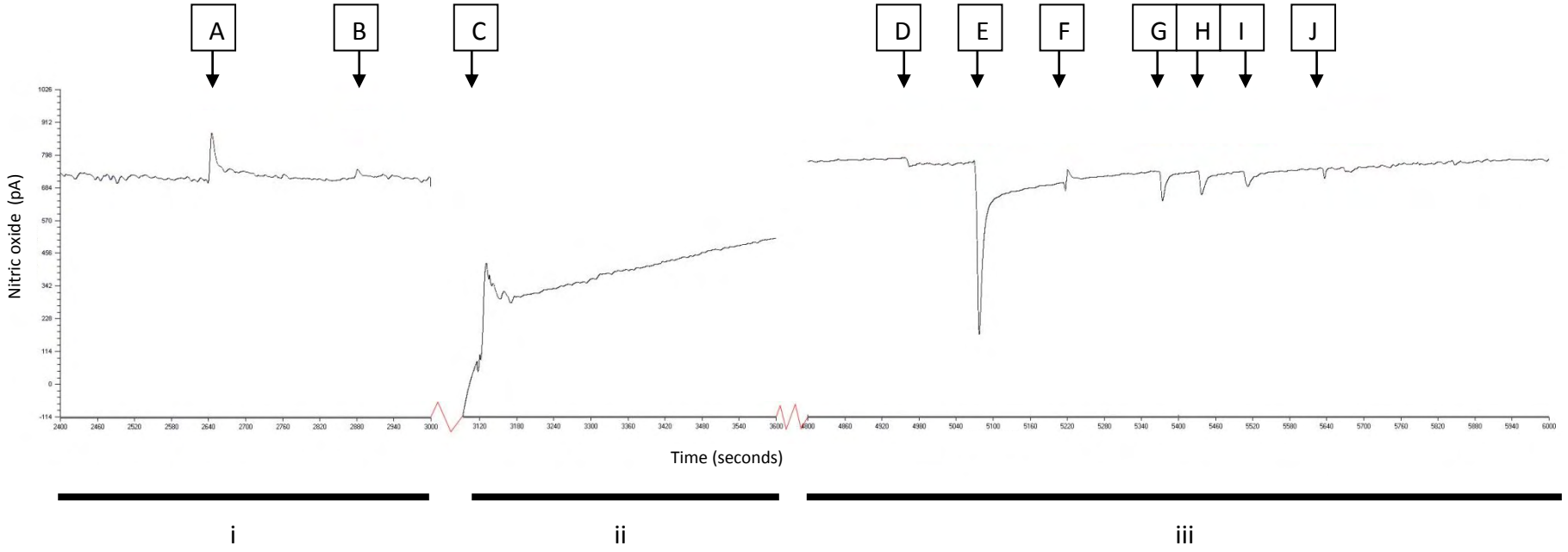


Fig. 2.14. Original trace showing the responses evoked by nitrite (region i), rat liver, bradykinin (BK), co-factors and glutathione (region iii), expressed in picoAmps. Region i shows that nitrite produces a response as described previously in the literature (Ray *et al*., 2002). However, responses evoked by the NOS-abundant liver, bradykinin, and co-factors of NOS activity elicit a response that is dissimilar to that evoked by nitrite. Region ii, leading to the early portions of region iii demonstrates the sensitivity of movement from one buffered solution to another. The responses evoked by all substances other than nitrite, suggest that the electrode is also sensitive to ions from other solutions and these are displayed as a reduction in the current. A= 1µM nitrite; B= 0.5µM; C= changed phosphate buffer solution; D= 100µl rat liver (50mg/ml); E= 500µl BK (5µM); F= PBS (50µl); G= 100µl BK; H= 100µl cofactors; I= 100µl glutathione; J= 20µl BK

Chapter 3 Shear stress and angiogenesis

3.1 Introduction

In humans, blood viscosity, the resistance of blood to flow, is increased in conditions such as polycythaemia (overproduction of erythrocytes) which occurs in response to chronic hypoxia, in tumours, myoproliferative diseases (excess cell production by the bone marrow), and in acute conditions such as dehydration (high haematocrit due to low plasma water content) (Levick, 2003). Blood may also become more viscous during chronic cold exposure, particularly at the extremities and in the skin microcirculation. Changes in environmental temperature can leave some species faced with suboptimal conditions, such as reduced tissue perfusion due to increases in blood viscosity, and reduced kinetic energy of oxygen molecules leading to reduced rate of cellular diffusion (see Chapter 1). As there is strict thermoregulation in mammals, cold-induced increases in blood viscosity are short-lived. In contrast, a lack of well-controlled thermoregulation has meant that ectotherms need to accommodate changes in environmental temperature, and have developed methods of offsetting the adverse effects of viscous blood on effective convective transport. Elucidation of the mechanisms by which ectotherms acquire reduced thermal sensitivity may help identify mechanisms that may be exploited by mammals to cope with extreme temperature changes. In addition, identifying mechanisms of viscosity-induced angiogenesis will provide us with a more complete understanding of physiological control of angiogenesis, and help uncouple mechanical angiogenesis from metabolic-driven

angiogenesis. Given that mammals usually resist temperature change, and prolonged increases in blood viscosity cannot be achieved by cold-acclimation, fish, which have comparable elements of their cardiovascular system (see Chapter 1), are an ideal model for this investigation.

In trout, although total cardiac output (CO) decreases with temperature, the distribution of CO to red muscle increases at low temperature (Barron *et al.*, 1987), so despite an observed bradycardia in cold-exposed animals, we hypothesised that with the combined influences of increased blood flow and blood viscosity, shear-induced angiogenesis will occur at low temperatures. At high temperatures, despite a high CO, we propose that reduced blood flow through red muscle as a result of differential tissue perfusion may be a cause of capillary regression in warm-acclimated animals.

Rainbow trout are a temperate fish species that naturally inhabit waters ranging from 4-18°C. In this study on skeletal muscle, the effects of long term acclimation, to their extreme thermal tolerance levels were investigated. The parameters measured were capillarity, red and white muscle fibre size, and the condition factor of the fishes. The condition factor, which is influenced by age, sex, maturation, fullness of gut, fat reserve, muscular development and temperature was used as an indication of the health of the fish, as fish with a high mass for their length are considered to be in good physiological condition (Bolger & Connolly, 1989).

In the wild, rainbow trout show little overall phenotypic changes with temperature (for example capillarity changes are slight compared to laboratory-acclimated fish (Egginton & Cordiner, 1997)), although an increase in the activity of key metabolic enzymes such as citrate synthase in cold-acclimatised fishes, suggest that metabolic adaptation takes place (Cordiner & Egginton, 1997; Somero, 1995). Previous acclimation studies have shown that fishes respond markedly to chronic temperature decrease, with angiogenesis (Day & Butler, 2005; Egginton & Cordiner, 1997; Egginton & Sidell, 1989; Johnston, 1982). An increase in C:F, by 107%, in crucian carp (Johnston & Maitland, 1980) acclimated to 5°C compared to 25°C, a 47% increase in rainbow trout (Egginton & Cordiner, 1997) acclimated to 11°C compared to18°C, a 41% increase in striped bass acclimated to 5°C compared to 25°C (Egginton & Sidell, 1989) and a 18% increase in brown trout acclimated to 5°C compared with 15°C (Day & Butler, 2005), suggests that the scope for capillary growth is species-dependent, though the direction of growth is similar i.e. capillarity increases at low temperature. However, in some cases, despite an increase in C:F, fibre hypertrophy with cold-acclimation can bring about a decrease in CD (Egginton & Cordiner, 1997). In the latter studies, fish were acclimated to a common (11°C) tank temperature for different durations that were unrecorded, before being exposed to their experimental temperature. In addition, photoperiod was not controlled in order to investigate the influence of seasons on cold-induced angiogenesis (Cordiner & Egginton, 1997; Day & Butler, 2005; Egginton & Cordiner, 1997)). In the current study, animals were allowed to acclimate to a

common temperature for 4 weeks prior to the experimental start date, and placed in the tank in which they were to be kept for the duration of the experiment to minimise the likelihood that adaptations were stress-related. Furthermore, the influence of season was minimised by maintaining the same photoperiod by artificial lighting under controlled laboratory conditions during the course of the study.

The effects of alterations in vascular tone, by the inhibition of archetypal examples of vascular control mechanisms- including blockade of NO synthesis (using L-NNA to block nNOS and eNOS), α-adrenoceptor (with prazosin) and prostaglandin synthesis (cyclooxygenase inhibition using indomethacin)- were investigated to identify the effects of temperature-induced changes in blood flow. Prazosin is a selective competitive α_1-adrenoceptor antagonist, which has previously been used to induce peripheral vasodilatation in hypertensive humans; indomethacin is a COX-inhibitor and blocks the synthesis of all COX-dependent proteins including prostaglandins and thromboxanes; L-NNA is a non-selective reversible competitive NOS inhibitor, which blocks eNOS and nNOS far more effectively than it does iNOS (Furfine *et al.*, 1993). Shear stress is more easily pharmacologically modulated in conditions where blood vessels may already be experiencing elevated shear stress (cold-induced increased blood viscosity) or where shear stress is likely to be low (warm-acclimated animals are expected to have less viscous blood).

It was hypothesised that causing vasoconstriction by NOS blockade or COX inhibition at low temperatures would reduce shear stress on the capillary endothelium and inhibit cold-induced angiogenesis. Conversely, we expected that warm-acclimation would cause capillary regression due to reduced blood viscosity, therefore reduced shear stress. We hypothesised that warm-induced capillary rarefaction could be reversed by increasing shear stress by increasing blood flow through capillaries using the arteriolar vasodilator, prazosin. Based on the current literature, we also hypothesised that cold-acclimation would induce hypertrophy causing stretch and strain on endothelial cells. As such, we expected to observe a cold-induced splitting and sprouting (demonstrated by cell proliferation) angiogenesis.

3.2 Methods

3.2.1 Animal husbandry and drug administration

For animal husbandry conditions, refer to General Methods (Chapter 2). Trout (for fish numbers see Appendix A) were acclimated to 11°C for four weeks prior to acclimation to experimental temperatures. As metabolic rate, food consumption and by extrapolation, growth is proportional to temperature in fishes, trout were selected so that those with lower starting weights were acclimated to higher temperatures, and those that had higher starting masses were cooled, in an attempt to compensate for differences in growth rate and hence normalise experimental body mass. However, this was unsuccessful and the variation of sizes within and

between groups was large and many of the individual trout seemed to be in poor condition - their responsiveness to human presence had declined markedly and their feeding had reduced. There were many unexpected fish deaths during the course of this study and it is suggested that these trout may have been suffering from tank stress or bacterial infection. There was no evidence for the latter in the prazosin and LNNA-treated trout. However, there were infection markers on the skin of many fish in the indomethacin-treated trout.

Where drugs (L-NNA, prazosin or indomethacin) were administered *per os* (p.o.), the drugs were dissolved (L-NNA and prazosin in Birmingham tap water, and indomethacin in ethanol) and poured into homogenised and gelatinised fish food, which were subsequently re-pelleted. It was not expected that drugs would leach out of the food before it was eaten, because under normal circumstances, when pellets are put into water, a feeding frenzy ensues immediately. Dose estimates were based on average food consumption rates of 1.5g of food a day and the amounts of each drug combined in the food were based on previous studies (in brackets) that demonstrated an effect of the drugs when administered p.o. in mammals:

Prazosin- 116µg/g food (Williams *et al.*, 2006a)

LNNA 233µg/g food (Williams *et al.*, 2006a)

Indomethacin 1mg/g food (Pearce *et al.*, 2000)

During the course of the indomethacin study, it was discovered and verified by the animal technicians that some of the trout had been removed from the tanks and replaced at a later date. As such, some of the trout had not been exposed to the full length of treatment. It was not possible to identify and separate those that had been added to each group later than others as fish were collected randomly from each tank and were not individually tagged.

3.2.2 Winter and summer trout

Trout in this study were separated and described according to the time of year at which they were brought into the laboratory from their source. Winter trout are those that were brought into the laboratory in December and January, and summer trout are those that were brought in during May and June.

3.2.3 Cooling regime and histochemistry

Animals were cooled and warmed as described in section 2.1. Briefly, all animals were acclimated to 11°C and they were cooled (to 4°C) or warmed (to 18°C) at a rate of 1°C/hr. Animals were sacrificed and samples taken at selected time points as described in section 2.2. Tissues were stained for alkaline phosphatase (section 2.5.1) for capillary visualisation, tissue VEGF (2.6.1) to demonstrate angiogenesis, and PCNA (2.6.2) to identify proliferating cells.

3.2.4 Statistical analysis

Capillary density (CD), capillary to fibre ratio (C:F) and mean fibre cross-sectional area (MFA) were quantified as described in section 2.6.3 and are expressed as mean ± SEM. For acclimation experiments with and without drugs (Figs. 3.1- 3.5 and Tables 3.1 and 3.2), data were analysed using GraphPad Prism software. Comparisons between all conditions were made by analysis of variance (ANOVA) and $P < 0.05$ was considered significant. Bonferroni's post-test was carried out when ANOVA showed $P<0.05$, to identify which conditions were significantly different from one another. Biological replicates (number of trout used) ranged from 2-7, and technical replicates (number of images from each trout sample) ranged from 7-8. Due to the low number of biological replicates and unequal sample sizes, tests for normality or equal variance were not carried out.

3.3 Results

3.3.1 Cold-acclimation (L-NNA groups)

3.3.1.1 Red muscle

Summer trout that were cold-acclimated (CA; Fig. 3.1A) had an increase in C:F from 1.0 ± 0.04 to 1.2 ± 0.07 after 1 week of cold acclimation, and an increase in CD (from 664.9 ± 94.9 to 804.9 ± 39.9mm^{-2}), and a further increase in C:F (1.4 ± 0.2) and CD (990.03 ± 30.5) by 4 weeks. By week 8, the C:F decreased to 1.1 ± 0.06, although the decrease in CD was minor (925.6 ± 80.3 mm^{-2})

(Fig. 3.1A). This can be explained by the decrease in MFA seen at this time-point (1121.9 ± 14.6 μm^2) compared to all other time-points (which ranged from 1439.7 ± 229.1 to 1471.4 ± 96.7 μm^2). Therefore, there was not a true capillary rarefaction; instead a decrease in MFA increased the number of capillaries per fibre (C:F) but not the fibre area served by a capillary. MFA remained unchanged at all temperature and time-points (without drug treatment) (Fig. 3.1A). None of the changes described above were statistically significant and the low number of biological replicates (n=2-3) used because of unexpected fish deaths during the course of the experiment was insufficient to draw conclusions.

In the winter trout (Fig 3.1B), CA did not increase C:F over the 8 week acclimation period, although CD increased from 1119.3 ± 65.4 to 1233.8 ± 124.2 mm^{-2} from 1 week of CA to 4 weeks CA (note: the 11°C control is absent in this group of trout, thus is not used as a comparison point). In this group, temperature acclimation had no effect on MFA (Fig. 3.1B).

In summer trout, there was a significant decrease in C:F in the red muscle of L-NNA treated trout that were cold acclimated for 4 weeks (0.8 ± 0.12) compared with untreated CA trout (1.41 ± 0.18, $P< 0.05$) (Fig. 3.1A), and a non-significant decrease in CD (685.9 ± 100.5 vs 804.9 ± 39.7 mm^{-2}). However, C:F and CD increased non-significantly in 11°C trout treated with L-NNA

(CF: 1.25 ± 0.03, CD: 846.9 ± 38.9 mm^{-2}), compared with 11°C untreated (CF: 1.0 ± 0.04, CD: 644.91 ± 94.9 mm^{-2}).

CA did not induce an increase in C:F in winter L-NNA-treated trout. It is unclear whether there was an increase in C:F in L-NNA treated fish at 11°C compared with untreated fish, due to the absence of the latter group. The CD of L-NNA treated trout at 4°C was lower, although this was again not statistically significant and was not as pronounced as that observed in summer trout (1233.8 ± 124.2 vs. 1008.7 ± 105.9 mm^{-2}), which was also not statistically significant. The MFA of L-NNA-treated trout at 11°C (1455.3 ± 160.3 μm^2) and 4°C (1365.7 ± 17.7 μm^2) were higher than in any other condition in this group of trout, again not statistically significantly.

Although trends towards an increase or decrease in capillarity were sometimes observed, the changes described above were not statistically significant as analysed by ANOVA, unless stated otherwise, and this is due to the low number of biological replicates (n=2-3). Repetition of the above experiments was not possible owing to time and financial constraints.

3.3.1.2 White muscle

This study was carried out in collaboration with S. Rehman, University of Birmingham. In summer CA trout, there was little change in C:F until 8 weeks, when C:F increased by 29% (n.s.).

However, there was a 24% increase in CD from 11°C to 1 week CA and a 26% increase in CD by 8 weeks (Table 3.1; n.s.). Within 1 week of CA, MFA decreased by 13%, but then increased by 8 weeks to control levels (n.s.). There was no effect of L-NNA treatment in these trout.

In winter trout, the C:F and C:D were consistently higher than summer trout (Table 3.1), and neither CA, nor LNNA affected either estimate of capillarity. Unlike in summer trout, in winter trout, MFA increased from 1 -4 weeks CA by 20%, but decreased to week 1 levels by 8 weeks. In this study, there was no 11°C (control) group for comparison. Neither summer nor winter trout were affected by L-NNA treatment at low or high temperatures. The changes described here were not statistically significant, and the inconsistency between individual trout renders this study invalid and the findings inconclusive.

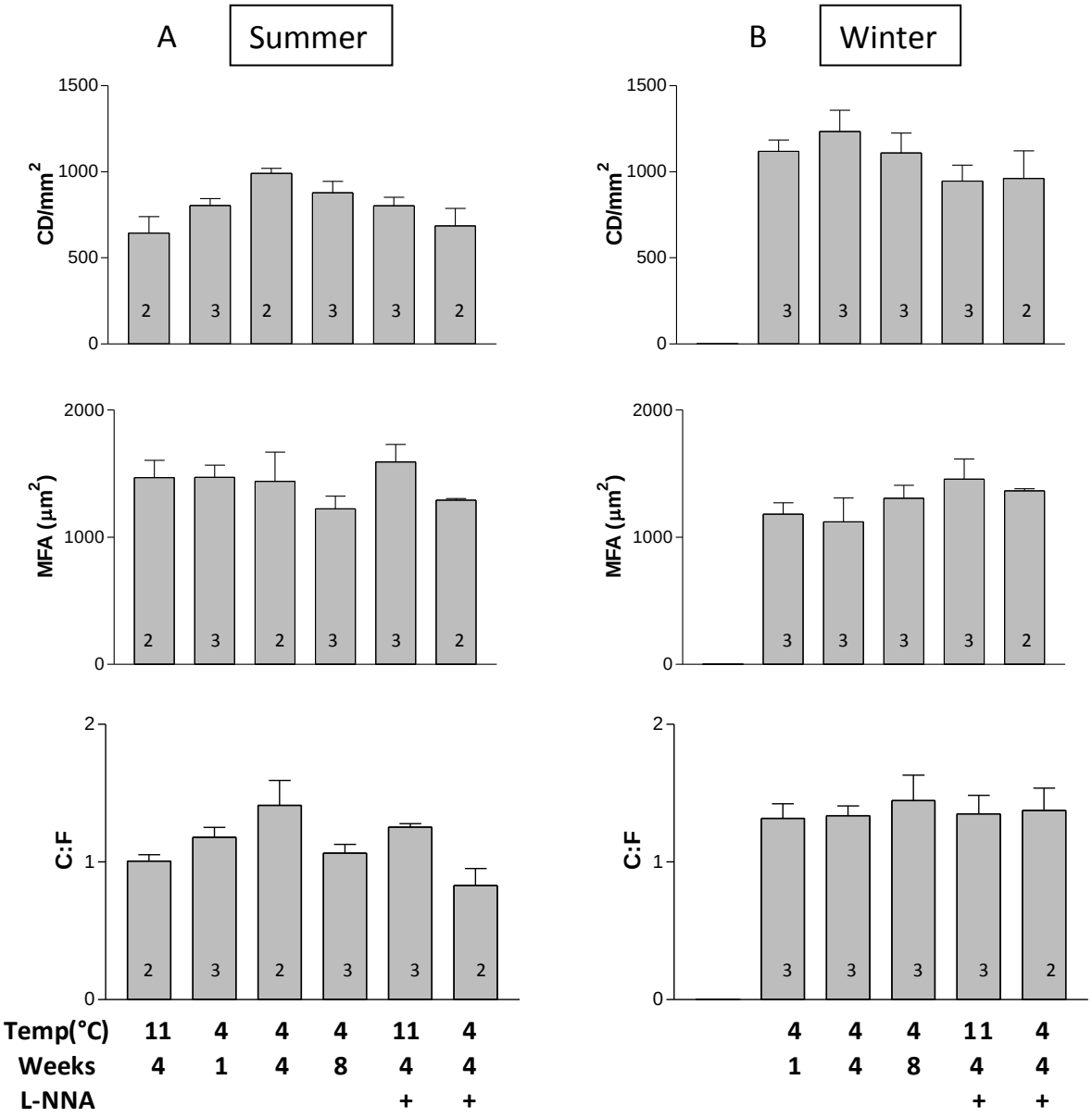


Figure 3.1. Capillary: fibre ratio (C:F), capillary density/mm^2 (CD) and mean fibre area (MFA; µm^2), in red muscle of summer and winter cold-acclimated trout, with and without L-NNA. Biological replicates (n) = 2-3, technical replicates= 7-8. Numbers within the bar= n value. Data are expressed as mean ± SEM. ANOVA was followed by Bonferroni's post-test *P<0.05

			11°C (week 4)	4°C (week1)	4°C (week4)	4°C (week8)
	summer	C:F	0.31 ± 0.02	0.32 ± 0.05	0.33 ± 0.04	0.40 ± 0.11
		CD	517 ± 30.5	639 ± 127.1	633 ± 5	652 ± 2
		MFA	5968 ± 90	5177 ± 256	5255 ± 248	6167 ± 1510
L-NNA		C:F	0.36 ± 0.04	–	0.35 ± 0.04	–
		CD	615 ± 38	–	589 ± 25	–
		MFA	5976 ± 822	–	5980 ± 671	–
	winter	C:F	–	0.49 ± 0.01	0.48 ± 0.02	0.44 ± 0.00
		CD	–	894 ± 72	724 ± 44	904 ± 37
		MFA	–	5603 ± 330	6726 ± 351	5583 ± 348
L-NNA		C:F	0.46 ± 0.01	–	0.49 ± 0.03	–
		CD	892 ± 47	–	795 ± 62	–
		MFA	5263 ± 328	–	6317 ± 844	–

Table 3.1. Capillary: fibre ratio (C:F), capillary density/mm^2 (CD) and mean fibre are (MFA; μm^2), in white muscle of summer and winter cold-acclimated trout, with and without L-NNA. Biological replicates= 3-4, technical replicates= 7-8. Data are expressed as mean ± SEM. ANOVA showed no significant difference between means. Horizontal lines denote unavailable data.

		11°C (week 4)	4°C (week1)	4°C (week4)
	C:F	0.86 ± 0.07	0.71 ± 0.030	0.66 ± 0.035
	CD	631 ± 23	471 ± 7	566 ± 5
	MFA	1394 ± 151	1520 ± 329	1185 ± 71
Indo	C:F	0.75 ± 0.02	–	0.71 ± 0.07
Indo	CD	560 ± 16	–	554 ± 30
Indo	MFA	1353 ± 588	–	1295 ± 121

Table 3.2. Capillary: fibre ratio (C:F), capillary density/mm^2 (CD) and mean fibre are (MFA; µm^2), in red muscle of cold-acclimated trout, with and without indomethacin (Indo). Biological replicates= 3-4, technical replicates= 7-8. Data are expressed as mean ± SEM. Horizontal lines denote unavailable data. ANOVA showed no significant difference between means.

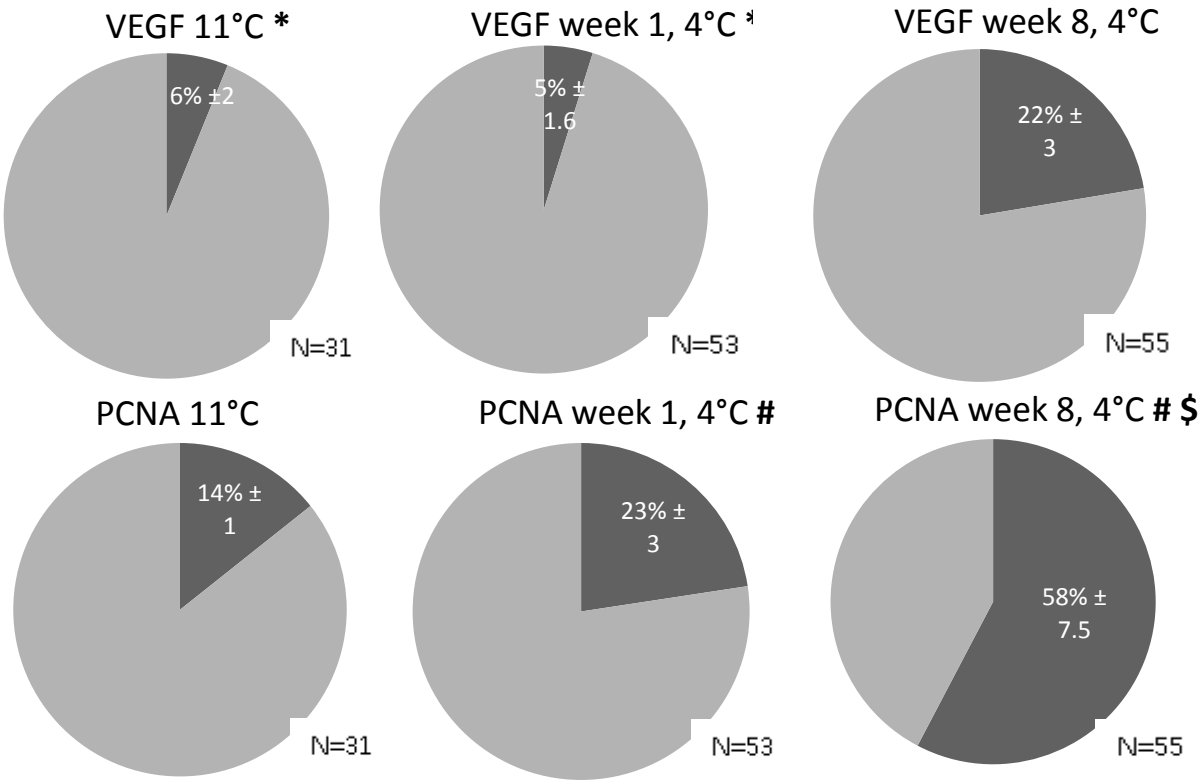


Fig 3.2 Percentage VEGF-positive capillaries and PCNA-positive capillaries in pooled white muscle samples from summer and winter cold-acclimated trout at 1 and 8 weeks, compared to control (11°C).Values expressed as mean ± SEM. Biological replicates (n) = 3. N= mean number of capillaries per field. *P<0.05 vs VEGF week 8. # P< 0.05 vs. PCNA 11°C, $ P< 0.05 vs. PCNA week 1, 4°C.

Despite the lack of a statistically significant increase in capillarity, the number of VEGF-positive capillaries increased from a baseline of 6% VEGF staining at 11°C to 22% by 8 weeks CA in white muscle from pooled summer and winter trout (Fig. 3.2). This suggests increased VEGF production may have been stimulated in this muscle although not sufficient to trigger a visible capillary growth. PCNA- positive capillaries increased from 14% at 11°C, to 58% at 8 weeks of CA, showing that substantial cell proliferation had occurred in CA trout but the origin of the PCNA was not identified so the cell type undergoing proliferation is unknown (Fig. 3.2).

3.3.2 Cold acclimation (indomethacin group)

This study was carried out in collaboration with S. Rehman (University of Birmingham). Analysis of the effects of CA in the presence and absence of indomethacin was carried out in red muscle. There was some suggestion of a decrease in CD and C:F by 1 week of CA, followed by an increase in CD, and a decrease in C:F by 4 weeks (Table. 3.2), but these changes were not statistically signficant. MFA transiently increased at 1 week CA but this was followed by a 22% decrease in MFA, by 4 weeks, perhaps explaining the decrease in C:F despite an increase in CD at 4 weeks CA. Indomethacin did not significantly influence C:F, CD or MFA.

3.3.3 Cold acclimation (prazosin group)

This study was carried out on the red muscle of summer trout. In this group, CA did not cause any significant changes in C:F, CD or MFA (Fig. 3.3). Although there is an increase in the mean CD in CA prazosin treated trout compared to CA untreated trout at 4 weeks, the SEM is high, thus this was not statistically significant.

3.3.4 Warm acclimation (L-NNA group)

This study was carried out on red muscle of winter trout. In this group (Fig. 3.4), warm acclimation (WA) did not change C:F, CD or MFA. LNNA treatment caused a non-significant increase in mean CD at 11°C (969.6 ± 91 vs. 1213.9 ± 9.2 mm^{-2}) and 18°C (941.9 ± 61.7 vs. 1154.9 ± 158.1 mm^{-2}).

3.3.5 Warm acclimation (prazosin group)

This study was carried out on red muscle of winter trout. In this group (Fig. 3.6), WA brought about a non-significant decrease in C:F by 4 weeks, but increased after 8 weeks WA. Prazosin did not influence C:F, CD or MFA.

3.3.6 Condition factor and C:F, CD and MFA

There was no correlation between the condition factor and C:F, CD and MFA in any of the trout used in this study. Furthermore, the range of condition factors was large (between 7 and 15), so

there was little to no consistency in the health status of the animals rendering this study invalid (Fig. 3.6).

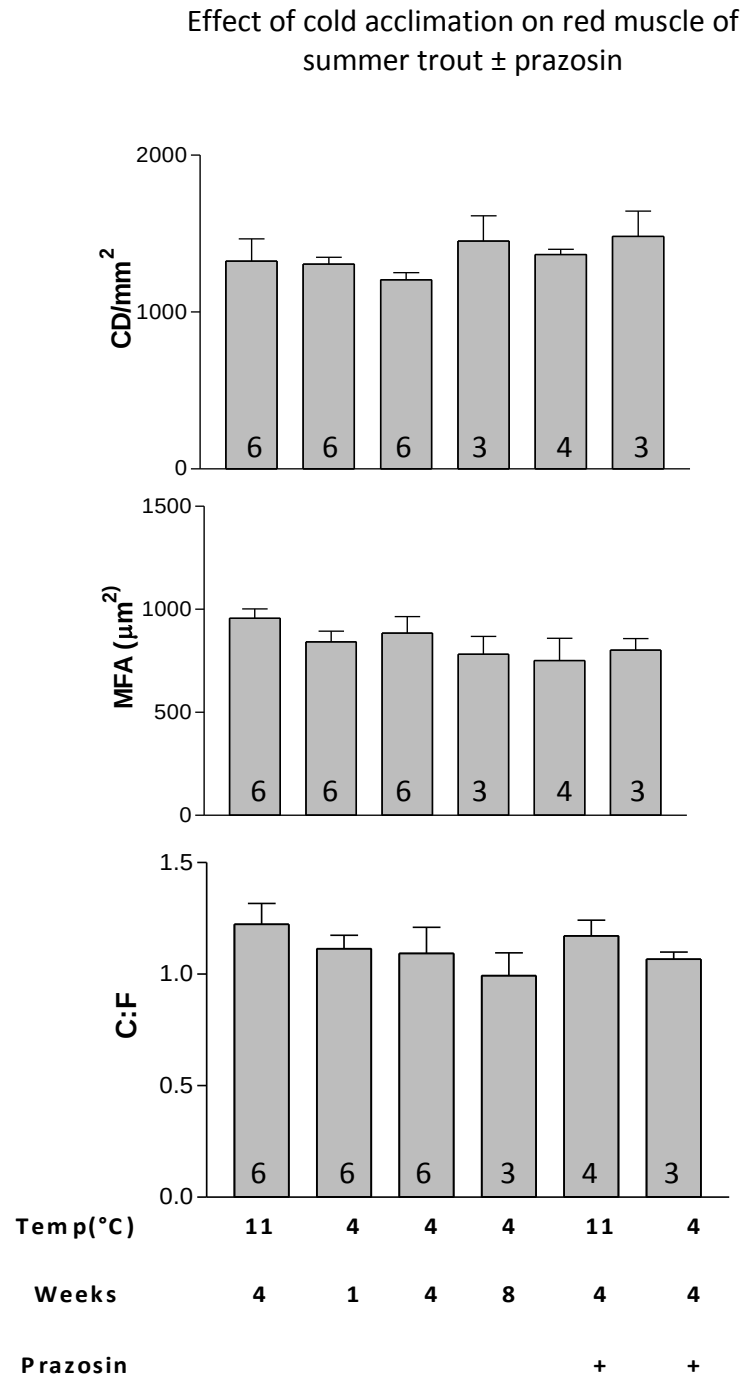


Figure 3.3 Capillary: fibre ratio (C:F), capillary density/mm^2 (CD) and mean fibre cross-sectional area (MFA; µm^2), in red muscle of summer cold-acclimated trout, with and without prazosin. Biological replicates(n; numbers in bars represent n for that condition)= 3-6 , technical replicates= 7-8. Data are expressed as mean ± SEM. ANOVA showed no significant difference between means.

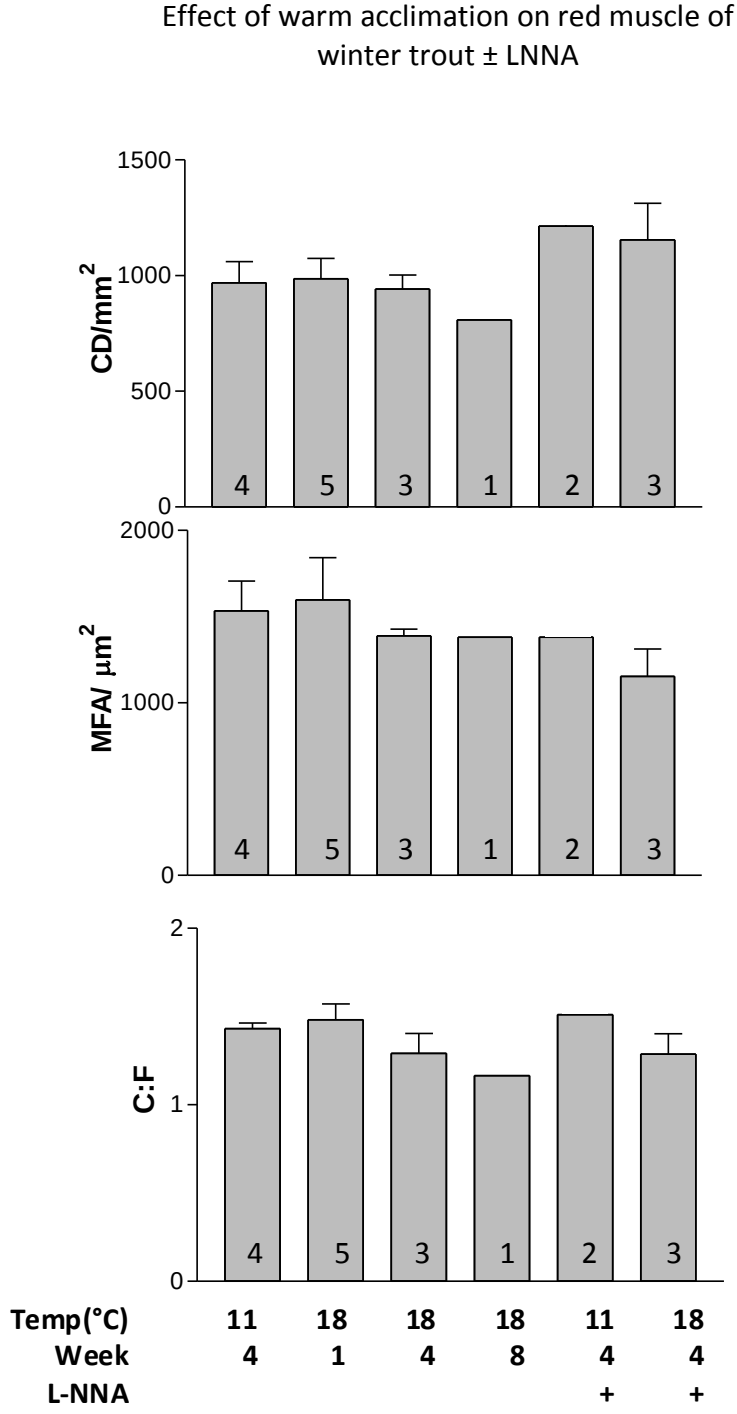


Figure 3.4 Capillary: fibre ratio (C:F), capillary density/mm^2 (CD) and mean fibre cross-sectional area (MFA; µm^2), in red muscle of winter warm-acclimated trout, with and without L-NNA. Biological replicates= 2-6, except 8 weeks at 18°C, where there was 1 biological replicate, technical replicates= 7-8. Data are expressed as mean ± SEM. ANOVA showed no significant difference between means.

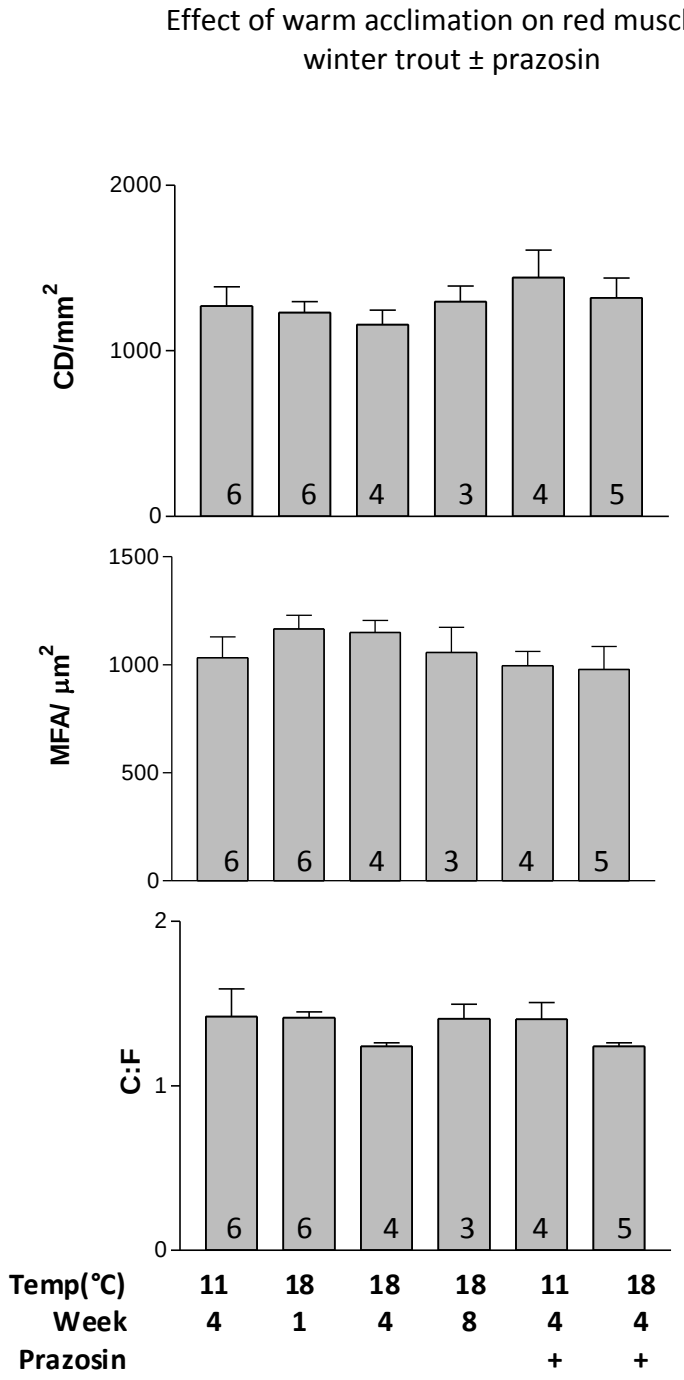


Figure 3.5 Capillary: fibre ratio (C:F), capillary density/mm^2 (CD) and mean fibre cross-sectional area (MFA; µm^2), in red muscle of winter warm-acclimated trout, with and without prazosin. Biological replicates= 3-6, technical replicates= 7-8. Data are expressed as mean ± SEM. ANOVA showed no significant difference between means.

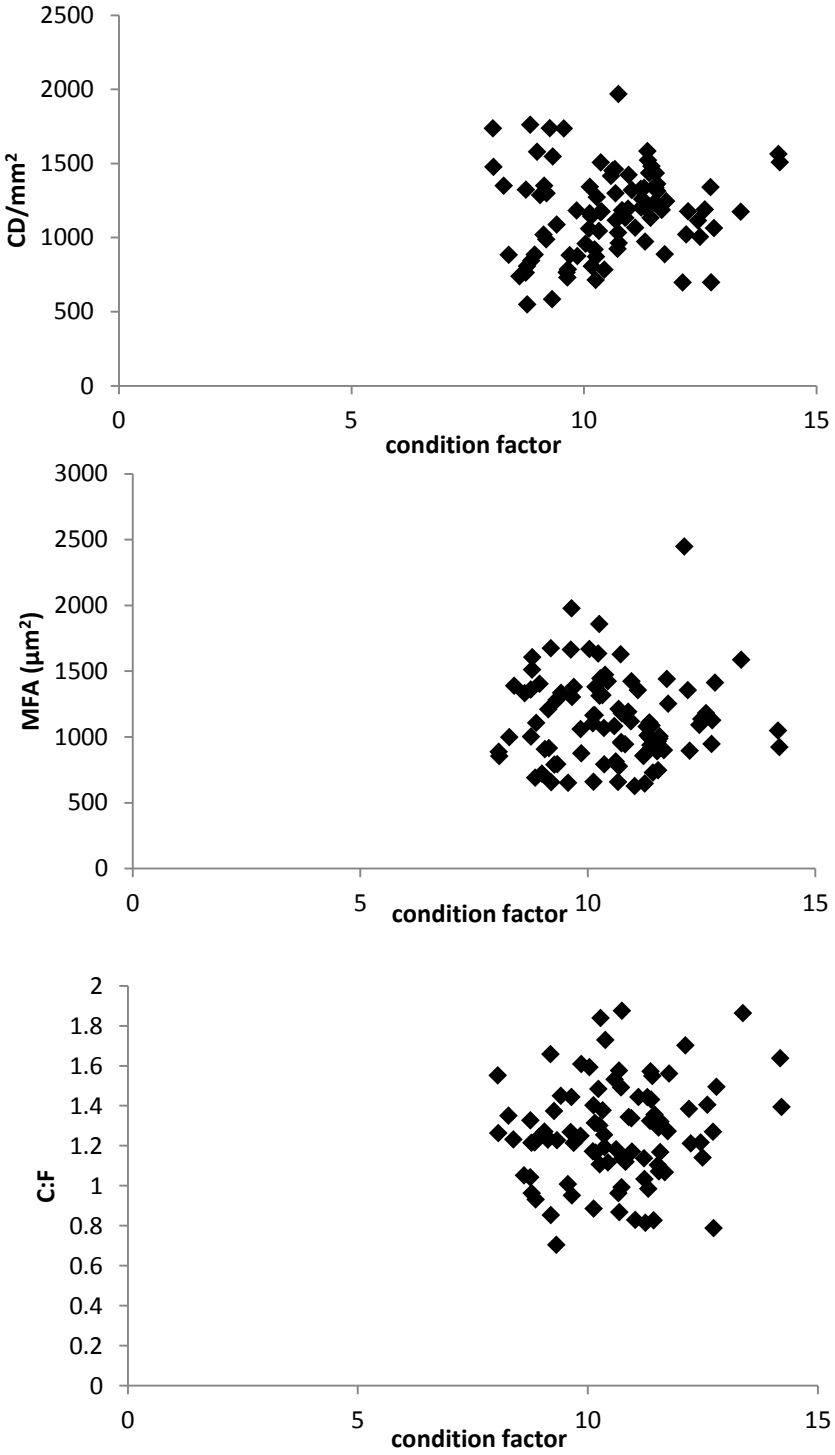


Figure 3.6 Relationship between condition factor and capillary density (CD), mean fibre area (MFA) capillary: fibre (C:F),. No correlation is seen: R^2= 0.031, 0.006 and 0.026 respectively

3.4 Discussion

Angiogenesis is triggered by local metabolic and mechanical factors. In this study, the effects of some mediators of vascular tone (nitric oxide, noradrenaline, and prostaglandins), therefore blood flow and shear stress were investigated in trout at different temperatures, in order to identify the role of mechanical stresses caused by increased viscosity in cooled blood. It was hypothesised that cold-acclimation would increase capillarity due to elevated shear stress in capillaries which would be inhibited by vasoconstrictor drugs and that it would cause fibre hypertrophy. It was also hypothesised that warm-acclimation would cause capillary rarefaction and that this would be reversed by increasing blood flow using a vasodilator. This study demonstrated that NOS blockade can cause capillary rarefaction (Fig. 3.1) but probably not as a direct result of vasoconstriction, as blocking the complementary prostaglandin pathway by indomethacin was unable to do the same. It also showed that the effects of NOS blockade on capillarity varied with temperature, muscle type and batch of trout used. Further, the results show no effects of chronic vasodilatation.

3.4.1 The effects of cold-acclimation on capillarity and fibre morphology

The effects of temperature on capillarity on skeletal muscle were varied and probably dependent on the muscle type (oxidative or glycolytic), and the season the trout were acclimatised to immediately prior to the study. There was a minor increase in capillarity (though

this was not statistically significant), in the red muscle of summer trout but not winter trout. This began within a week of acclimation and reached its peak by 4 weeks. The lack of a cold-induced increase in capillarity of fibre size in winter trout could have been because they had been pre-exposed to cold, and a further reduction in temperature was required to induce further capillary growth. Although the C:F decreased by 24% by 8 weeks of cold acclimation, the CD value remained similar to the value at 4 weeks. This follows the trend towards a reduction in mean fibre size observed with increased duration of cold-acclimation, which is at variance with previous studies demonstrating cold-induced muscle hypertrophy. However, previous studies showing an increase in red muscle mass in cold-acclimated animals may be showing the effects of hyperplasia in red muscles. The reduction in mean fibre size in red muscle suggests that cold-acclimated trout may be undergoing red muscle hyperplasia as newer fibres are likely to be smaller in size, thus reducing the mean fibre area. Longer acclimation may have allowed restoration of the mean fibre size to that prior to acclimation when new muscles had had time to grow. A reduction in mean fibre size were seen in some of the studies but not in others, and in such cases this may have been because there was no response to cold-acclimation as is evidenced by the lack of angiogenesis in winter cold-acclimated trout (Fig 3.1) and some of the summer cold-acclimated trout (Fig 3.3).

Cold-acclimation did not induce a significant increase in C:F or CD in glycolytic muscle although there was a trend towards an increase. The increase in VEGF expression seenin the absence of

angiogenesis, when summer and winter trout were pooled, suggests that an angiogenic stimulus was present in this tissue but the level was not sufficient to promote angiogenesis to an observable level. The increased expression of PCNA, a marker of proliferating cells, co-localised with capillaries (as demonstrated by parallel staining with alkaline phosphatise) suggest that some cell growth occurs between muscle fibres, but whether this is growth of endothelial cells or peri-endothelial cells such as pericytes or macrophages was not determined as the magnification used for microscopy in this study was not adequate to identify the cell type associated with the PCNA. Electron microscopic examination to assess changes in pericyte or macrophage levels around the capillaries may have clarified this. The VEGF and PCNA synthesis profile seen in physiological angiogenesis in mammals is the reverse of that observed in this study, i.e. in mammals, VEGF production precedes angiogenesis and cell proliferation (Milkiewicz *et al.*, 2006). Stromal cells, particularly fibroblasts clearly have a role in the control of angiogenesis (Egginton *et al.*, 2001) but it is also possible that the origin of the PCNA is the skeletal muscle nuclei as these lie on the periphery of the muscle fibres and may protrude into the interstitial space, which would suggest that glycolytic muscle also undergoes hyperplasia with cold-acclimation. Identifying new fibres in glycolytic muscle may not be possible by simply measuring the changes in total white muscle mass because white muscle mass makes up most of the total body mass in fish, and it may be difficult to calculate numerically small but physiologically relevant changes in total mass. In order to identify hyperplastic fibres

determining the size and the identification of the presence of a transcription factor that is involved in myocyte development but not hypertrophy.

3.4.2 The effects of warm acclimation on capillarity and fibre morphology

Warm acclimation of fishes reduces blood viscosity and so probably shear stress on the vessel walls. As shear stress is essential for endothelial cell survival (Dimmeler *et al.*, 1996), it was hypothesised that warm-acclimation would cause capillary regression. Furthermore, as the capillarity in fish red muscle fluctuates between seasons, it was reasoned that during the progression from winter to summer, where capillarity is at its lowest, capillary regression occurs. The current study revealed a transient but non-significant increase in capillarity at 1 week after warm-acclimation, followed by a gradual trend (also not statistically significant) towards reduction in C:F and CD. The muscle capillarity was higher at the start of the experimental period than the summer trout, as they were purchased after winter-acclimatisation when cold-induced angiogenesis was expected to have already occurred, so it was expected that a significant capillary regression would be observed with warm acclimation. However, the lack of a marked response suggests that reduced shear stress alone may not be sufficient to cause capillary regression or that humoral responses play a greater part in the response to photoperiodicity-related changes in capillarity. Investigation into the longevity of action of hormones (such as the pineal melatonin), or changes in the pineal hypothalamic pituitary-adrenal axis (Falcon *et al.*, 1994) that are elevated in the winter, on angiogenesis may

be a useful means by which to determine the role of photoperiodicity compared with temperature. It is known that the physiological effects of shortened daylengths can often mimic the effects of cold acclimation for example increased SR Ca^{2+}, in myocytes (Dibb *et al.*, 2005) see section 1.3.6. Moreover, the stress of confinement in tanks and rapid temperature change may have induced the release of stress hormones such as cortisol in both cold and warm-acclimated trout which has been previously been shown to inhibit cell proliferation and VEGF synthesis in human cancer cell lines (Sakayama *et al.*, 2008) and *in vitro* models of angiogenesis (Jaggers *et al.*, 1996).

3.4.3 The effect of NOS-blockade on angiogenesis and capillary maintenance

Increased blood viscosity is expected to cause shear stress-induced release of NO by the endothelium (Davies, 2009) . Nitric oxide causes arteriolar vasodilatation to counter elevated shear stress on vessel walls, leading to an increase in velocity through the downstream capillaries which do not dilate because they lack smooth muscle cells, so shear stress on the capillary endothelium is maintained for prolonged periods. The NO released by the endothelium is also a potent direct trigger for angiogenesis (Ziche *et al.*, 1994). The blockade of NO synthesis is expected to reduce the angiogenic effects of viscosity-induced shear stress by a) inhibiting arteriolar vasodilatation and b) reducing NO produced by capillary endothelium. In this study, it NOS inhibition with the non-selective NOS inhibitor, L-NNA, inhibited capillary growth at low temperatures in 2 trout, causing capillary regression below control, but statistical analysis was

not possible (Fig 3.1). The slight reduction in capillarity seen with prolonged reduction of shear stress by NOS blockade, is consistent with previous findings that reduced shear stress triggers cell apoptosis (Dimmeler *et al.*, 1996). However, NOS inhibition consistently but not statistically significantly, increased capillary growth at 11°C and showed a trend towards capillary growth at 18°C, suggesting a temperature-dependency of NO action on capillaries (Fig 3.4). Temperature acclimation and NOS blockade did not have any effect on capillarity on winter-acclimatised, 4°C acclimated or 11°C trout. The trout used in this study did not feed consistently (evidenced by a lack of feeding frenzy and settling of food at the bottom of the tank) so it is probable that *in vivo* L-NNA concentrations were not maintained at the levels intended. In addition, temperature acclimation is only seen in fed animal as hypophagia will result in hypometabolism. As cell proliferation is an energetically costly event, one may speculate that physiological feedback would suppress proliferative activity and protein turnover (Loughna & Goldspink, 1984). The temperature-dependency of NO on blood vessels indicates that the role of NO in angiogenesis is complex and non-specific NOS-blockade has effects beyond that on vessel tone and shear stress alone. NO is a ubiquitous molecule and is likely to affect the entire cardiovascular system and neurons associated with it e.g. NO modulates cardiac ion channels responsible for generation of action potentials (Musialek *et al.*, 1997) and interferes with the mitochondrial respiratory chain (Brown, 1999) thus affecting contractility and tonus.

3.4.4 The effects of α-adrenoceptor antagonism on angiogenesis and capillary maintenance in warm and cold acclimated trout

α-adrenoceptors are a receptor for adrenaline and noradrenaline, which causes arteriolar constriction. α-adrenoceptor blockade using prazosin brings about relaxation of pre-capillary arterioles, increasing flow through capillaries, thereby elevating shear stress on capillary endothelium. It was hypothesised that prazosin administration would cause angiogenesis in the fish in the same way that it does in mice (Williams *et al.*, 2006a). However, prazosin administration had no effect on capillarity at any temperature suggesting that the role for α-adrenoceptors in trout may not be as important as in mice, as fish have a lower sympathetic vasoconstrictor tone (Evans & Claiborne, 2006). Alternatively, due to lack of feeding, prazosin may not have been administered successfully, so again it is difficult to draw any conclusions.

3.4.5 The effects of COX-inhibition on angiogenesis and capillary maintenance in cold acclimated trout

Prostaglandins (PG) are thought to be the predominant endogenous vasodilator in fishes (Evans & Gunderson, 1998; Kagstrom & Holmgren, 1997). As such, it was hypothesised that inhibition of the enzyme that synthesises PGs (cycloooxygenase; COX) would causes a significant and consistent reduction in shear stress therefore cause capillary regression in fish red and white muscle. However, in this study, angiogenesis was not observed and indomethacin had no effect on capillarity although it has previously been shown to block angiogenesis in rats (Pearce *et al.*,

2000). Furthermore, the fish appeared to have been subjected to stress from bacterial infection. It is possible that bacterial infection caused prostaglandin release to such an extent that the dose of drug administered was unable to block PG synthesis sufficiently enough to cause vasoconstriction, thus invalidating the experiment.

3.4.6 Methodological considerations

Two indices of capillary estimates were used- the C:F ratio which tell us the number of capillaries per fibre, but does not reveal with certainty whether there is true angiogenesis or whether the number of capillaries per fibre in a unit of area has increased because of fibre hypertrophy, which would reduce the number of fibres per unit area but not the space occupied by fibres in that area. Capillary density provides a measure of true angiogenesis because it refers to the total number of capillaries per unit area of fibre, irrespective of the number of fibres. Mean fibre area was also measured to identify whether there was any change in fibre size; an increase indicates fibre hypertrophy, whereas a decrease indicates atrophy or hyperplasia. Distinction between atrophy or hyperplasia could only be made by measuring change in total mass of the fibre type in the whole animal. Additionally, assessment of the distribution of fibre size is needed.

The condition factor of the fish, an indication of their health status (Bolger & Connolly, 1989), did not influence fibre size, C:F or CD (Fig. 3.6). Therefore, despite the variation in the condition

factor of the trout (range 7-14), this was unlikely to have had any effect on the results. However, it is clear from the analyses that C:F, CD and mean fibre size of one muscle type, exposed to identical conditions can vary markedly/significantly between fish. This may have been due to the fact that size rather than age was used to select fish for this (Kiessling *et al.*, 1991). As with previous studies in the literature, one single area of the muscle fibre was chosen in this study. Although in mammals, there is a heterogeneity in fibre size and capillarity between different regions of the same tissue (Deveci *et al.*, 2001), in fish, muscle types are separated into discrete regions, so heterogeneity is unlikely (see Fig. 2.2). Minimising heterogeneity by choosing one region meant that it was possible to minimise regional variation as a contributing factor to the variability between individual species.

The C:F and CDs in this study were lower in the current study compared with previous studies at comparable temperatures in rainbow trout (Egginton & Cordiner, 1997). As the capillarity at 11°C was lower in this study, it may have been expected that the angiogenic potential (the capacity for an increase in capillarity) would have been greater, given that the literature suggests that C:F can reach 2.5 at 4°C and CD can be as high as 2300mm^{-2} at 11°C in rainbow trout red muscle (Egginton & Cordiner, 1997). However, the maximum C:F observed in this study was 1.44 and the maximum CD, 1480mm^{-2}. This demonstrates that despite the fact that the source of the trout was identical in the two studies, the capacity for capillary growth may differ between batches of fish and there may be some genetic drift over some years (in this

case, approximately a decade). Further, the trout in this study were acclimated to a common temperature and their respective tanks for 4 weeks prior to exposure to the experimental conditions, whereas in the study by Egginton & Cordiner (1997), the animals were exposed to their environmental temperatures immediately after transportation to the University. However, this is at variance with the findings of this study that the trout may not exceed their intrinsic angiogenic potential, regardless of the extent and the duration of mechanical stresses. As such, it is proposed that batch-differences in intrinsic angiogenic potential may exist.

In this study, neither temperature acclimation, nor pharmacological intervention (with LNNA, prazosin or indomethacin) had any significant effect on the capillarity or the fibre size of the trout

3.4.7 Conclusions

The capillarity of fish and the effects that temperature acclimation has on capillarity depends on the pre-existing state of the trout and whether their full angiogenic potential has already been reached, for example in CA winter acclimatised animals. Alternatively, stress-induced cortisol release in all conditions may have suppressed any possible angiogenic stimuli. Additionally, although the condition factor of individual fish bears no influence on the capillarity and fibre size, stressed animals may not respond to mechanical stresses such as increased blood viscosity or blood flow through capillaries, in order to preserve energy, for example by reducing protein

turnover, similarly to the energy preservation seen with heat stress (Buckley *et al.*, 2006). In this study, there was some indication that p.o. NOS blockade may have a temperature-dependent effect on capillary growth and maintenance suggesting that the role of NO in fishes is complex and may have significant impact on other cardiovascular parameters such as heart rate (Tota *et al.*, 2005) which is also heavily influenced by temperature (Clark, 1920). The lack of a response to prazosin, contrary to studies on mammals (Williams *et al.*, 2006b), may have been a true lack of response because there is comparatively low α-adrenoceptor density in fishes, which have lower vascular tone than mammals, or it may have been that very little drug was administered due to lack of feeding.

Although all experiments were started with 6 animals per group, unexplained deaths and time constraints (each experiment takes 4 months), have hindered our endeavour to achieve a more complete understanding of the effects of mechanical stresses on angiogenesis. Ideal future experiments would require that the animals were tagged, and their start and end body masses monitored individually. This would enable the identification of fish that had not fed properly (so individuals in which drugs had not properly been administered may be identified), and those fish that had grown little, indicating stress and an impact of the territorial nature of fishes. The dose per gram of feed could be increased to ensure that at least the minimum amount of the required drug is administered, although this may introduce the additional problem of drug toxicity if the fish feed well. Additionally, it may be useful to find an alternative method of drug

administration as it is difficult to be certain whether the animals have fed (a major weakness in this study), although body mass may be an indication. Intramuscular or intraperitoneal administration of drugs is possible but this involves handling stress, which is best avoided in the investigation of angiogenesis, a phenomenon that may be influenced by stresses external to the shear stress under investigation here. A better option for drug administration in unrestrained fish may be the implantation of mini-osmotic pumps which continuously infuse drug (Riley *et al.*, 2005). However, these are expensive, the kinetics of drug release at low temperature may mean that animals at different temperatures may receive different doses, and the mass of osmotic pumps is significant compared to the body mass of the fish used in this study and would disrupt swimming ability and could stress the fish.

Chapter 4 Temperature, heart rate and oxygen consumption

4.1 Introduction

As ectotherms lack the strict thermoregulation that exists in endotherms, temperature change also has a direct and profound effect on central cardiovascular function such as heart rate (HR) and metabolism. As described in greater detail in Chapter 1, the intrinsic HR of teleosts, as in mammals, is set by the pacemaker cells, and subsequently modulated by the autonomic nervous system. Neural and humoral adrenergic stimulation increases HR and neural cholinergic (vagal) stimulation decreases HR, the balance between the intensity of these antagonist systems determining the physiological HR observed. The influence of the various mediators of HR have long been established; however their temperature-sensitivity is not fully understood.

Cold-induced bradycardia occurs because at low temperature the time taken for the unstable pacemaker potential to reach threshold is increased (Clark, 1920). However, in cold-acclimated trout, the basal HR is higher than in warm-acclimated trout, at a common temperature (Aho & Vornanen, 2001). This is because, with time, compensation for reduced speed of intrinsic rate of pacemaker activity at low temperatures takes place, by increasing the density of the inward rectifier K^+ channels, and a reduction of the action potential duration (Haverinen & Vornanen, 2007). In addition Ca^{2+} influx into the cells (Shiels *et al.*, 2010), and sensitivity to adrenergic stimulation is increased (Shiels *et al.*, 2003). In the current study, we attempted to record acute

changes in HR along with the long-term response to parallel the 8-week acclimation study reported in Chapter 3.

In fishes, changes in HR with temperature follow the classic thermodynamic response (approximately doubles with every 10°C increase in temperature, i.e. Q_{10}~2). In most fishes, including rainbow trout, HR reduction leads to reduced cardiac output (CO = HR x stroke volume), which is tightly coupled to O_2 consumption, $\dot{M}O_2$ (Webber *et al.*, 1998) The mechanisms of central control of the cardiorespiratory interaction are not well understood (Levick, 2003), again especially so at low temperatures. Hence, MO_2 was also measured to assess any changes in cardiorespiratory coupling.

HR is controlled locally and remotely, with an important role for noradrenaline acting through β-adrenoceptors and α-adrenoceptors (see chapter 1). The β-adrenergic system is upregulated in cold-acclimated temperate species such as the trout (Aho & Vornanen, 2001; Hanson *et al.*, 2005) so the influence of the β-adrenergic system on the HR of cold-exposed trout was investigated. The influence of cholinergic input on cold-exposed trout was also explored because in Antarctic species high vagal tone is thought to be a characteristic of cold-adaptation (Axelsson *et al.*, 1992) whereas in other, temperate species, such as the sole, it is possible that vagus activity varies directly with temperature (Sureau *et al.*, 1989).

Heart rate itself varies from beat to beat (known as the heart rate variability, HRV) as the sinoatrial (SA) node rintegrates a combination of inputs and each R-R interval therefore represents the sum of the autonomic tonus. In the time domain, such variation in cardiac rhythm can be assessed by simple statistics, eg. standard deviation of the R-R interval (SD), The standard deviation of the changes in successive R-R intervals (SDNN), and the percentage of RR intervals that are different from the previous interval, by a predetermined value, in clinical studies usually 50% (NN_{50}). The 'N' in this standard notation refers to 'normal', to distinguish the pattern involving ectopic beats. In addition, by power spectral analysis of the variability in the R-R intervals in the frequency domain, it is possible to isolate the effects of the individual inputs such as sympathetic & parasympathetic activity and ventilation rate on the heart rate. In mammals there are three distinct components, high (associated with ventilation, mainly parasympathetic mediated), mid (peripheral blood pressure, mainly sympathetic mediated) and low (assumed to be thermoregulation, but in reality of unknown origin) (Akselrod, 1981). In the rainbow trout, there are two components with the low frequency (LF) component being dominant at low temperature and the high frequency (HF) component, at high temperature (Devera & Priede, 1991). It was previously thought that as parasympathetic activity in the heart is more apparent at low temperatures (Priede, 1974), that the low frequency component represents parasympathetic tone and the high frequency component represents sympathetic tone. However, later studies utilising pharmacological blockade suggest the opposite is true (Devera & Priede, 1991). Tachycardia, along with reduced HRV, may be an indicator of stress in

post-surgical recovery in fishes. This tachycardia is probably due to withdrawal of parasympathetic activity as bilateral cardiac vagotomy in the sculpin evokes a similar response to post-surgical handling stress (Campbell *et al*, 2004).

To further our understanding of the findings of Chapter 3, where the effects of nitric oxide (NO) seemed to be temperature-dependent with little influence of α-adrenergic blockade at any temperature, the possible influences of NO and the α-adrenergic system on HR and oxygen consumption were assessed. Based on previous studies (Sandblom & Axelsson, 2005) it was hypothesised that α-blockade would have little effect on HR in these trout. In addition, the roles of sympathetic and vagal tone at different temperatures were addressed. In previous studies on rainbow trout, resting HR differed from 47 beats per minute (bpm) 4 hours post-surgery in trout weighing between 200-600g (Stevens & Randall, 1967) to 62.4 bpm at 3 days in trout weighing more than 600g (Sandblom & Axelsson, 2005). Anaesthesia alone has previously been reported to have little or no effect on HR on fish such as the salmon, but handling fish prior to anaesthesia increased HR (Hill & Forster, 2004), so in the current study, the HR while the trout were still under the influence of anaesthetic, and the time taken for HR to stabilise following recovery was monitored, in order to identify a suitable time to begin experimental procedures post-surgery, in trout weighing approximately 500g.

4.2 Methods

4.2.1 Surgical procedures

Rainbow trout were anaesthetised one at a time in recirculating filtered Birmingham tap water containing MS222 (0.1g/L) and $NaHCO_3$ (0.2g/L), to neutralise the otherwise acidic solution. When righting reflex was lost, the fish were transferred to a table and positioned ventral side upwards. The gills were irrigated with aerated, lightly anaesthetised water (approx 0.01g/L MS222), cooled by immersed chiller blocks. Two ECG electrodes (Teflon-coated, 7 stranded stainless steel wires; AM Systems, USA) enclosed in PP50 tubing (Portex, UK), were inserted percutaneously using a 10G needle, close to the heart, after exposing 1cm at both tips of the wire and hooking one end in to the needle. The electrodes were then secured to the body of the fish with 5-0 silk sutures, in several places. Heparinised catheters (PP10) were prepared for intraperitoneal implantation by heat-flaring one end. The catheters were inserted intraperitoneally by creating a small incision at the base of the pectoral fin into which the catheter was pushed through and secured by purse-string sutures. On some fish, the area was then dried and VetBond ('superglue') applied and allowed to dry for 3 minutes, as this improved the security of the catheter. The catheter was then flushed with heparinised saline. Trout were transferred to sealed holding tubes for the experimental procedure (Fig 4.1).

4.2.2 ECG and O_2 consumption recording from holding tubes

ECG signals were amplified and continuously recorded using a PowerLab Module running Chart software (AD Instruments, Oxford), at a sampling frequency of 40/s. Positive and negative leads were secured to the pair of ECG leads from each fish using crocodile clips when recordings were taken with a reference lead placed in the tank water. Water samples were withdrawn for 30 minutes every 3 hours (Fig. 4.1 (A)) from the holding tubes to measure oxygen content, using the Strathkelvin 949 oxygen system. (Strathkelvin Instruments, Glasgow). The oxygen content gradient in the sample during interrupted flow through the holding tube was used as an index of oxygen consumption. Oxygen consumption and ECG recordings (Fig. 4.2) were taken from each trout, immediately post-surgery to assess recovery time (Fig 4.4 & 4.5). This was followed by daily recordings for one week to allow full recovery of the trout before pharmacological interventions.

4.2.3 Experimental plan

After the 7 day of recovery at 11°C, propranolol (1mg/kg) and atropine (1mg/kg) was administered. Allowing one day for recovery from atropine (as a competitive antagonist of the muscarinic acetylcholine receptors, its effects are long-lasting) , the tank was cooled or warmed at a rate of 1°C/hr over a day. Two drugs were administered per day, the drugs with a higher clearance rate (propranolol and L-NNA) in the morning, and those with a lower clearance rate (atropine and prazosin) in the afternoon. The effects of the drugs on HR were recorded from 0-

10minutes after administration (labelled 0 mins); 25-35 minutes after administration (labelled 30mins) and 50-60 minutes (labelled 60 mins) after administration.

4.2.4 Datalogger preparation and implantation

For datalogger preparation and programming instructions, see Appendix B. The datalogger records HR at intervals and duration set by the user, at a sampling rate of 512/sec. Trout and carp were anaesthetised as described in 4.2.1. Where loggers were externally placed, the loggers were secured to the flank of the fish using rubber saddles, and the ECG electrodes were exposed at the tips and inserted percutaneously close to the heart using a 10G needle. The electrodes were secured to the body of the fish in several places using sutures. For internally implanted loggers, an anterio-posterior incision was made on the ventral side of the fish, to allow access to the heart and peritoneal cavity. The two ECG electrodes were hooked directly on to the pericardial sac using a 19G needle and the logger implanted intraperitoneally. The incision was closed using a 3-0 silk suture with blanket stitches. Following datalogger attachment, the trout or carp were monitored until they recovered from anaesthesia and regained their swimming ability. Representative traces obtained from carp using the dataloggers are shown in Fig. 4.3. Analysis of the data obtained from the loggers was not carried out as the study was not completed either in the carp or trout.

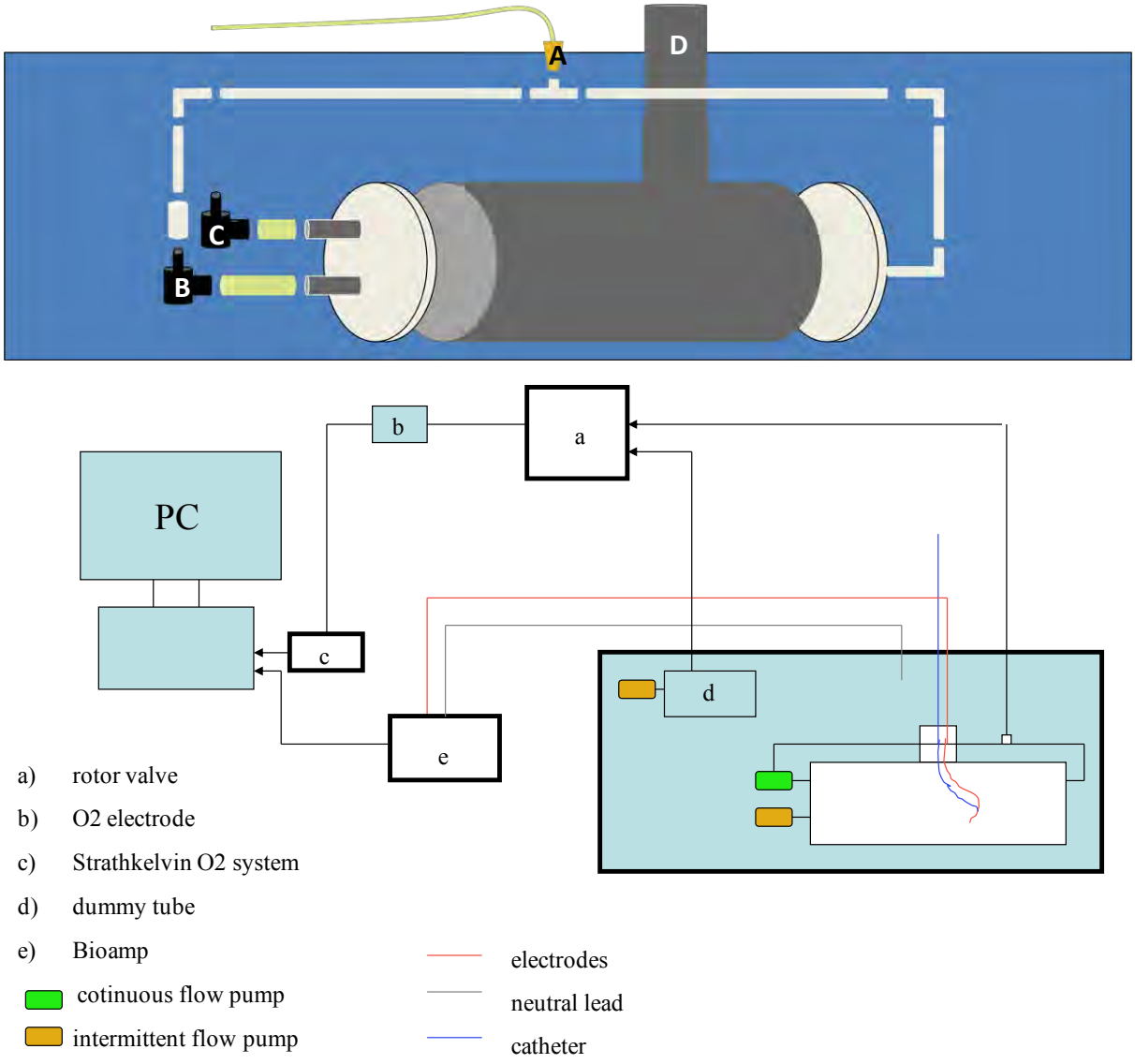


Figure 4.1. Sealed fish-holding tube (top) with **A** tubing for water sample withdrawal, **B** continuous pump for circulating water, **C** intermittent pump for periodic replacement of tube water with oxygenated water from the tank, and **D** overflow. Everything except the overflow was placed below the water line. This was a sealed unit; gaps in this image denote detachable parts of the setup designed for ease of parts replacement and positioning of the tube after insertion of the animal. The trailing ECG wires and i.p. catheter from the animal were led out through the overflow. The circular lids at either of the end of the body of the tube were lined with rubber O-ring seals.

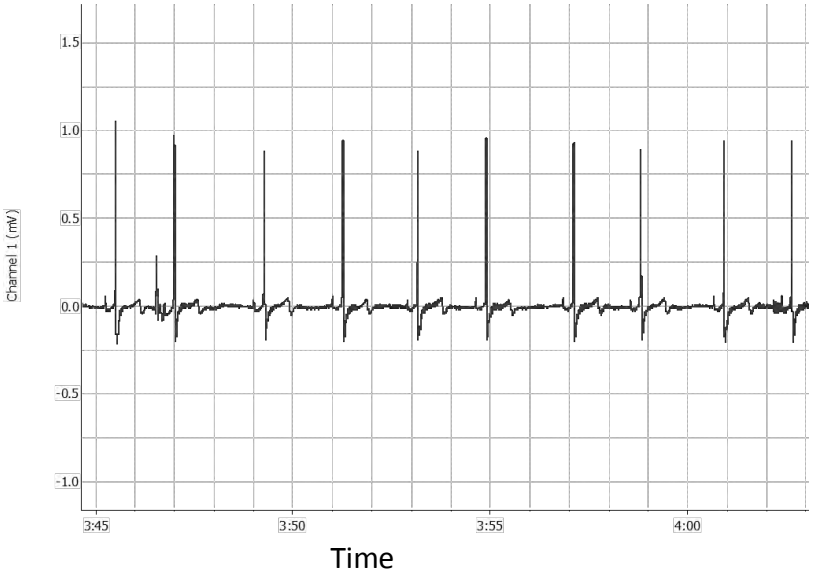


Figure 4.2 Representative ECG trace from rainbow trout obtained from fishes held in a tank and recorded using PowerLab. Here, the signal amplitude is approximately 1mV but the amplitude varied between fishes depending on electrode placement.

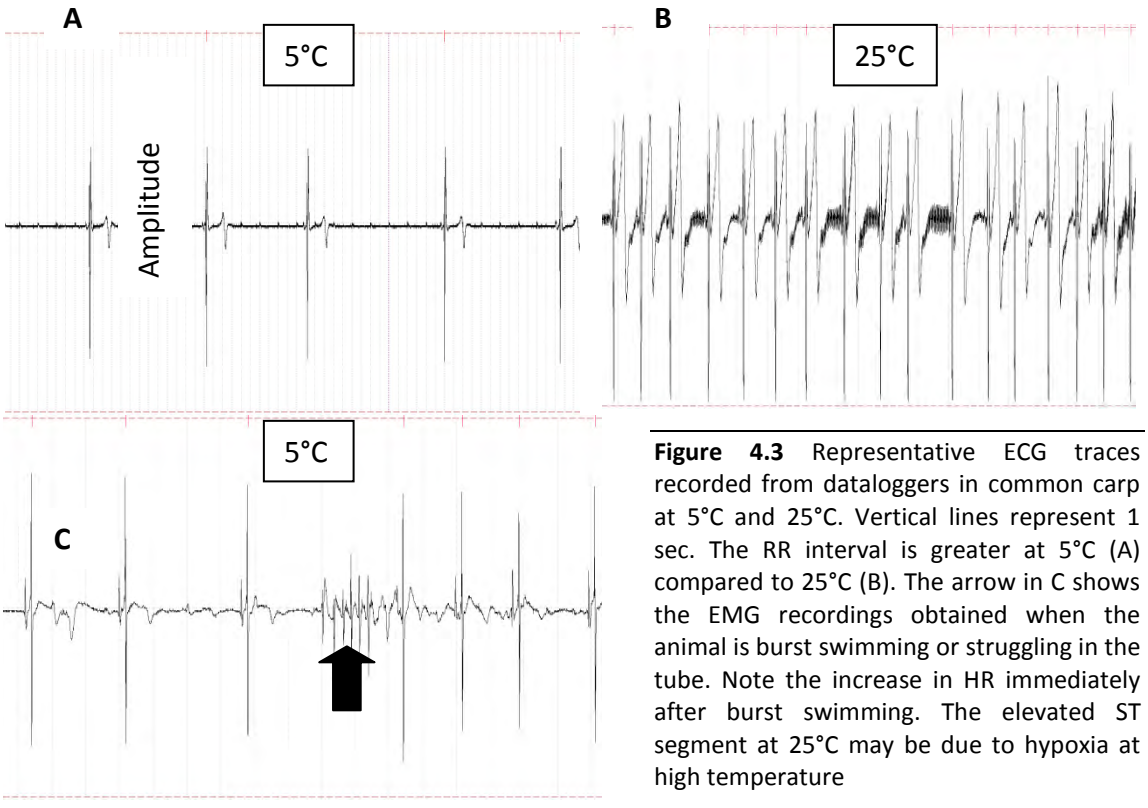


Figure 4.3 Representative ECG traces recorded from dataloggers in common carp at 5°C and 25°C. Vertical lines represent 1 sec. The RR interval is greater at 5°C (A) compared to 25°C (B). The arrow in C shows the EMG recordings obtained when the animal is burst swimming or struggling in the tube. Note the increase in HR immediately after burst swimming. The elevated ST segment at 25°C may be due to hypoxia at high temperature

4.3 Results

4.3.1 Recovery time after surgery

Mean HR was 40 ± 3.2 bpm (n=10) immediately after surgery, while the trout was still under the influence of anaesthesia. After 30 mins, when the trout had regained consciousness and its righting reflex was restored, there was a 35% increase in HR (n=9), which was maintained at 60mins post-recovery (n=8). Mean HR then reduced to 42 ± 3.9 bpm (n=10), at 24 hours after surgery and HR fluctuated between 41-34 bpm (n=4-11) over the next 6 days (Fig. 4.4, inset). It is therefore possible to interpret day 2 as a suitable time-point to begin experimental procedures. However, the Poincaré plots (Fig 4.5) demonstrate that HRV was not restored fully until 6 days post-surgery. Immediately post-surgery, while the trout was still under anaesthesia, the HRV was high and the mean HR was low, suggesting that the central tranquilising effects of MS-222 were still in place. However, at 30 minutes, much of the HRV was lost, and by 60 minutes, there was a further reduction in variability, suggesting a combination of full withdrawal of vagal tone and imposition of maximal sympathetic tone. At 24 hours post-surgery, the HRV increased but was not fully restored (compared with 6 days). This suggests that although the HR may appear to have returned to normal 24 hours, or even 4 days post-surgery, the HRV does not, so the trout is under some degree of stress and there is a sympathovagal imbalance. As such, experimental procedures should not begin until at least 6 days post-surgery. A similar recovery time for cholinergic tone was recorded in the sculpin (Campbell *et al.*, 2004)

LF (sympathetic)/HF (parasympathetic) increases with the HR in trout recovering from anaesthesia, and is shown in Fig. 10. Immediately after surgery, the ratio is approximately 1, indicating an equal input. As the trout recovers from anaesthesia, the parasympathetic tone is withdrawn and as such the HF signal increases relatively and the ratio doubles after 30 minutes and increases 2.5 fold by 60 minutes. The withdrawal of vagal tone reduces by the second day and oscillates around 1 until 6 days post-surgery.

4.3.2 β-adrenergic and cholinergic blockade at 11°C and 4°C

Fig. 4.6 shows a trend towards a decrease in mean HR in trout treated with propranolol (34 ± 5 bpm (control) to 28 ± 1.1 bpm) 7 days after surgery, but this was not statistically significant. Atropine administration approximately 5 hours after propranolol administration, had little effect on HR. This was probably because the HR prior to atropine administration was higher than the pre-propranolol HR (40 ± 6 bpm). This suggests that the trout was stressed, possibly due to the morning's i.p. injections, and as such vagal tone had already withdrawn a significant amount so any further cholinergic blockade had a minor effect. This is supported by Poincaré analysis (Fig. 4.7), showing the difference between consecutive R-R intervals, which reveals that the HRV is clearly higher in the morning and propranolol did not have the expected effect of reducing variability. HRV is attenuated markedly in the afternoon and the effect of atropine is minor, though not absent. The difference between the resting HR (34 ± 5 bpm) and the

maximum HR after atropine administration (45 ± 4.1 bpm) represents the total vagal tone. All individual trout in this study responded in much the same manner i.e. showed a minimal response to drugs.

Unexpectedly, acclimation to 4°C (Fig 4.8) did not attenuate HR (34 ± 5.0 bpm and 34 ± 4.5 bpm). However, given that it is clear from the datalogger pilot studies (Fig. 4.3) that, that cold-exposure increases R-R interval, it seems that some other influences, for example tank stress or starvation (fish were starved for the full duration of these experiments) may be overriding the kinetic influences of cold temperature by either withdrawing vagal tone or increased sympathetic tone. Again, in this experiment, propranolol causes a small but statistically insignificant decrease in HR (-15%), approximately 30mins after administration. There was a 9% increase in HR 60 minutes after atropine administration; again, this is not statistically significant.

Fig. 4.11A suggests that there may have been some reduction in sympathetic tone by the 67% reduction in LF/HF 30 minutes after propranolol administration. At 4°C, the LF/HF increases by 30% and following propranolol administration, the ratio is reduced again by 43% (Fig. 4.11B). Atropine appears to cause an increase in the LF/HF ratio, as expected, albeit with very high SEM so not statistically significantly, despite that the physiological HR is not increased under these conditions. It is suggested, as previously, that these trout are stressed and the variability

between trout, hence the high SEM is likely due to the inconsistency caused by the stress response, in addition to the low number of replicates in some cases.

4.3.3 NOS and α-adrenoceptor blockade at 18°C

Warm-acclimation (to 18°C) causes a significant, 44%, increase in mean HR (Fig. 4.9A). In these experiments, the trout reached the maximum HR seen in the entire set of studies, suggesting that high temperature has a greater influence on increasing HR than sympathetic tone alone. Neither L-NNA nor prazosin had any effect on the mean HR of these trout, but on closer inspection, it became clear that only one trout in the study was responsive to L-NNA and prazosin, which was fish 3 (Fig. 4.9B). On completion of the experiment, it was observed that fish 3 was the only fish in the study that had a patent catheter. This revealed that the other trout were not being subjected to pharmacological interventions and this explains why they responded to warm acclimation but not the drugs. These experiments were repeated but were unsuccessful, owing to fish deaths. In fish 3, NOS blockade with the non-specific NOS inhibitor, L-NNA, caused a biphasic response; a transient elevation in HR (by 12bpm) followed by a longer-lasting attenuation by 20bpm. This is likely to correspond with transient vasoconstriction caused by L-NNA (possibly caused by central inhibitory effects of NO on the vagus) followed by the longer lasting reduction in HR. Contrary to a previous study by Sandblom *et al* (2005), prazosin caused a short-lived elevation in HR which was followed by a reduction 30 minutes later, which is unexpected given that its clearance rate is expected to be low. However, reflex reduction of HR may be a consequence of the baroreceptor reflex. The increase in the LF/HF seen in 18°C-

acclimated trout (Fig. 4.11C) suggests that increased temperature causes either increased sympathetic tone or decreased vagal tone, but it is difficult to draw any reliable conclusions.

As many of the trout in this study died and whether this was due to tank stress or surgery stress is unclear, the MO_2 and HR of individual fishes at different time was collated to gather an overall picture of the relationship between metabolism and HR. This study demonstrates a very weak positive correlation (r= 0.08) between HR and metabolism and is not as clear as the current literature suggests (Fig. 4.13).

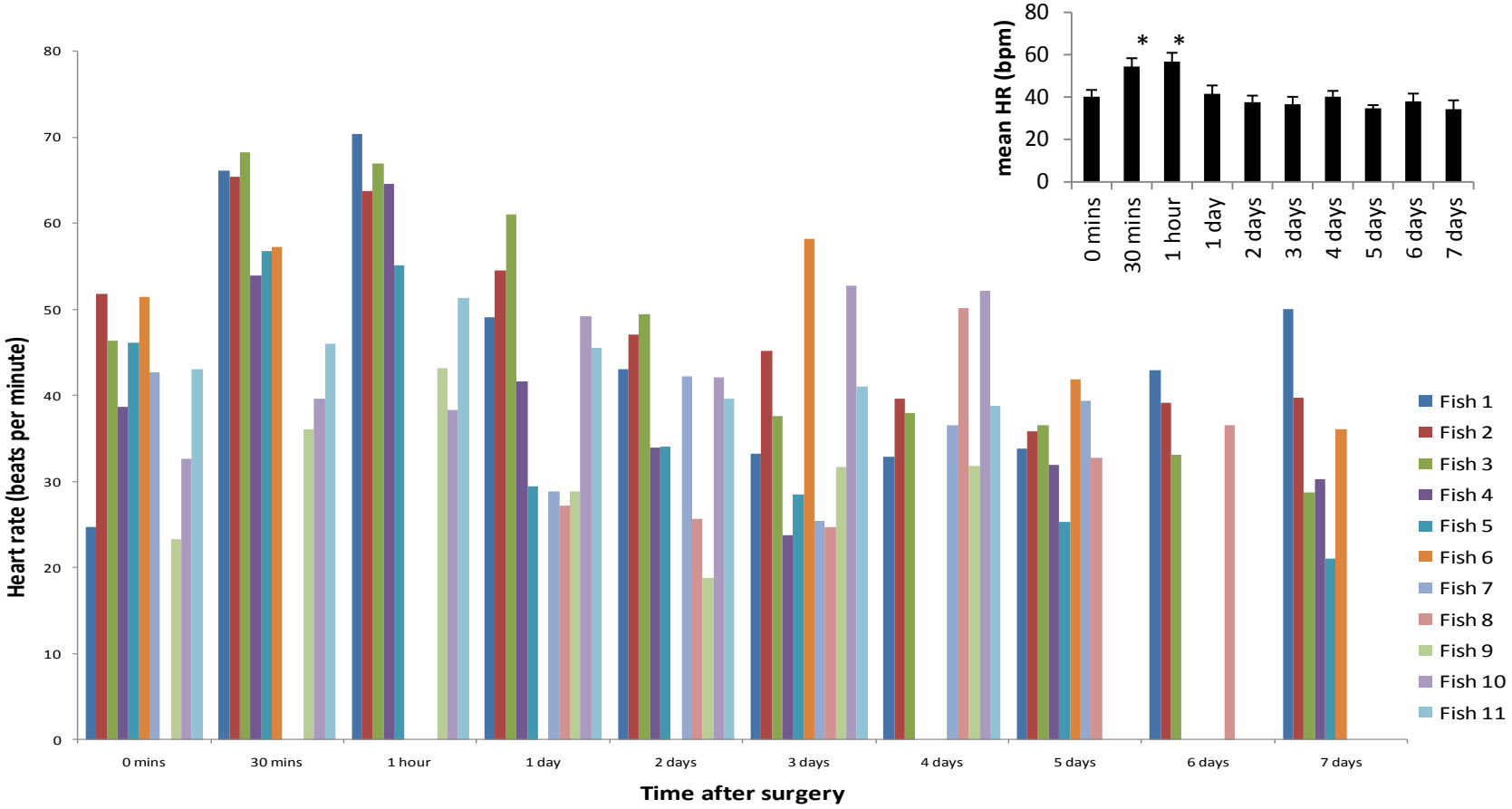


Figure 4.4 Heart rate (bpm) of individual trout immediately post-surgery and at several time points up to 6 days. Where the bars are missing at some time-points for individual trout, HR was not calculated either due to the close proximity in time between the surgery of two trout, trout deaths, or poor ECG signals. Means ± SEM (n= 4-11) are displayed in the black and white chart, inset. Means were analysed by ANOVA. *P<0.01 compared to 0 mins. These recordings are from animals held in tubes.

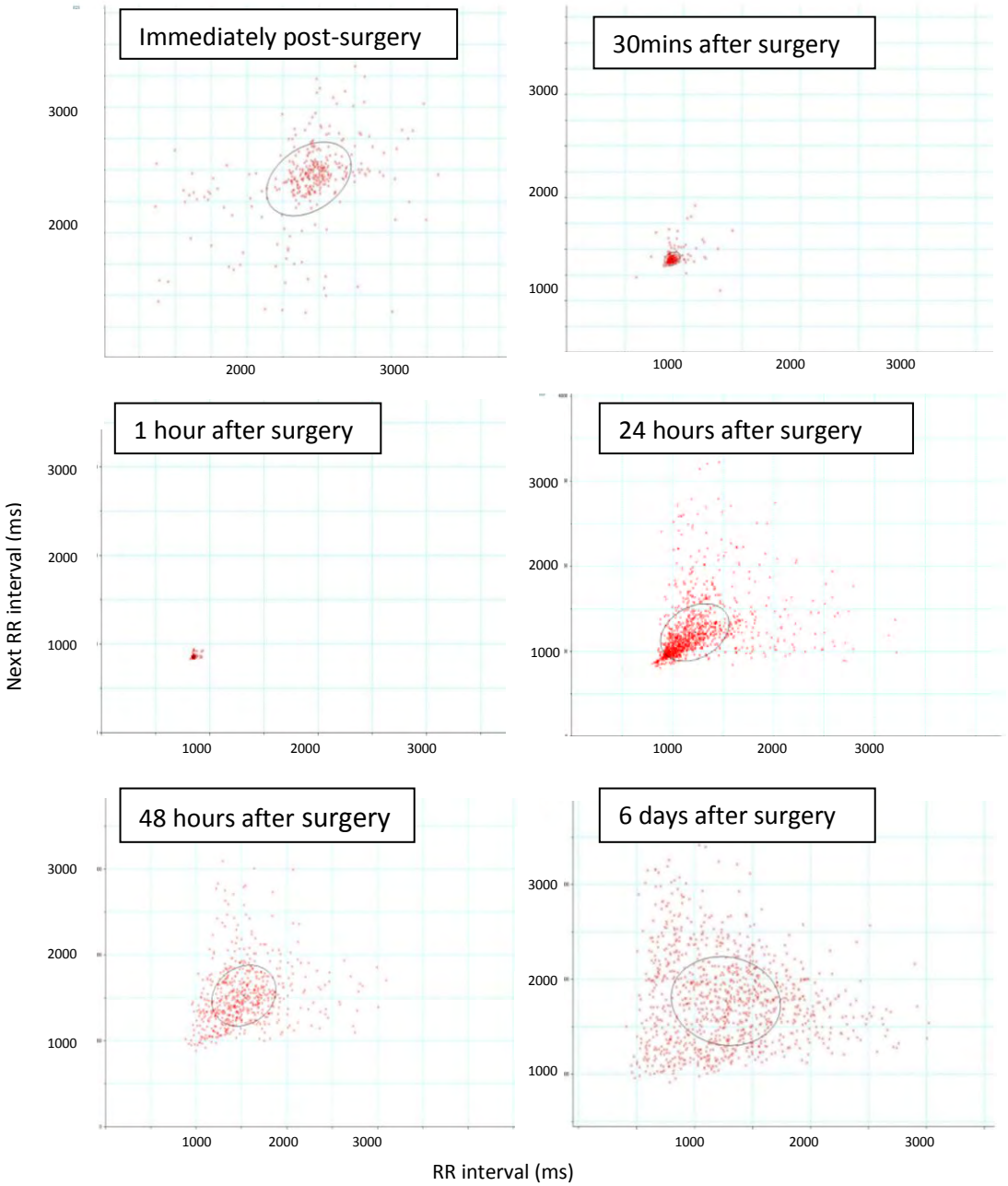


Figure 4.5. Poincaré plots demonstrating the heart rate variability (HRV) in a representative trout at various time-points post-surgery. The graph plots the R-R interval of one beat against the R-R interval of the following beat. The plot demonstrates that there is high variability in animals that are still under the influence of anaesthesia but this is lost when the animals have recovered from anaesthesia, suggesting they are stressed on recovery. The HRV increases to a maximum by 6 days, as the animals recover from surgical stress. These recordings are from tube-held animals.

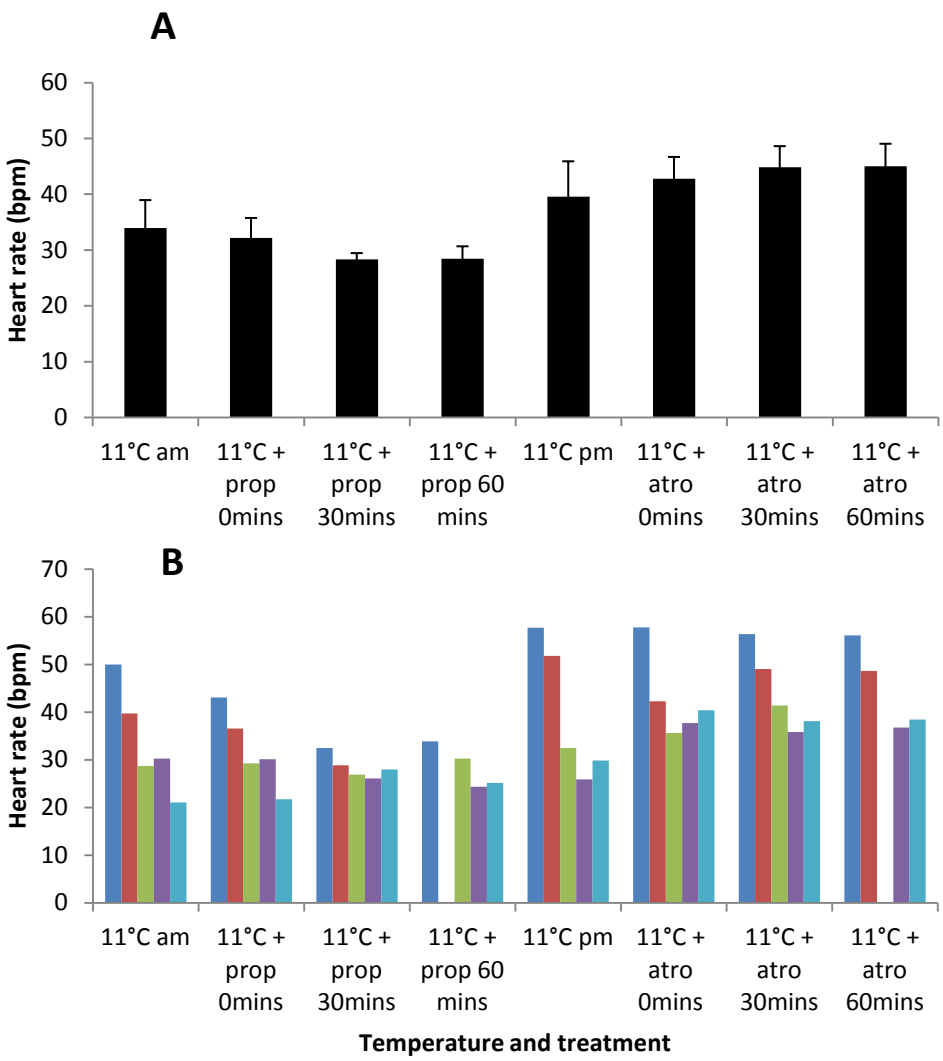


Figure 4.6. A) Mean heart rates (bpm) of trout exposed to propranolol (am) followed by atropine (pm). There is a trend towards a decrease in HR following propranolol treatment (N.S., n=4-5) By the afternoon, prior to atropine treatment, trout were considerably stressed, and their HR was high compared to that morning. Treatment with atropine had no significant effect (P>0.05, n=5). **B)** HR from individual trout show that in one trout (dark blue), propranolol reduced heart rate but atropine had no effect on any of the trout. Statistical analysis by ANOVA showed no significant difference between means.

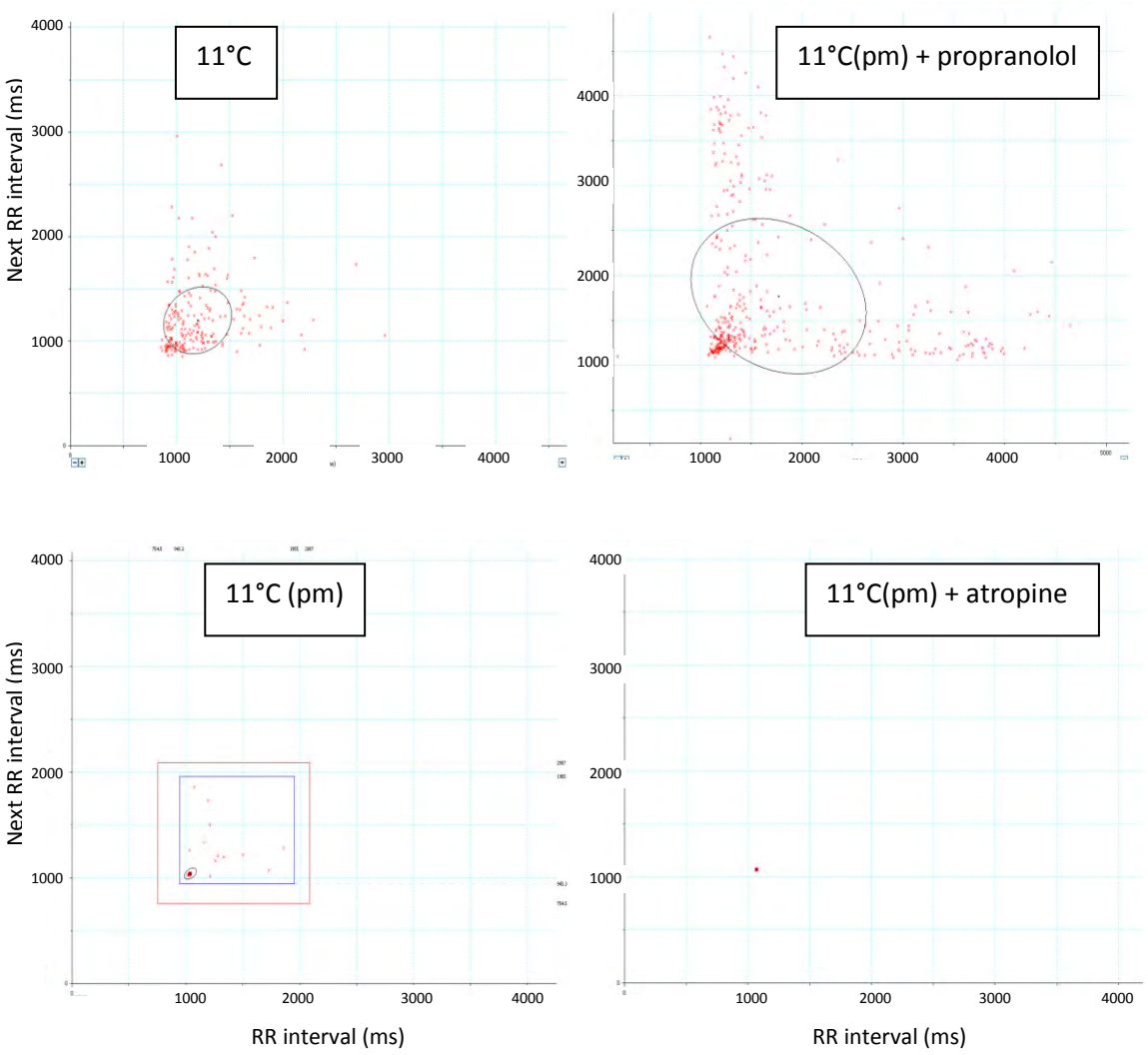
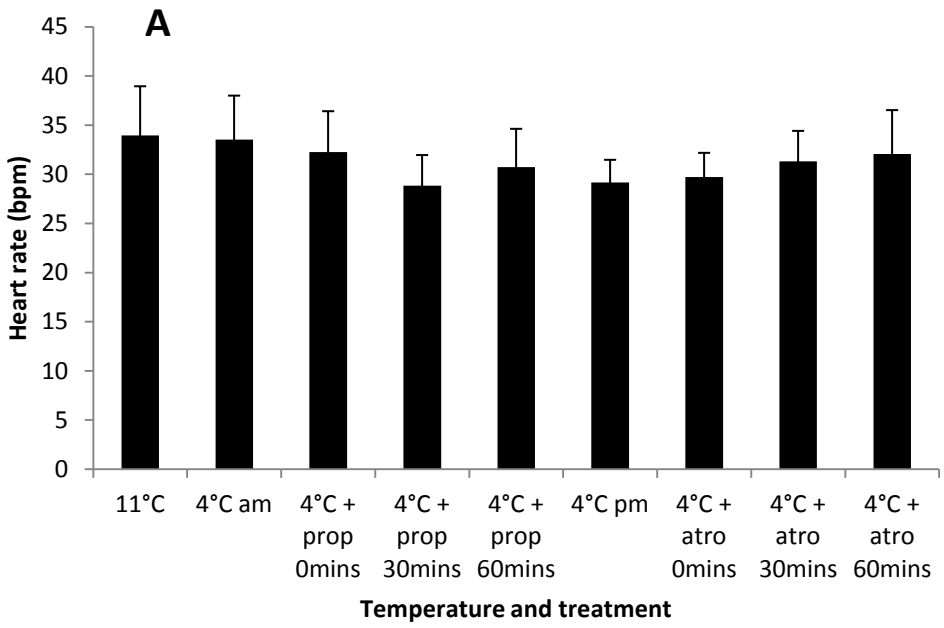


Figure 4.7 Representative images from Poincaré analysis of the trout that were treated with propranolol and atropine. This shows that whilst the trout were relatively unstressed in the morning prior to propranolol treatment, they were severely stressed in the afternoon prior to atropine treatment. As vagal tone was almost fully withdrawn, any subsequent cholinergic blockade had little or no effect on the trout.



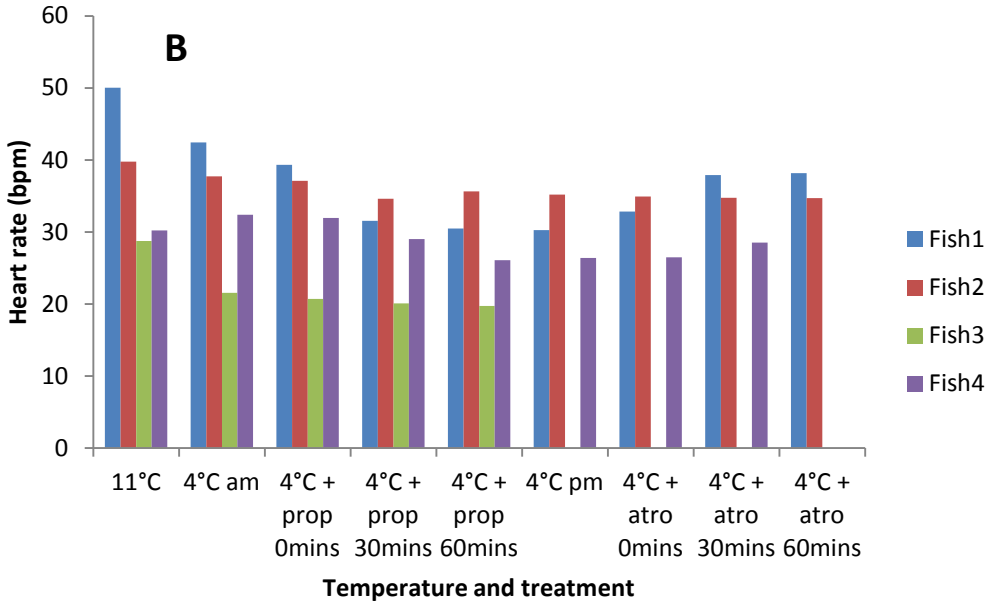
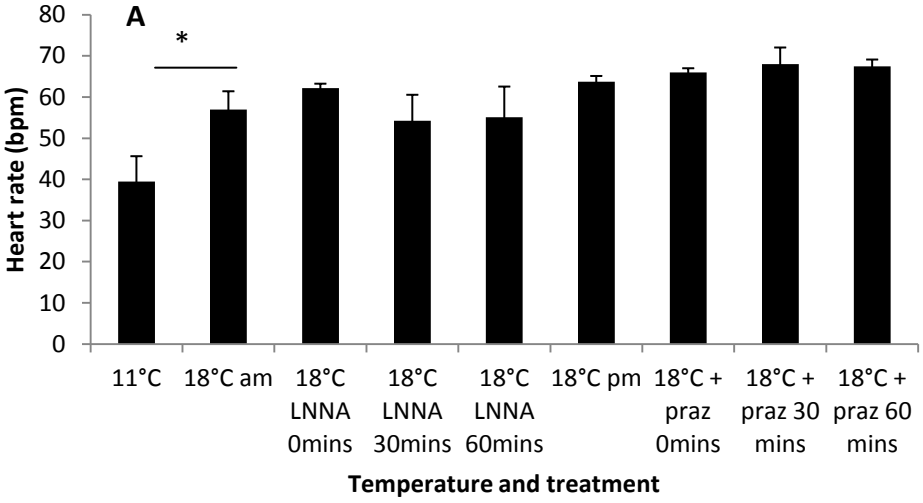


Figure 4.8. **A** Mean heart rates (bpm) of trout exposed to propranolol (am) followed by atropine (pm), at 4°C. There is a trend towards a decrease in HR following propranolol treatment (N.S., P>0.05, n=2-4) By the afternoon, prior to atropine treatment, trout were considerably stressed, and their HR was high compared to that morning. Treatment with atropine had no significant effect (P>0.05, n=2-4) **B** HR from individual trout show that in fish 1, propranolol reduced heart rate but atropine had no effect on any of the trout. ANOVA showed no significant difference between means.



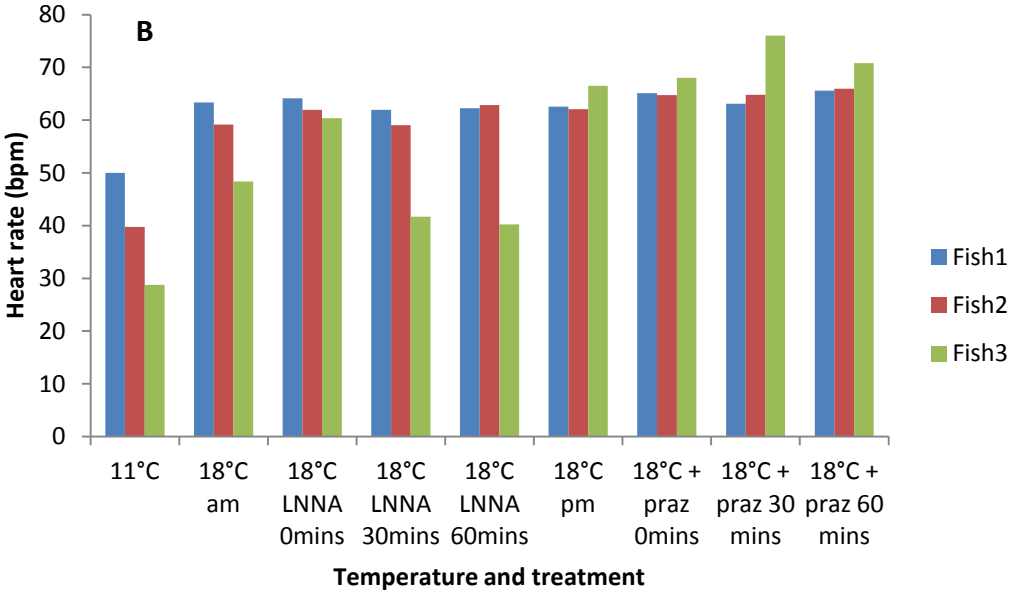


Figure 4.9 A Mean heart rates (bpm) of trout exposed to LNNA (am) followed by prazosin (pm), at 4°C. There was an increase in HR (*P<0.05 vs. 11°C) following warm-acclimation but no significant difference between means before and after prazosin treatment. Data are expressed as means ± SEM. **B** HR from individual trout show that in fish 3, there was a biphasic reponse to LNNA, with a transient increase in HR followed by a reduction, at 30 mins and 60 mins. After the experiment, it was discovered that fish 1 and 2 did not have patent catheters so it was likely that drugs were not successfully administered, which may explain their lack of drug-responsiveness.

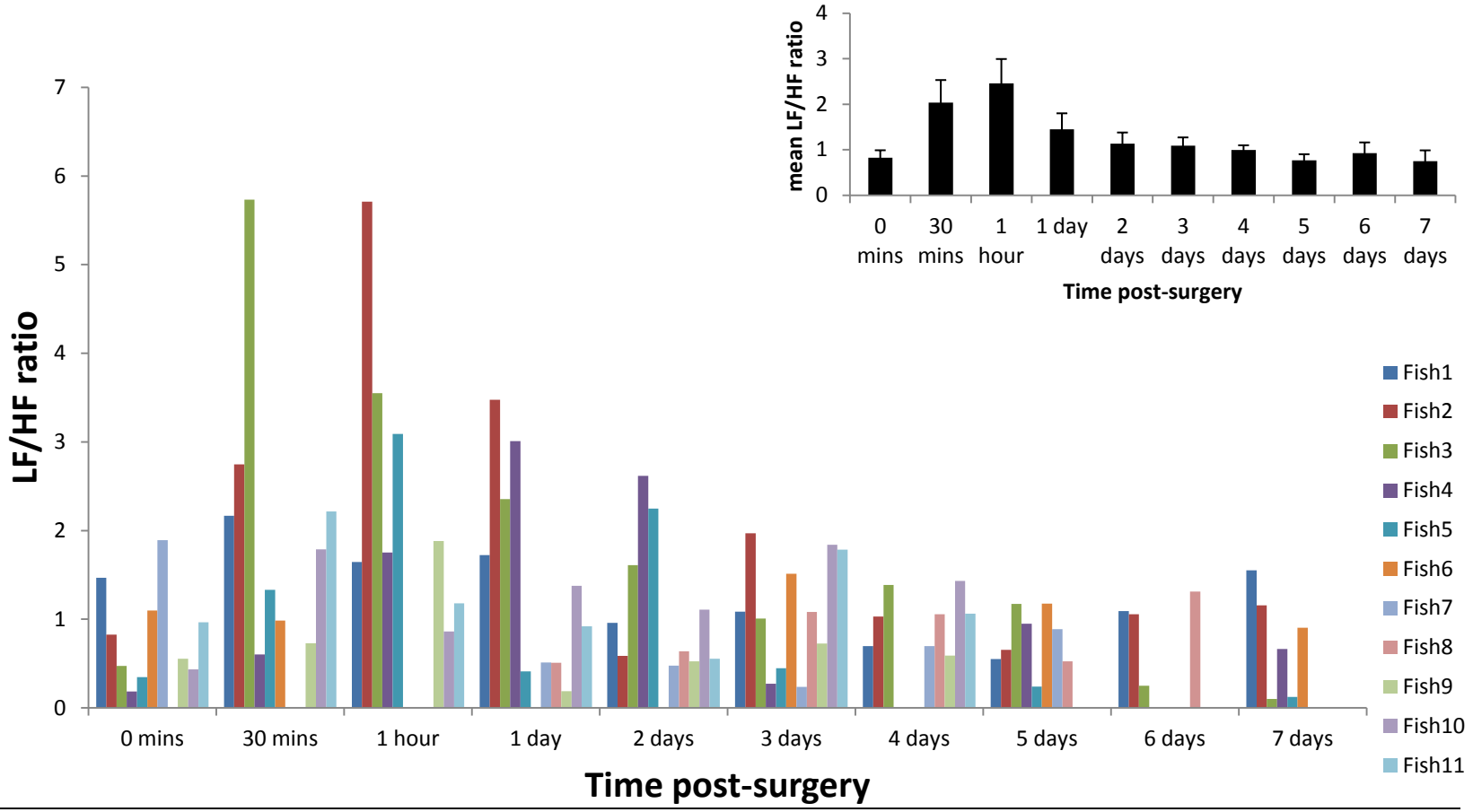


Figure 4.10. LF/HF ratios of ECG signals from trout following surgery at 11°C. This shows the ratio between sympathetic (LF) to parasympathetic (HF) activity. Mean ± SEM LF/HF ratio is also shown (inset). ANOVA showed no significant difference between means. These values are pooled from all trout used in Figs 4.5-4.9

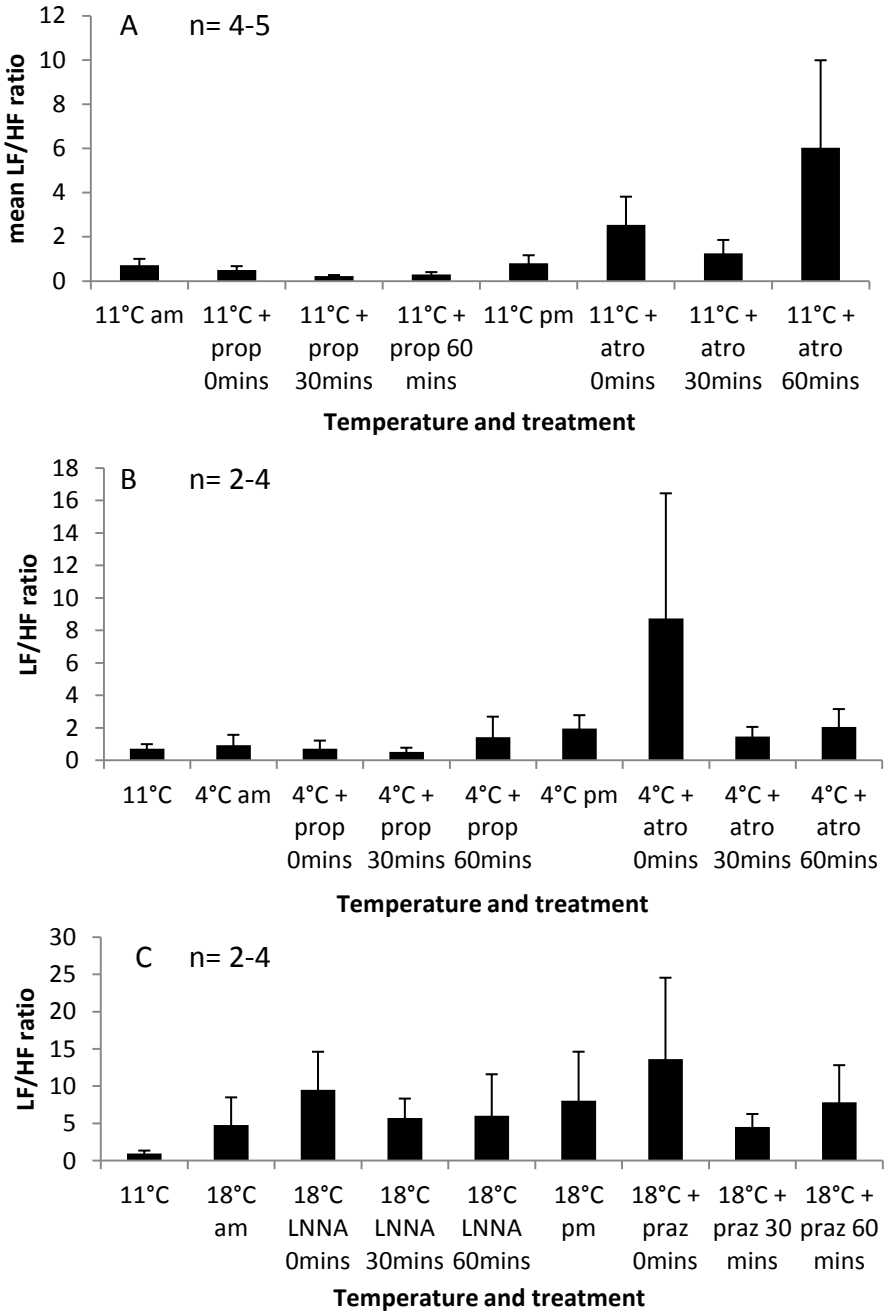


Figure 4.11. Mean ± SEM LF/HF ratios for trout exposed to 4°C, 11°C and 18°C in the presence of **A** (n= 4-5)and **B** atropine and propranolol (n=2-4) and **C**, LNNA and prazosin (n=3). These values are from the same trout used in the Figs 4.6-4.10. ANOVA showed no significant difference between means. Atro= atropine; prop= propranolol; praz= prazosin. Data collected from fish held in tubes.

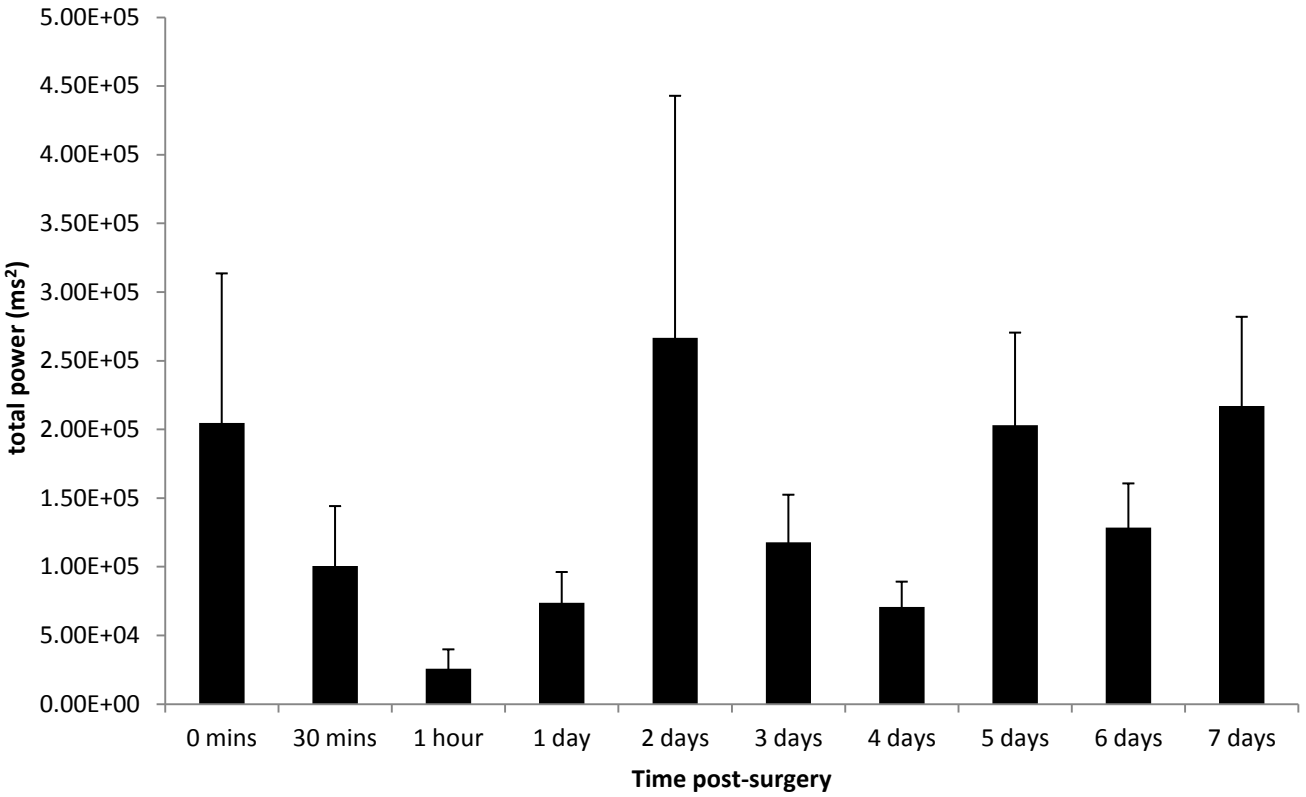


Figure 4.12. Total power analysis from the recovery stages of all trout. The trend here is demonstrative of a stress response. Immediately following surgery, there is significant sympathetic and parasympathetic tone, but with recovery from the central tranquilising effects of MS222, neural and hormonal activity is lost, and the trout's heart rate is influenced by intrinsic pacemaker activity. In the 6 days following surgery, the total power oscillates, probably the result of tank stress and starvation. These values are from the same animals used in Figs 4.5-4.11. ANOVA showed no significant difference between means. Data collected from trout held in tubes.

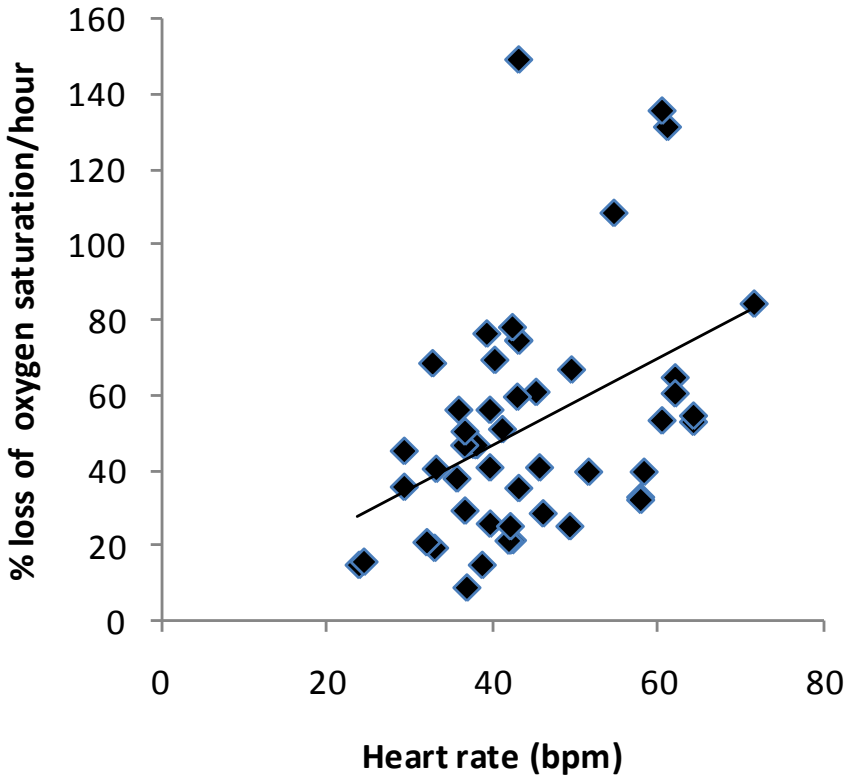


Fig 4.13 Heart rate (beats per minute) plotted against oxygen consumption. This demonstrates the overall, HR corresponds with MO_2, irrespective of the condition of the trout, although this correlation is weak (r=0.08). These values are from the same animals used in Figs 4.6-4.12. Data collected from fish held in tubes.

4.4 Discussion

The purpose of this chapter was to further explore the possible reasons for our findings in chapter 3 that neither cold-induced angiogenesis nor warm-induced capillary regression is consistent. In this chapter, the influences of temperature on the HR and HRV, and the relative influences of sympathetic and parasympathetic tones in these varied conditions are measured in a different set of trout. The possibility that the temperature-dependence of L-NNA activity is due to its effects on HR at high temperature was addressed.

It was hypothesised that cold-acclimation would decrease mean HR as low temperature increases the time taken for the threshold of an action potential to be reached (Clark, 1920), and that conversely, warm-acclimation would lead to tachycardia. The effects of NOS blockade were uncertain as NO has been shown to have different effects on HR, for example, NOS blockade may cause a decrease in HR as eNOS-derived NO causes tachycardia by activating the hyperpolarisation-activated inward current of pacemaker cells (Musialek *et al.*, 1997). However, nNOS-derived NO may decrease HR by facilitating acetylcholine release from the vagus, causing bradycardia (Herring *et al.*, 2002). In this study it was found that where a response to L-NNA was observed, a transient tachycardia was followed by bradycardia. This may be explained by the blockade of nNOS-dependent bradycardia (Herring *et al.*, 2002), followed by the baroreceptor

reflex whereby increases in arterial pressure caused by LNNA-induced vasoconstriction, causes a reduction in sympathetic stimulation and increase in vagal stimulation on the heart, thereby reducing heart rate. As yet, there is little convincing evidence for the presence of eNOS in fishes (see chapter 6), so an eNOS-dependent tachycardia may not exist in fishes.

The response to prazosin in one of the trout was somewhat unexpected given that prazosin has previously been shown to have no effect on HR in trout (Sandblom & Axelsson, 2005). However, reduced peripheral resistance by α-blockade is also detected by baroceptors in mammalian vessels and may have caused reflex tachycardia by triggering an increase in sympathetic activity and reduced vagal activity, but there is some evidence from European eels (*Anguilla Anguilla, L)* (Soulier et al., 1988) and rainbow trout (Wood & Shelton, 1980) suggesting the baroceptor reflex to hypotension in fishes is weak compared with the mammalian response.

The findings in this study corroborated the findings of previous studies in rainbow trout, suggesting the low frequency component of the power spectrum represents adrenergic activity as β-blockade demonstrated a trend towards a reduction in the low frequency component (Devera & Priede, 1991). The data in this study also agrees that vagal cholinergic activity is withdrawn in stressed trout as there was a non-significant increase in LF/HF ratio in those trout in which the HRV was reduced dramatically, and atropine had no further effect on the HR.

The two methods used in this study to investigate changes in HR with exposure to high and low temperatures and with pharmacological interventions each have their own disadvantages, as well as advantages. The study in which the trout are held in tubes, allows simultaneous recording of MO_2 and HR in order to understand the effects of the different conditions on HR and metabolism and the relationship between the two. Additionally, using the PowerLab to record HR allows power spectral analysis to be carried out with greater ease, to isolate the effects of sympathetic and vagal tones. Furthermore, these fish are contained, so drugs can be administered when needed. Finally, the surgery carried out for HR recordings from trout contained within tubes are less invasive and traumatic for the trout so recovery should occur more rapidly. However, despite the more invasive surgery required for intraperitoneal datalogger implantation, using this method, the animals are released back into a tank and are not isolated, thereby allowing interventions to be made under normal 'resting' conditions. In addition, the reasoning behind this study was to carry out a study parallel to that in Chapter 3 in which trout were acclimated to their experimental temperature for up to 8 weeks. However, Home Office regulations do not permit the keeping of animals in tubes for longer than 2 weeks because fish held in tubes cannot be fed and the maximum permitted food withdrawal time is 2 weeks. As such, the experiments with dataloggers are more suitable because the fish can be kept and recordings taken, for extended periods of time. Furthermore, the drugs may be administered with the fish feed in those fish carrying dataloggers, as there is no restriction on

feeding these trout. However, as seen in Chapter 3, food intake cannot be predicted in unrestrained fishes. Unlike that which was suggested previously, the implantation of mini-osmotic pumps in these trout would be problematic given that implanted loggers will have already filled the peritoneal cavity, and externally placed loggers reduce buoyancy of the trout significantly and any additional weight on the trouts could severely stress them.

4.4.1 Conclusions

This study showed that LNNA reduced HR and therefore cardiac output at high temperature in one fish. This suggests that any capillary growth that may occur in the presence of L-NNA is unlikely to be a result of the inhibition of a positive inotropic effect of NO at high temperatures. It also shows that although, contrary to studies on mice, prazosin does not seem to promote angiogenesis (Chapter 3), when administered i.p., it may be causing reflex tachycardia secondary to reduced peripheral resistance. This may be expected to increase cardiac output and therefore shear stress on the vessel wall, but this does not seem to cause angiogenesis in fishes. This may be because despite the increased cardiac output and increased blood flow due to vasodilatation, the blood viscosity at high temperature is reduced to such an extent, that prolonged increases in cardiac output and blood flow only serve to restore the shear stress levels required for maintenance of existing capillaries.

Two methods for HR investigation were attempted in this study, and due to the stress and deaths caused by food withdrawal and isolation in fish kept in tubes, in addition to the fact that drugs can be administered through food in free-swimming fish, it is suggested that the use of dataloggers for long-term recordings is more suitable for this study. Due to time and financial constraints, only pilot experiments to assess the health and survival of trout and carp with dataloggers have been carried out thus far.

Chapter 5 Transcriptional responses to altered environmental temperature

5.1 Introduction

Microarray analysis of gene expression makes use of Watson-Crick complementary base pairing to determine transcript abundance, to quantify expression of thousands of genes in a particular cell type or organism, at a particular time, under particular conditions, in a single experiment. cDNA arrays consist of spotted PCR amplicons that represent specific genes on a solid matrix. The first cDNA microarray was developed using the plant *Arabidopsis thaliana* owing to its small genome (Schena *et al.*, 1995). Fluorescent-labelled (e.g with cyanine dyes) antisense RNA or complementary DNA from the experimental samples is applied to the array to hybridise with the immobilised DNA samples. If the DNA on the array is double stranded, sense RNA or DNA may be used (Southern *et al.*, 1999). The intensity of the labels at each spot is used as an index of gene expression- greater intensity represents a greater expression of the gene. Microarray analyses carry the advantage that genome-wide integrative pathways of responses can be identified. Further, they may highlight the importance of genes that had not previously been considered to play a part in the responses under investigation (Duggan *et al.*, 1999).

Though the entire genome for the zebrafish, pufferfish species, fugu and the Japanese medaka have been sequenced, limitations lie in the use of microarrays for studying non-model species such as common carp because, often, for these species the entire genome has not been

sequenced. Microarrays with non-model species therefore often use expressed sequence tags (ESTs), which may not be annotated and where much of the annotation is based on zebrafish sequences (Herbert *et al.*, 2009). ESTs are a sequence of nucleotides transcribed from mRNA. As each gene may not correspond with a unique EST, false positives may arise. Additionally, as ESTs are based on expressed genes, genes with a low copy number, or genes specific to a minor tissue compartment, such as those from endothelial cells in skeletal muscle, may be underrepresented.

Increased thermal tolerance, seen with temperature acclimation in temperate fishes is accompanied by complex changes in gene expression that aid processes such as skeletal muscle remodelling (Cossins *et al.*, 2006; Gracey *et al.*, 2004). Earlier investigations into temperature acclimation were hypothesis-driven studies looking at changes in single gene expression. Although these studies demonstrated important changes in gene expression for example an increase in anti-freeze protein genes expression in winter-acclimated winter flounder (Pickett *et al.*, 1983), an increase in delta-9-saturase gene expression, which increases cell membrane fluidity (Tiku *et al.*, 1996), and reduced β_2-microglobulin transcription (which may suggest a tendency towards reduced immune function, perhaps to conserve energy, but without comparable assessment of other immune-regulatory gene transcription, this is uncertain), in cold-acclimated carp (Rodrigues *et al.*, 1998). More recent studies have explored the global response to temperature change in fishes such as the goby, where microarray studies on heat-

stressed muscle show repression of many genes involved in promoting cell growth and proliferation, providing more convincing evidence of gene suppression for energy conservation (Buckley *et al.*, 2006).

The global transcriptional responses to cold-acclimation in common carp have been investigated in various tissues but not red muscle (Gracey *et al.*, 2004). Common carp is an ideal organism for studying transcriptional responses and coping mechanisms for extreme environmental challenges such as reduced oxygen availability (as these fish can tolerate anoxia for several months), and temperature acclimation as they have a wide thermal tolerance range. Additionally, the polyploidy of their genome increases the probability of seeing a genomic response to these challenges as there is a presumed plasticity of their genotype. The study by Gracey *et al* (2004) demonstrated the response in the pooled RNA from several carp that had been cooled to a common temperature, so variability between individual animals could not be assessed. That there may be differences in transcriptional responses at different time-points in the same tissue, was not considered. As such, their method of step-wise cooling to different temperatures over several days meant that those animals that were exposed to the coolest temperatures were also exposed to greater thermal stress because of both the duration and the extent of temperature change.

In this study, we focus on red muscle, as angiogenesis and hypertrophy takes place in this tissue on cold exposure (Egginton & Sidell, 1989). Furthermore, at low temperatures swimming speed is reduced and red muscle is more likely to be in continual use when swimming speed is reduced (Johnston *et al.*, 1977) and metabolic demand on white muscle in some fishes is expected to be minimal as they go into torpor (Guderley, 2004). Indeed transcription of white muscle contractility genes are repressed in cold-exposed animals (Gracey *et al.*, 2004). Tissue samples were taken at 3 temperatures, and at 2 time-points to assess thermal and temporal changes in gene expression.

The purpose of this chapter is to explore the generalised transcriptional responses to temperature acclimation in carp red muscle, with particular focus on endothelial genes. In order to do this, gene expression profiling by microarray was carried out. This enabled the investigation into changes in the vasculature during temperature acclimation, especially those changes that may be counter-intuitive. This includes, for example, exploring the seemingly paradoxical situation where capillary regression occurs at warm temperatures where it might be expected that hypoxia-driven angiogenesis would occur, and capillary growth occurs at lower temperatures where oxygen availability to fishes is increased and metabolism is reduced.

5.2 Methods

5.2.1 Temperature acclimation

Carp purchased from Sport & Leisure Fisheries Centre (Telford, UK) were acclimated to 15°C in 3 tanks of 10 animals, for 4 weeks prior to exposure to their experimental temperatures. After the initial 4 weeks, one tank was cooled, the second was warmed, and the final tank was unchanged. The temperature change regime was such that temperatures were either increased or decreased at 1°C/hour over 10 hours. Red muscle samples were collected as described in section 2.2 and stored in RNase-free sterile microtubes at 4 and 8 weeks after temperature acclimation. The two samples used in each microarray were paired according to the interwoven loop design (Fig 5.1).

5.2.1 RNA extraction and assessment of quality and quantity

Total RNA was extracted by homogenising frozen carp red muscle using Trizol reagent (2ml per 200-300mg tissue) by high-speed shaking (30 revolutions per second) of the tissue enclosed in a 2ml BioPur Eppendorf safe-lock microtube with a single, sterile, 5mm stainless steel bead (Qiagen) that had been pre-cooled in liquid nitrogen, using a Qiagen Tissuelyzer, for 4 minutes at room temperature. The lysate was incubated at room temperature for 5 minutes to allow complete dissociation of nucleoprotein complexes. Chloroform was added to the lysate (200µl per ml Trizol reagent) to separate the solution into an aqueous phase and an organic phase; the

RNA remained only in the aqueous phase. The aqueous phase was separated and washed with 75% ethanol and the RNA purified from solution using the Trizol® Plus RNA Purification System (Invitrogen), as per the manufacturer's instructions.

All RNA samples were quantified using the NanoDrop 2000 spectrophotomer and checked for quality using the Agilent Bioanalyzer (Appendix C). The Agilent Bioanalyzer provides an index of the RNA quality, by way of an RNA integrity number (RIN), ranging from 1-10, with a RIN of 1 indicating heavy degradation, and 10 indicating the most intact RNA (Schroeder *et al.*, 2006) As the quantity of RNA extracted from many samples was insufficient to carry out microarrays (<5µg), RNA amplification was necessary. In addition, it has been documented that the used of amplified RNA improves the sensitivity of the array analysis, which enables the identification of biologically regulated genes that might be missed if unamplified RNA is used (Polacek *et al.*, 2003).

5.2.1 RNA amplification

mRNA was amplified by the Van Gelder & Eberwine linear RNA amplification technique (Van Gelder *et al.*, 1990) using Ambion's Amino Allyl MessageAmp™ II aRNA Amplification Kit with a starting quantity of 1µg total RNA. Linear amplification is used as exponential amplification, as used in PCR, could exaggerate the difference in expression of two genes. The technique involved reverse transcription of mRNA templates with a reverse transcriptase, ArrayScript™, using an

oligo(dT) primer containing a T7 RNA polymerase promoter to synthesise full length first strand cDNA, at 70°C. mRNA, which constitutes 1-5% of total RNA, was used as the template as oligo(dT) primers bind to the poly(A) tail of mRNA. RNase H and DNA polymerase were used, at 16°C, to degrade mRNA and to synthesise second strand DNA, respectively. cDNA was then purified and *in vitro* transcription (IVT) was carried out at 37°C, to form aminoallyl-modified RNA (some of the UTP used in the IVT was modified aaUTP). The amplified RNA integrity was measured using the Agilent Bioanalyzer to ensure RNA quality was not lost during amplification.

5.2.1 RNA labelling

10µg Amino-allyl aRNA (aa-aRNA) was Cy3 or Cy5-labelled using the Amersham CyDye Post-Labelling Reactive Dye Pack, as per the manufacturer's instructions. Briefly, aa-aRNA was vacuum dried to 5µl, and made up to 40µl by adding 35µl 0.1M sodium bicarbonate buffer (pH 8.7) and mixing well. The aa-aRNA was then added to a vial of either Cy3 or Cy5 dye in DMSO, and incubated in the dark, at room temperature for 90 minutes to allow the dye to couple with aa-aRNA. The dye-labelled sample was then incubated for 15 minutes with 15ul 4M hydroxylamine after which the labelled aa-aRNA was purified using spin columns and resuspended in nuclease-free water. The purified labelled aa-aRNA was quantified using the Nanodrop and kept out of light until use.

5.2.1 Microarray preparation, hybridisation and quantification

Hybridisation to the microarrays was carried out according to standard protocol and in collaboration with the Liverpool Microarray Facility. Microarray slides, with 13,440 probes (Gracey *et al.*, 2004), were prepared for hybridisation by incubating slides in a pre-hybridisation buffer containing 3X SSC (saline-sodium citrate buffer; pH 7.0), 0.1% SDS (sodium dodecyl sulphate; Invitrogen) and 0.1mg/ml BSA (bovine serum albumin; Sigma) at 50°C for 60 minutes, washing three times in distilled water for 30 seconds at room temperature, dipping briefly in isopropanol (BDH), and drying by centrifugation, at 1000rpm, for 5 minutes at room temperature in a microtitre plate centrifuge. 2µg of the two labelled samples to be compared were combined according to the interwoven loop design (Fig. 5.1) and made up to 25µl. 25µl 2X hybridisation buffer (9X SSC, 0.3% SDS and 0.3mg/ml BSA), was added to the sample. They were then applied to microarray slides prepared according to the MAUI (MicroArray User Interface) system (Fig. 5.2), using a Gilson Microman positive displacement pipette and incubated in a MAUI® hybridisation chamber at 50˚C for 13 hours. The slide was then washed in heated 2X SSC + 0.1% SDS buffer at 42°C, for 5 minutes, 0.1X SSC + 0.1% SDS at 42°C for 5 minutes, once in 0.1X SSC at 42°C for 1 minutes, twice in 0.1XSSC at room temperature for 2 minutes, once in 0.01X SSC for 30 seconds and finally dipped in 0.01X SSC for less than 30 seconds, in the dark. The slide was then dried by centrifugation at 1000rpm for 5 minutes at room temperature. Slides were scanned using GenePix software and the image was quantified (fluorescence intensities measured and compared to background) using Bluefuse software (from BlueGnome).

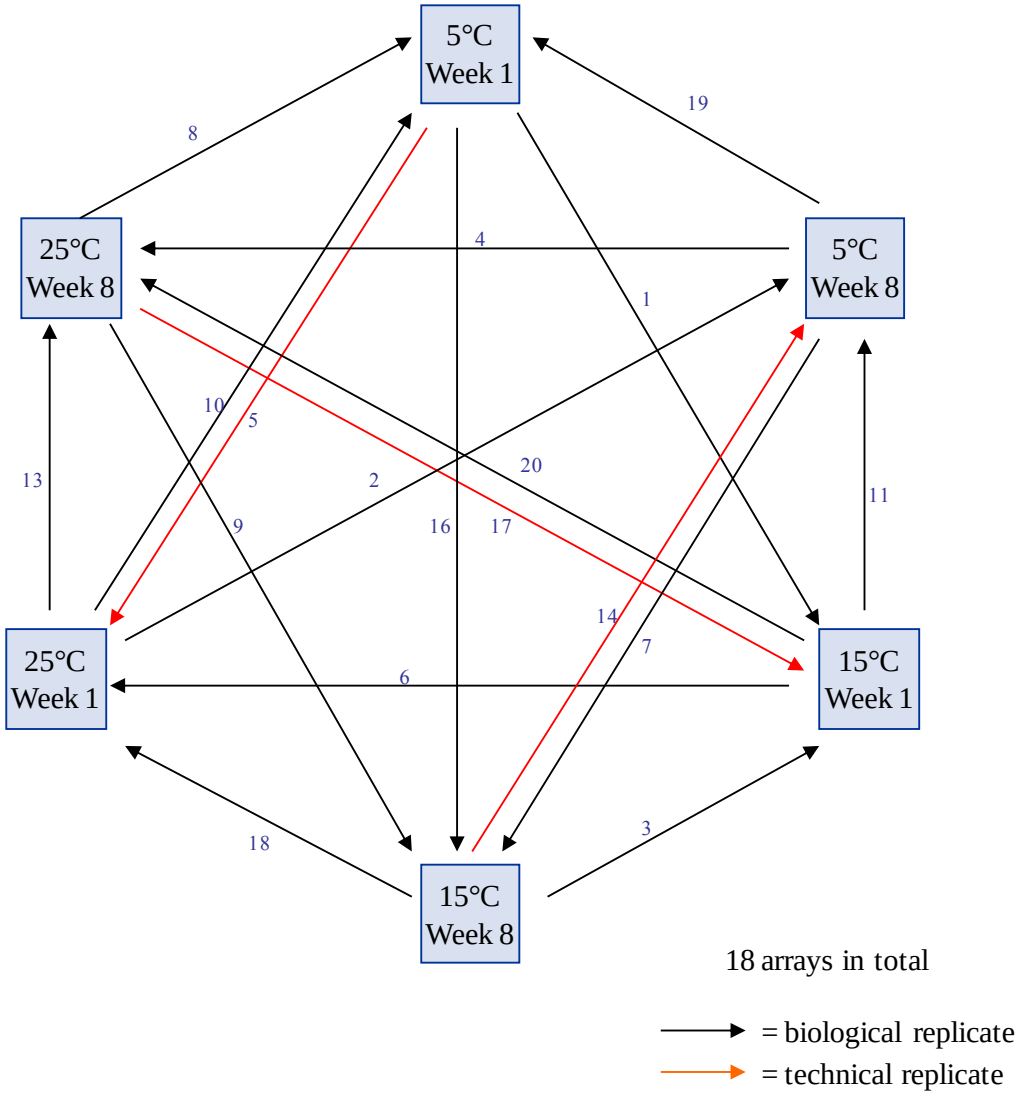


Figure 5.1 The interwoven loop design demonstrating the way in which samples were compared in order to minimise experiments, and statistical error associated with multiple t-tests. The values in the blue boxes indicate the acclimation temperature and the number of weeks after temperature acclimation the samples were taken. The numbers adjacent to the arrows depict the array number of the two samples joined by the arrows. 'Biological replicates' refers to the 5 arrays that tissue from each experimental condition, were used in. 'Technical replicates' refers to the arrays in which the array was repeated using the same two samples but where the dye-label was swapped.

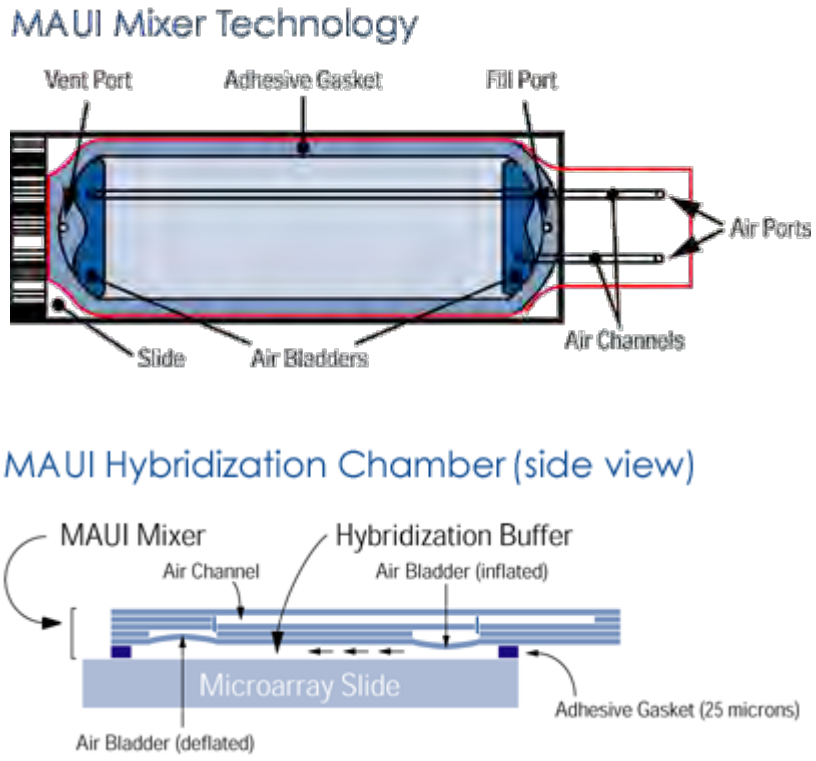


Figure 5.2. The MAUI mixer system. Tissue samples for hybridisation to the microarray slide are applied through the Fill Port (top image) using a Gilson Microman positive displacement pipette, so that the samples lie between the slide and the lifter slip (red outline). The air channels allow mixing and even distribution of the samples on the slide. The unit is sealed once it is within the hybridisation chamber (bottom image) so samples cannot evaporate and slides do not dry. Images reproduced from BioMicrosystems Inc.

5.2.1 Analysis (I)

Principal Component Analysis (Fig. 5.3) was carried out, with the assistance of F. Falciani (University of Birmingham), using TM4 Multi-experiment viewer (TMEV) software (http://www.tm4.org/mev/) to identify directions, i.e. principal components, along which the variation in the data is maximal, using three dimensions (components). This allows the highly variable data in microarrays to be simplified into meaningful groups, to identify those groups that are the most different from one another, giving an overview of the direction of change between experimental groups (Ringner, 2008). Experimental groups were then hierarchically clustered and displayed in the form of a heatmap using bootstrap analysis in TMEV (Fig. 5.4) to identify groups of genes that were consistently up-regulated or down-regulated at each temperature and time-point. Bootstrap analysis was carried out to calculate the relationship between differentially expressed genes, with the length of connecting lines being proportional to their similarity of response.

5.2.1 Analysis (II)

Normalisation was carried out with the assistance of Y.Fang (NERC Microarray Facility), according to the method of Fang *et* al (Fang *et al.*, 2003). Briefly, Dye-bias (Loess), to check responses were dye-independent, and spatial bias (2-D Loess), to ensure even probability of hybridisation across the array, corrections were carried out, followed by t-tests. M-A plots

(Dudoit *et al.*, 2002), where M (the log ratio of Cy3 to Cy5) are plotted against A (the sum of intensity of both dyes), were created to assess the intensity of the Cy3 and Cy5 dyes (Fig 5.6).

Arrays and samples were separated into 15 groups (contrasts) that were used to assess differential expression between all 6 conditions. The contrasts were numbered like so:

1. 2vs1 2. 3vs1 3. 4vs1 4. 5vs1 5. 6vs1 6. 3vs2 7. 4vs2 8. 5vs2 9. 6vs2 10. 4vs3 11. 5vs3 12. 6vs3 13. 5vs4 14. 6vs4 15 . 6vs 5

1=5^{o}C week 1 2=5 ^{o}C week 8 3= 15 ^{o}C week 1 4=15 ^{o}C week 8 5= 25 ^{o}C week 1 6=25 ^{o}C week8

529 differentially expressed (DE) genes were clustered into 15 clusters by a k-means cluster method (MacQueen, 1967). GO-stats analysis (Martin *et al.*, 2004)was carried out on the DE genes, to identify gene sets with functional similarity, and the significant GOs were extracted by both P-value <0.01, obtained from pairwise t-tests, and false discovery rate (FDR; q-values) < 0.1. FDR is the probability of the occurrence of false positives in the dataset. A high FDR was chosen because with an FDR of 0.05, few DE genes could be identified.

5.3 Results (I)

Principle Component Analysis (Fig. 5.3) revealed that transcription at 15°C (control) remained constant over the 8 week experimental period. Transcriptional changes at 5°C and 25° were time-dependent and occurred in all three components. The greatest overall variation occurred between 5°C and 15°C compared to 25°C, which increased with time. Some genes were consistently upregulated or downregulated at a given temperature, irrespective of time-point, whereas some genes were either up or down regulated at 1 week after temperature acclimation but the converse response occurred after 8 weeks. An attempt was made to identify these genes and group them according to function, but many of the DE genes were unannotated. In the list of 20 most differentially expressed genes, 10 of those were unannotated or labelled as an 'unclassifiable EST'. Many of the genes that were upregulated at high temperature were for ribosomal proteins. The upregulation of the β-actin gene here suggests some remodelling is occurring. Genes that are known to be upregulated (e.g. VEGF and angiopoietin-1) or downregulated (e.g. angiostatin and endostatin) during angiogenesis, were sought in the DE gene list but none were present. In addition there was no evidence for transcriptional responses to hypoxia for example through hypoxia up-regulated protein-1 (hyou-1) or hypoxia-inducible factors (HIFs), in warm or cold-acclimated animals. Although the latter is not thought to be transcriptionally regulated in mammals, there is some evidence for its transcriptional regulation at low temperature in insects (Morin *et al.*, 2005).

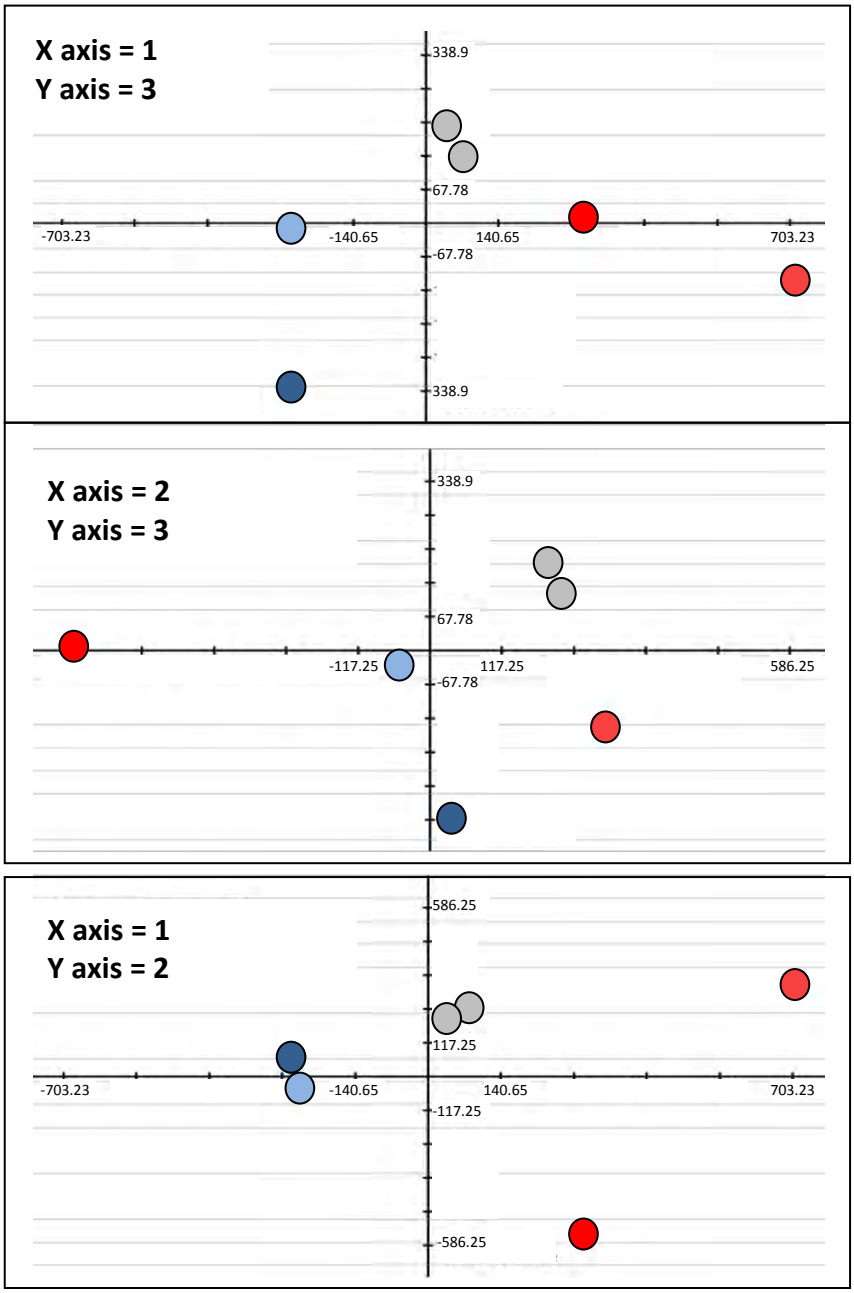


Figure 5.3 Principle Component Analysis of the overall transcriptional responses to temperature acclimation to 5°C (blue circles), 15°C (grey circles) and 25°C (red circles). Darker shades of each colour represent the longer acclimation period. This PCA shows the global discrimination among acclimation groups, revealing both thermal and temporal changes. It also shows that there is little change in global transcription in at the control temperature. Note: the 15°C samples remain constant over the 8 week period

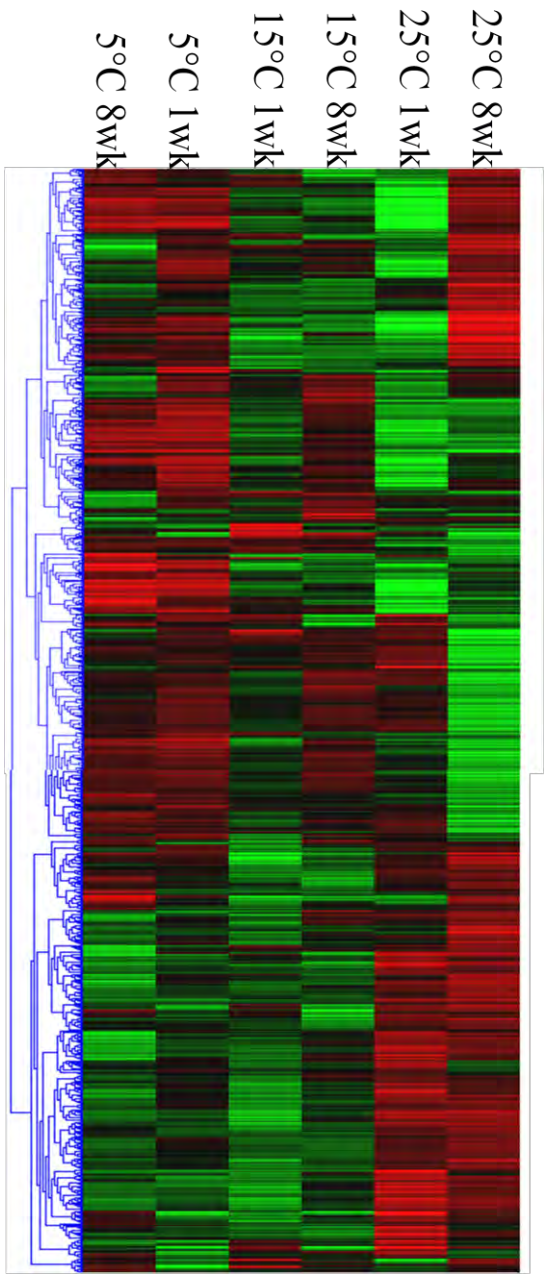


Figure 5.4. Heatmap demonstrating the genes that were consistently upregulated (red), downregulated (green) or unchanged (black) in all samples under the 6 different experimental conditions, 5°C, 15°C and 25°C at weeks 1 and 8. A bootstrap method of analysis to identify genes similar in expression patterns was used. The distance of the lines (distance tree-left hand side), are proportional to the distance between genes. These could not be separated by gene ontology, as the information is not publicly available so this could not be carried out outside the NERC Microarray Facility.

5.4 Results (II)

Comparison of Cy3 and Cy5 dye intensities before and after normalisation are shown in Fig 5.5. Normalisation of dye intensities is necessary to reduce the influence of the fluorescent label on the results, especially in a situation such as this, where dye swap experiments did not reach the same conclusion. Fig 5.6 demonstrates that normalisation of dye intensities did not correct the dye bias, instead it introduced greater variation in the low intensity spots. In many of the contrasts analysed, the fold ratio (increase or decrease) did not correlate well with statistical significance (P-value; Fig 5.7). The above demonstrates that, overall, the results of this set of microarrays are unreliable. Histograms (Fig 5.8) show that there is a low number of DE genes, and where the genes are DE, the p-value is low. A closer look at two contrasts- 25°C, week1 vs 5°C week 1 and 25°C week 8 vs 5°C week 1- where the DE genes were highest, shows that the FDR is 1 in 6, so in the approximately 360 DE genes, 60 are expected to be false positives (Fig 5.9). Furthermore, a heatmap constructed to visualise the overall picture of DE genes shows that in most cases there is little change in gene expression, and that the majority of the changes are seen in warm-acclimated carp compared with cold-acclimated carp (Fig 5.10). Table 5.2 lists the number of genes within a GO group that are differentially expressed in warm-acclimated animals at week 1 and week 8 compared with cold-acclimated animals at week 1. None of these genes are directly involved in angiogenesis.

Before normalization

After Normalization

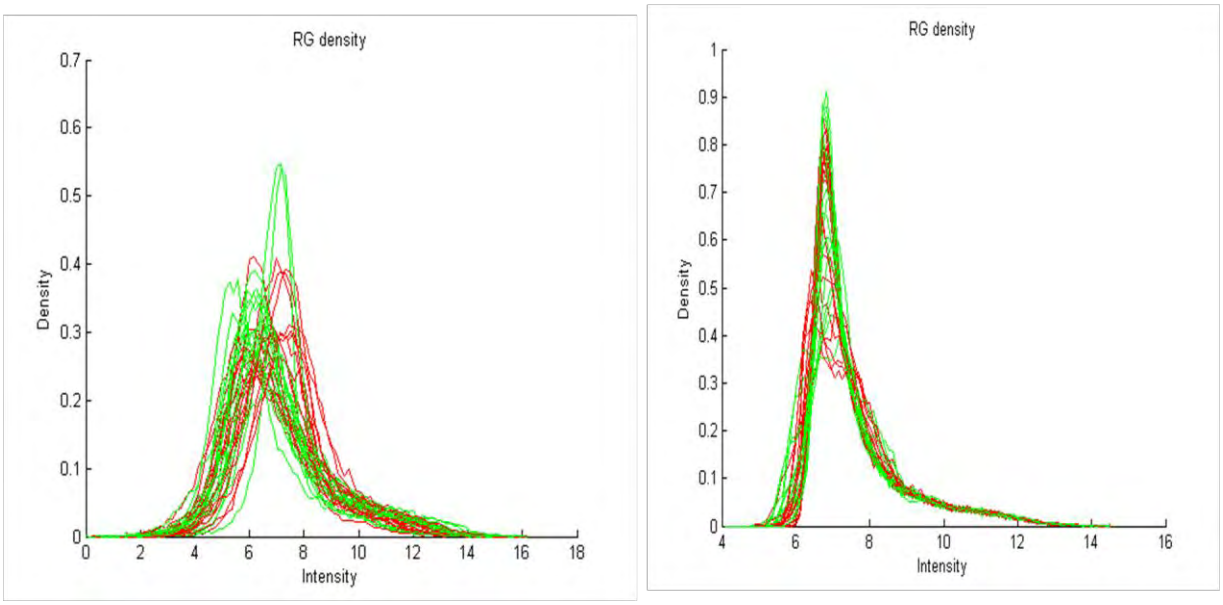


Figure 5.5. Comparison of total Cy3 (green) and Cy5 (red) intensities from all microarrays, to determine that the normalisation procedure was successful.

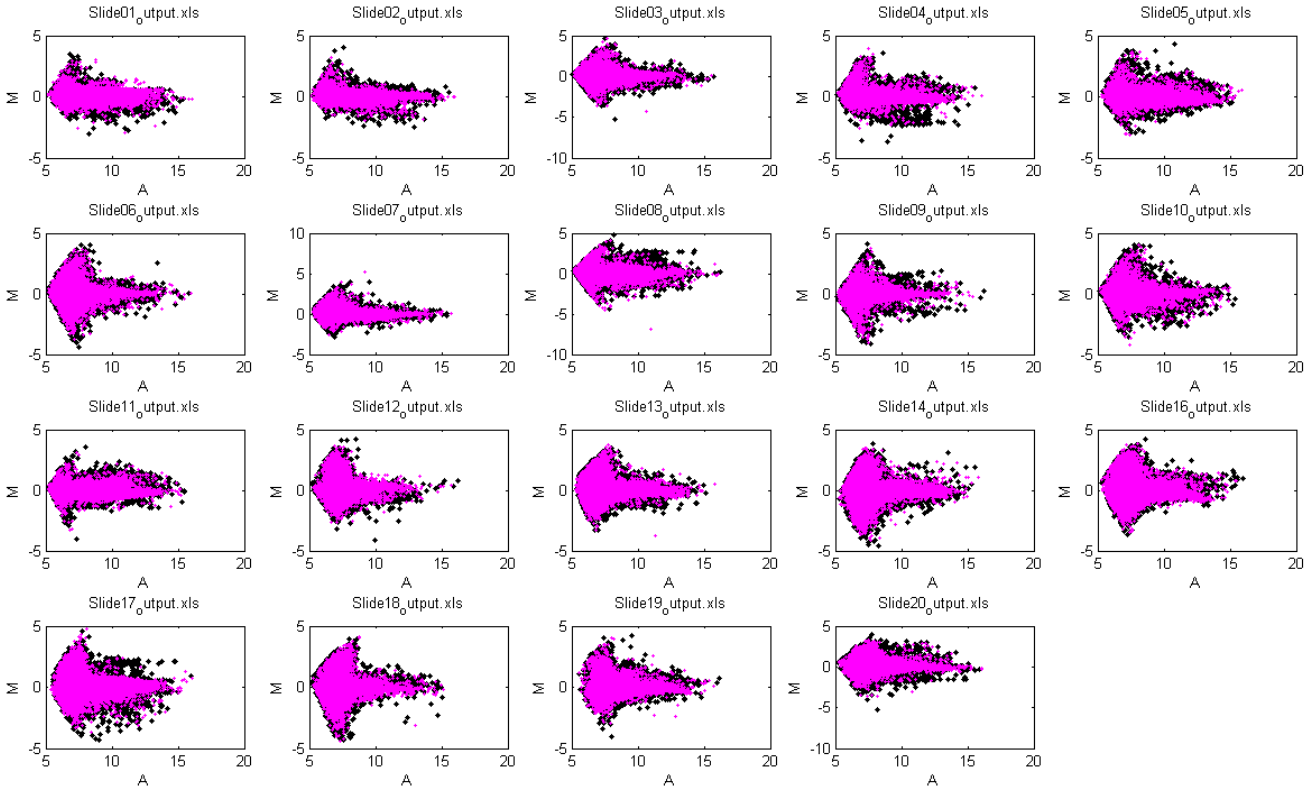


Figure 5.6. M-A plots demonstrating the intensity of the Cy5 and Cy3 dyes on each array, after normalisation. M= the log ratio of Cy3 to Cy5 dyes), A= the sum of intensity of both dyes. Where dye intensities are even, the line lies along 0 on the Y-axis. Here, the winged appearance at the low intensity regions suggests that normalisation introduced greater variability in the low intensity spots.

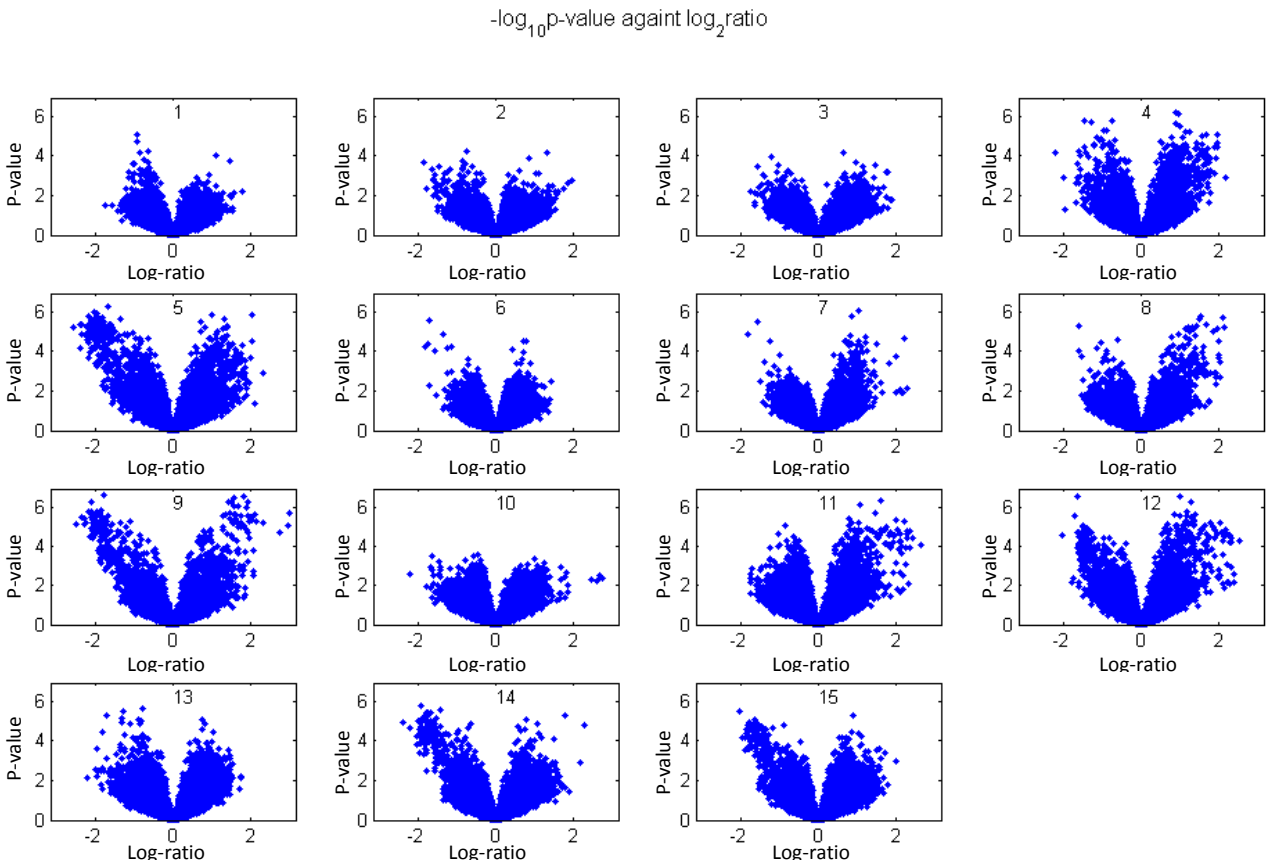


Figure 5.7 Volcano plot displaying significance (P-value) against log-ratio (fold change) to identify whether overall the fold-ratios seen are significant in each contrast. Contrasts 9, 11 and 12 show that fold changes correlate well with significance (i.e. that increased fold ratio, negative or positive, corresponds with an increased P-value. Contrasts 1,2,3,6,7 and 10 show a poor correlation.

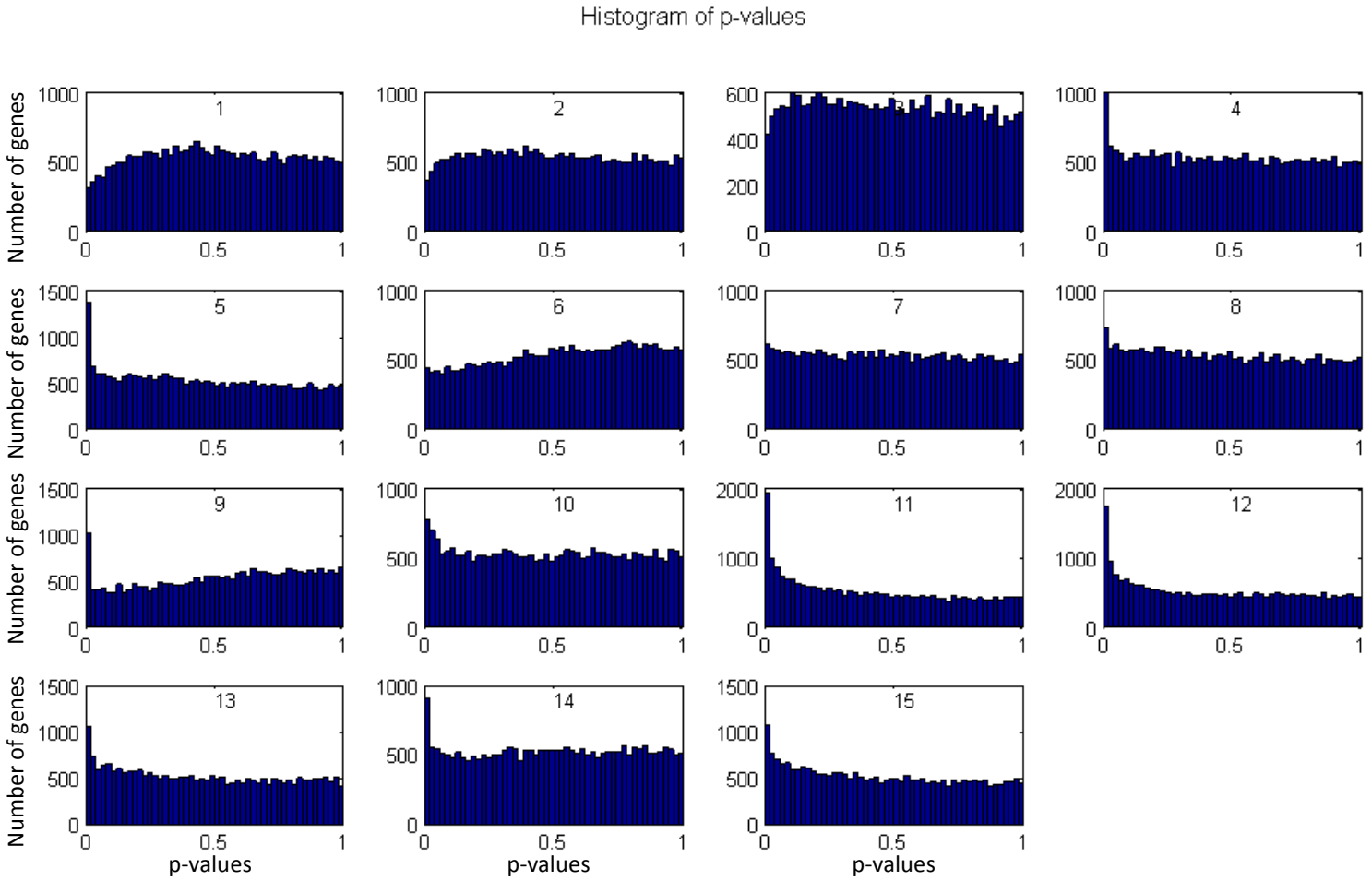


Figure 5.8. Histograms showing distribution of p-values of the individual contrasts. Anything that is greater than the height of the flat portion of the histogram (defined by the average height of all the bars in a plot), represents differential expression. As is evident, there is little differential expression for most of the contrasts. The contrasts are: 1. 2vs1 2. 3vs1 3. 4vs1 4. 5vs1 5. 6vs1 6. 3vs2 7. 4vs2 8. 5vs2 9. 6vs2 10. 4vs3 11. 5vs3 12. 6vs3 13. 5vs4 14. 6vs4 15 . 6vs 5; 1=5^{o}C week 1 2=5 ^{o}C week 8 3= 15 ^{o}C week 1 4=15 ^{o}C week 8 5= 25 ^{o}C week 1 6=25 ^{o}C week8

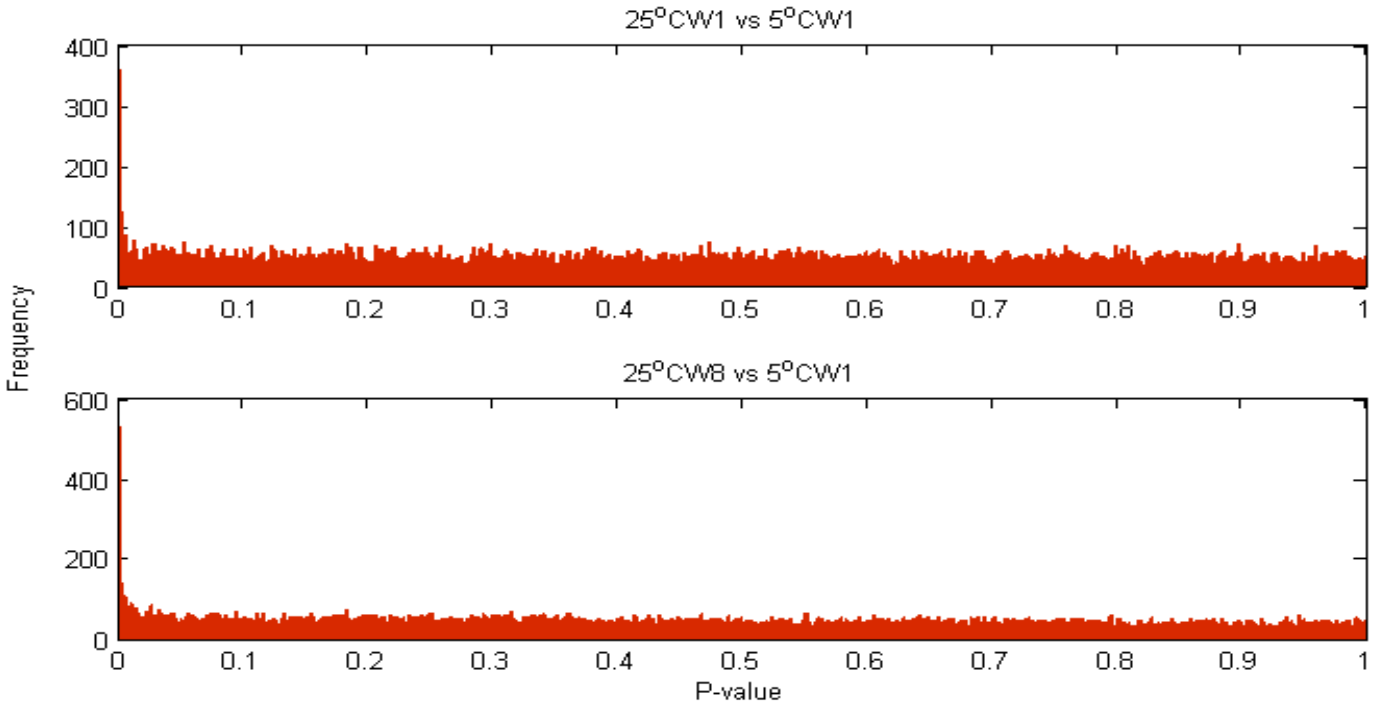


Figure 5.9. These histograms display the distribution of two contrasts (5°C week 1 compared with 25°C weeks 1 (top) and 8 (bottom) that have the greatest number of differentially expressed genes. It is seen that there are at least 360 differentially expressed genes but the false discovery rate is 1 in 6, so it is expected that approximately 60 of these genes are false positives.

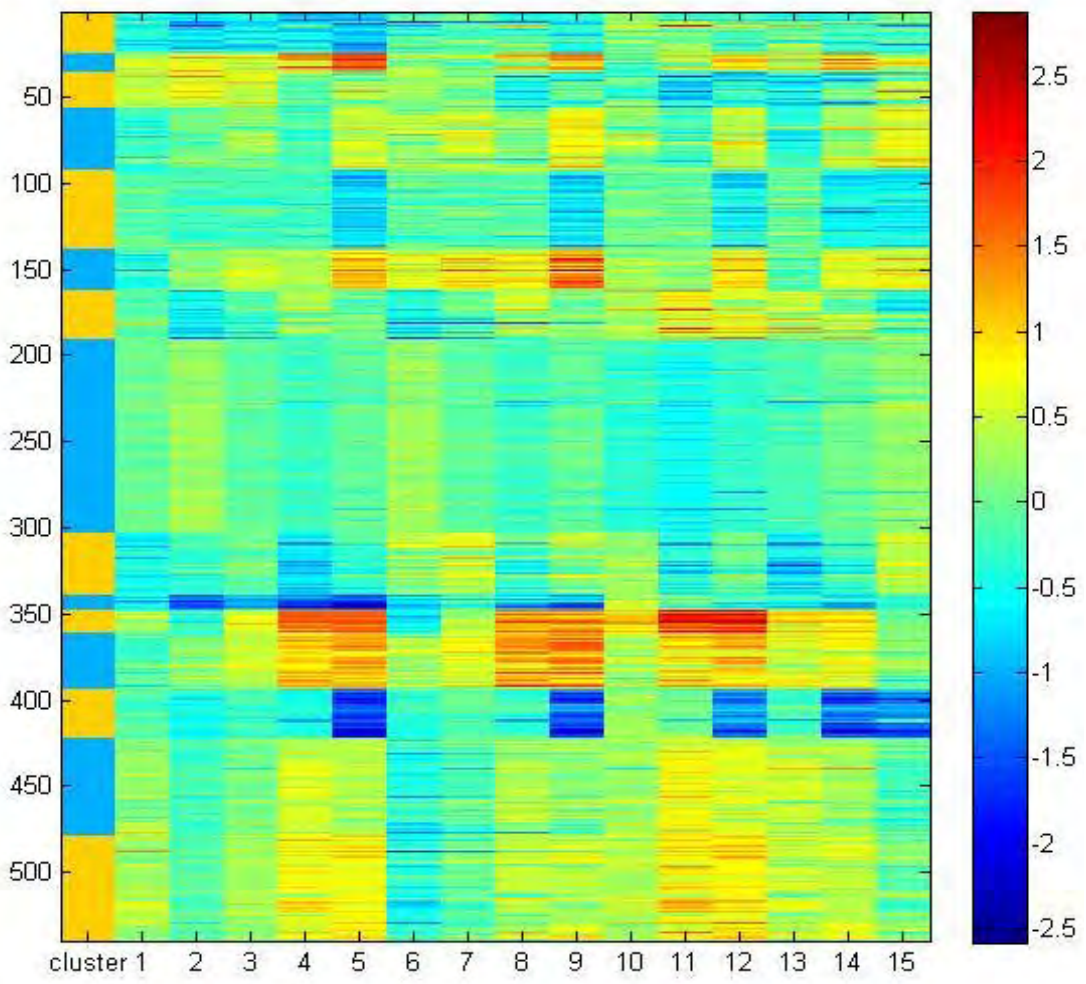


Figure 5.10. Heatmap of the 529 differentially expressed (DE) genes in all 15 contrasts. In some contrasts such as 1,2,3,6,7,10,13 and 15, the change in expression is minimal. The alternating blue and yellow boxes indicate clusters of similarly expressed genes within the 529 DE genes. The bar on the right shows the log ratio that each colour represents. The contrasts at the bottom of the heatmap are described as: 1. 2vs1 2. 3vs1 3. 4vs1 4. 5vs1 5. 6vs1 6. 3vs2 7. 4vs2 8. 5vs2 9. 6vs2 10. 4vs3 11. 5vs3 12. 6vs3 13. 5vs4 14. 6vs4 15 . 6vs 5, where 1=5^{o}C week1 2=5 ^{o}C week8 3= 15 ^{o}C week1 4=15 ^{o}C week 8 5= 25 ^{o}C week 1 6=25 ^{o}C week 8

GOName (LIST A)	P-value	Q-value	NumberGenes
regulation of transcription, DNA-dependent	0.87	0.99	293
oxidation reduction	0.87	0.99	176
signal transduction	0.83	0.99	192
metabolic process	0.80	0.99	99
protein transport	0.79	0.99	73
immune response	0.69	0.99	78
cell adhesion	0.60	0.98	97
ubiquitin-dependent protein catabolic process	0.48	0.96	132
multicellular organismal development	0.33	0.92	101
transport	0.31	0.90	164
biological_process	0.30	0.90	1607
protein amino acid phosphorylation	0.26	0.89	143
proteolysis	0.20	0.82	162
cell differentiation	0.12	0.70	80
apoptosis	0.10	0.70	88
mRNA processing	0.06	0.60	67
RNA splicing	0.03	0.46	76
translation	3.81E-07	0.00	145
translational elongation	3.35E-07	0.00	87

GOName (LIST B)	P-value	Q-value	NumberGenes
protein transport	0.99	0.99	73
regulation of transcription, DNA-dependent	0.93	0.98	293
cell adhesion	0.84	0.96	97
metabolic process	0.79	0.96	99
cell differentiation	0.77	0.96	80
small GTPase mediated signal transduction	0.63	0.94	66
mRNA processing	0.56	0.94	67
oxidation reduction	0.52	0.94	176
RNA splicing	0.43	0.92	76
multicellular organismal development	0.30	0.85	101
signal transduction	0.29	0.84	192
ubiquitin-dependent protein catabolic process	0.25	0.83	132
proteolysis	0.19	0.83	162
biological_process	0.17	0.79	1607
immune response	0.07	0.62	78
protein amino acid phosphorylation	0.06	0.59	143
apoptosis	0.01	0.16	88
transport	8.61E-05	0.01	164
translation	1.54E-20	2.14E-17	145
translational elongation	1.66E-27	4.6E-24	87

Table 5.1 DE genes grouped by GO and ranked by P-values to identify the pathways regulated by increased temperature. List A is the P-values for DE genes in 5°C week 1 versus 25°C week 1. List B is the P-values for DE genes in 5°C week 1 versus 25°C week 8.

5.5 Discussion

In the present study, the aim was to investigate the transcriptional responses to cold and warm acclimation. To minimise the stress response to rapid and acute temperature change, animals were acclimated to a common optimal temperature for that species and their tank temperatures changed uni-directionally, once. The animals were then allowed to equilibrate to that temperature before samples were taken. This is in contrast with the study carried out by Gracey *et al* (2004) where the fish were acclimated to temperatures at the upper limit of their thermal tolerance range, and the temperature was reduced in a step-wise manner. The upregulation of common stress protein genes in that study suggest that the cooling regime used caused a general stress response (Cossins *et al.*, 2006), thus may not be possible to separate 'stress' from a 'true' cold response. We reasoned that a smaller temperature change from a common temperature would improve this discrimination. Furthermore, other studies have demonstrated that cold-induced increases in enzyme activity are far less pronounced in animals which have been acclimatised to changes in seasonal temperatures (a gradual process) than those animals that have been exposed to rapid temperature changes and acclimated under laboratory conditions (Johnston & Sidell, 1984; Kleckner & Sidell, 1985). This suggests that acclimation studies demonstrating a substantial change in the skeletal muscle morphology (Johnston & Maitland, 1980), including vascularity (Johnston, 1982), may represent the combined effects of laboratory stress, cold-acclimation, and seasonal endogenous rhythms.

Principal component analysis (Fig. 5.3) showed that the control (15°C) animals were stable over the 8 week experimental period, and that there was a time and temperature-dependent shift in global gene expression. However, closer inspection using an alternative form of analysis, targeting individual genes demonstrated that most of the differential expression was in the warm-acclimated carp, suggesting that transcriptional responses to increased temperature are more pronounced than responses to lowered temperature. Many of the differentially expressed genes were associated with embryonic development, muscle remodelling, cytoskeletal reorganisation and stress (e.g. beta actin, alpha actin,) and apoptosis (e.g. janus kinase 1, alpha actin). The time-dependent shifts in transcription reveal that the consideration of temporal responses to cold-acclimation is essential, something that was not taken into account in the study by Gracey *et al* (2004), where the differences between the temperature were in addition to the time the animals were exposed to temperature changes.

In this study, there was no evidence that genes switched on by hypoxia (e.g. hyou-1 and HIF) were upregulated, despite our finding in chapter 4 that warm-acclimation of carp may cause hypoxia, as demonstrated by the ST segment elevation of the carp ECG at high temperature (Fig. 4.3B). Little change in transcriptional response in cold-acclimated animals at 1 week suggests that homeostasis is restored within that time and it may be that alterations in gene expression had taken place within a few days after acclimation, or that transcriptional responses to thermal

acclimation take longer than 8 weeks to be initiated. Alternatively, low temperature may have suppressed any molecular metabolic response in order to conserve energy, as has been suggested to occur in goby (Buckley *et al.*, 2006). That angiogenesis-related genes were not upregulated at 1 or 8 weeks of cold-acclimation, a time-point at which angiogenesis has previously been reported (Johnston, 1982), suggests that the cooling regime used in this study more closely resembles seasonal acclimatisation than cold-acclimation. Seasonal acclimatisation has been shown to often involve subtle changes in structure and metabolism, such that although the cumulative response may be biologically significant, individual elements may change very little (Cordiner & Egginton, 1997). We were unable to quantify capillary supply in the carp used for this study because we were unable to find a suitable endothelial marker (see Chapter 2).

Another possibility is that there was too low a signal:noise ratio to generate an adequate signal, as endothelial cells comprise only a minor part (less than 5%) of total muscle volume so the expression levels, though they may be biologically significant, may be undetected as their tissue expression levels fall below the false discovery rate. This problem could be overcome by endothelial cell isolation from the muscle, but this is technically difficult and has not yet been achieved in fish. In order to carry out PCR analysis of known endothelial genes to validate this

study, endothelial gene sequences need to be known but at the current time, the sequences are not publicly available.

Genes associated with glycolytic enzymes were upregulated in this study, despite their relatively low abundance in red muscle. However, oxidative phosphorylation genes were unchanged suggesting no real change in metabolic demand, or ability to synthesise ATP despite reduced enzyme kinetics at low temperature, supporting our hypothesis that cold-induced angiogenesis is mechanically stimulated because metabolic demand does not increase because of the onset of torpidity in fish at low temperature. Extensive tissue remodelling genes are upregulated suggesting that there is some strain such as shear stress, or stretch due to altered fibre size, on the endothelium that may be contributing to angiogenesis (Egginton, 2002). In order to investigate the possibility of viscosity-related shear-stress on endothelial cells, we sought to identify whether any genes with shear stress response elements (SSREs) were upregulated. However, a literature search on NCBI demonstrated that thus far, no genes with SSREs homologous to mammalian genes with SSRES have been identified in common carp, though some exist in zebrafish, such as tumor necrosis factor (TNF) and ras homolog gene family, member A (RhoA).

5.5.1 Conclusions

Microarray analyses of red muscle from common carp that were cold and warm-acclimated revealed that the method of temperature-acclimation could have an impact on transcriptional responses. It is proposed that the transcriptional response seen to gradual, uni-directional change in temperature is limited compared to the response to severe temperature change, and that there is no evidence for transcriptional responses suggesting an angiogenic response. Further, this method of laboratory acclimation produces results that are more similar to the responses to seasonal acclimatisation than those seen in previous laboratory acclimation studies.

5.5.2 Future directions

The cooling regime used in this study was more representative of the changes in temperature common carp are exposed to in their natural environment than previous studies on temperature acclimation in common carp; therefore the results seen were probably less likely to be associated with a stress response. The interwoven loop design used for this experiment was ideal for stringent statistical analysis compared with comparing all conditions to a reference point, which would have required multiple t-tests that increase the room for statistical error. However, the success of this method was dependent on perfect hybridisation to arrays, which

was not the case in this study. Furthermore, the RNA extraction and amplification methods used produced good quality RNA and aRNA. However, despite these stringent quality control methods, hybridisation to the microarray slides was uneven and less reliable than expected. In future studies, more focus needs to be put on microarray spot generation and hybridisation techniques used.

Chapter 6 Identifying eNOS in fishes

6.1 Introduction

The structure and functions of the three known nitric oxide synthase (NOS) isoforms have been described in Chapter 1. The importance of eNOS in regulating vascular tone in mammals has been demonstrated in an enormous number of studies since the identification of NO as an endothelium-derived relaxing factor (Palmer *et al.*, 1987). Gene knockout studies show that mice lacking an eNOS gene have high blood pressures and low heart rates (Shesely *et al.*, 1996), but this does not apply for iNOS knockout (-/-) mice (Laubach *et al.*, 1995). The co-expression of nNOS and eNOS in human endothelial cells suggests that nNOS may play a supporting role in controlling vascular tone (Bachetti *et al.*, 2004). Indeed, ablation of the nNOS gene increases baseline blood pressure and hampers the baroreflex response in mice (Carvalho *et al.*, 2006). In some instances, such as during retinal vascularisation, eNOS deficiency is compensated for by increased nNOS activity (Al-Shabrawey *et al.*, 2003). At the current time, gene ablation is difficult in adult mammals that are non-murine, and much of the existing literature relies on non-specific NOS blockade using the L-arginine analogues, L-NAME (L-nitro-arginine methyl ester) and L-NNA (NG-nitro-L-arginine). Some selective nNOS antagonists such as 7-NI (7-nitroindazole) and TRIM (1-(2-trifluoromethylphenyl) imidazole) have been synthesised and their selectivity for nNOS over eNOS has been determined using binding studies and comparing their effects on physiological phenomena including control of vascular tone, which have not yet

been unequivocally attributed solely to eNOS (Handy *et al.*, 1996), so whether the criteria used for selectivity are suitable, has been questioned (Reiner & Zagvazdin, 1998). As such, although the role of NOS in the vasculature is very clear in mice, in some species -particularly lower vertebrates- which NOS isoform, if any, is responsible for maintenance of vascular tone remains unclear.

Angiogenesis is reduced during wound healing in $eNOS^{-/-}$ mice (Lee *et al.*, 1999) and eNOS is essential for splitting angiogenesis (Baum *et al.*, 2004; Williams *et al.*, 2006a), caused by increased blood flow (Zhou *et al.*, 1998a). So, we attempted to ascertain the role of NOS in cold-induced angiogenesis, which we hypothesised to be a consequence of blood viscosity-related shear stress. In this study, as with others in the literature, non-specific blockade with LNNA meant it was impossible to ascertain which NOS isoform would have been responsible had there been a response to NOS blockade (chapter 3)

Despite the common use of fish species, particularly zebrafish, as models for the study of vascular development, to our knowledge, eNOS RNA expression has not yet been studied. Studies apparently demonstrating eNOS activity, including that carried out by Fritsche *et al* (2000) in the developing zebrafish, used L-NAME to cause vasoconstriction in the vasculature (Fritsche *et al.*, 2000), concluding that L-NAME was inhibiting eNOS to inhibit vasodilatation. Given that the sequence for nNOS is known for this species (Accession no: NM_131660.1), gene

silencing using Morpholinos (anti-sense oligonucleotides used to block gene expression) (Summerton & Weller, 1997) for nNOS could have been used to eliminate the role of the nNOS isoform in the study. In that and other studies, immunohistochemistry has been used to localise eNOS, using antibodies raised against mammalian eNOS protein (Amelio *et al.*, 2006; Ebbesson *et al.*, 2005; McNeill & Perry, 2006). In the current study, some of these antibodies have been used for western blotting in trout, carp, zebrafish and pufferfish, and immunohistochemistry in trout, to assess their cross-reactivity.

More direct studies suggesting the possible role of endothelium-derived NO in the vasculature of trout show that chemical denudation of the large (conduit) coronary artery endothelium using CHAPS 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Volff, 2002)and L-arginine (Mustafa & Agnisola, 1998). However, in the carp aorta, vasodilation using the calcium ionophore, A23187, is endothelium-dependent, but not through the actions of NO. This was demonstrated using a rat-carp combination where A23187-stimulated EDRF release from the rat failed to elevate cGMP levels in the adjacent carp vessel, and where A23187 was unable to stimulate EDRF release from the carp to dilate adjacent rat vessels. However, directly applied acetylcholine and sodium nitroprusside were able to elevate cGMP in rat vessels (Park *et al.*, 2000). Similarly, Evans and Gunderson (1998) showed in the spiny dogfish shark, that exogenous SNP and L-arginine failed to cause vasodilation. Additionally, A23187-induced vasodilation was endothelium-dependent and was inhibited by indomethacin, but not L-NAME. Interestingly, in

the eel, NO causes vasodilatation in the aorta (Evans & Harrie, 2001), but vasoconstriction in the branchial circulation (Pellegrino *et al.*, 2002), further confusing the role for NO in the vasculature.

To address the lack of direct evidence for eNOS in fishes, eNOS mRNA and protein expression patterns in tissues from various fishes, were studied. Tissues from zebrafish (*Danio. rerio*; gifted by R. Bicknell, Uni. Birmingham*)* , pufferfish (*Tetraodon nigroviridis*; gifted by F. Mueller, Uni. Birmingham), common carp (*Cyprinus carpio*) and rainbow trout (*Onchorhynchus mykiss*) were used to clarify the presence of eNOS. Zebrafish and pufferfish were selected as suitable additional species for this investigation because these species are of general interest as zebrafish are model species for investigation into vascular development, and pufferfish are studied due to interest in its exceptionally short genome, thus having low occurrence of non-coding sequences.

6.2 Methods

6.2.1 Search for an eNOS gene in fish species

To investigate eNOS transcription in fishes, amplification of mRNA by polymerase chain reaction (PCR) was carried out using custom-made primers (Invitrogen custom primers). In order to design the primers, eNOS nucleotide and amino acid sequences were sought.

6.2.1.1 Bioinformatics

To identify an eNOS homologue and the appropriate method for designing primers for PCR, the following steps were taken:

A. A literature search was carried out for submitted nucleotide and amino acid sequences in public databases

B. Sequences were aligned to determine homology and sequences from non-mammalian vertebrates were closely examined to determine orthology

C. Degenerate primers were designed from amino acid sequences.

D. PCR was performed using degenerate and non-degenerate primers.

E. Microsynteny (genes situated on the same chromosome) search was performed.

A)

All searches for sequences were carried out using an iterative search on the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/) within the 'nucleotide' databases for nucleotide sequences and 'protein' database for amino acid sequences, and within the Ensembl genome browser (http://www.ensembl.org). For eNOS, search terms used were "eNOS", "endothelial NOS", endothelial nitric oxide synthase", and "NOS3", with the species of interest, where appropriate.

B)

A few species randomly selected from each mammalian subclass (human, rhesus monkey, chimpanzee, mouse, Norway rat, cow, dog, horse, sheep, boar), and the *Xenopus. tropicalis* sequences were aligned. Amino acid alignments were carried out using *H. sapiens* [accession number P29474], *M. mulatta* [*XP_002803570*], *P.troglodytes* [XR_02287], *M. musculus* [NP_032739], *R. norvegicus* [AAT99567], *B. taurus* [NP_8513380], *C. lupus familiaris* [NP_001003158], *E.caballus* [XP_001504700], *O. aries* [NP_001123373], and *S. scrofa* [NP_999460]. Nucleotide sequences alignments were carried out using *H.sapiens* [accession number NM_00603.4], *M. mulatta* [XR_012468.1], *P. troglodytes* [XR_02287], *M. musculus* [NM_008713], *R. norvegicus* [NM_021838.2], *B.taurus* [NM_181037.2], *C. lupus familiaris* [NM_001003158.1], *E.caballus* [XM_001504649], *O. aries* [NM_001129901], and *S. scrofa* [NM_2142951. The *X. tropicalis* accession numbers for protein and nucleotide were XP_002943058 and NW_003164518, respectively. Neighbour joining distance trees, bootstrapped with 1000 resamples were created in ClustalW (Fig. 6.4); the distance representing the fraction of mismatches in aligned sequences. The possibility that the xenopus sequence was annotated incorrectly was investigated by aligning with mammalian iNOS and nNOS and xenopus nNOS (xenopus iNOS has not been sequenced) to identify whether the submitted sequence was in fact an nNOS or iNOS orthologue or another previously undescribed

NOS isoform (Fig. 6.5). The nucleotide sequence was also compared with all other species using the BLAST (Basic Local Alignment Search Tool) program.

C) Primers were designed using the CODEHOP (Consensus Degenerate Hybrid Oligonucleotide Primers) programs on http://bioinfo.weizmann.ac.il/blocks/codehop.html, which makes use of multiple aligned amino acid sequences. The output files (.aln) were used to create blocks using the Multiple Alignment Processor and the alignment was submitted for production of forward and reverse primers. Primers with melting temperatures of 60 ± 2°C, and sizes 18-24bp, were selected as this has been shown to be the optimal size for primers for ensuring stringency without having large primers (Lodish, 2003). A selection of primer pairs with estimated product sizes ranging from 100bp- 1000bp, were tested (Table 6.1) to optimise the product size for PCR. Primers were selected so they did not overlap regions that were conserved between the three NOS isoforms (Fig. 6.7). This reduced the likelihood of amplifying nNOS or iNOS.

D)
PCR was carried out on RNA from pufferfish liver, zebrafish liver and spleen, and carp and trout red muscle. RNA from human umbilical vein endothelial cells (HUVECs) was used as a positive control where possible. RNase-free water was used as a negative control. Liver samples were used because of their high vascularity.

E)

Microsynteny refers to the presence of two or more genes on the same chromosome, irrespective of whether they are related with one another. This allows the comparison of genes in different species to identify whether there has been some gene deletion or insertion. In this study, the eNOS gene in mouse, human and zebrafish was investigated.

6.2.1.2 RNA extractions

RNA extractions from all tissues were carried out using the RNeasy Mini Kit from Qiagen with some modifications depending on the homogenisation requirements of the tissue. Samples were homogenised in RLT buffer (600µl) with β-mercaptoethanol (10µl; Sigma) and stored on ice for 10mins to allow full lysis and dissociation. Disruption and thorough homogenisation of skeletal muscle and liver samples was achieved by high-speed shaking, by a beadbeater, of the tissue enclosed in a 2ml BioPur Eppendorf safe-lock microtube with a single, sterile, 5mm stainless steel bead (Qiagen) that had been pre-cooled in liquid nitrogen. After 10mins on ice, samples were diluted (1:1) with double distilled water and collagen was digested using 10µl Proteinase K (Qiagen) per 1200µl diluted sample, at 55°C for 10mins. This homogenisation method produced higher RNA yields than when carried out with a homogenising blade or mortar and pestle. HUVECs were homogenised by aspirating the cells thoroughly in RLT buffer only. 70% (v/v) ethanol equal to 50% of the sample volume was added and the sample was transferred to spin columns, and RNA was extracted according to the manufacturer's

instructions in 30-40µl suspensions. Samples were exposed to DNase for 15mins at room temperature, whilst on the column membrane, to remove genomic DNA contamination.

6.2.1.3 PCR

PCR was carried out on the ThermoHybaid PCR Express Thermal Cycler. Each PCR amplification was carried out in a 25µl reaction with 50ng RNA and 1ug primers per reaction, which was found to be optimal using Qiagen's OneStep RT-PCR kit. Using this kit, cDNA synthesis from the template mRNA took place in the same reaction. The cycling conditions were 30mins reverse transcription (50°C) with Sensiscript and Omniscript reverse transcriptases, 15mins Hotstart PCR activation (95°C) with HotStarTaq®DNA polymerase, 35 cycles of 1 min denaturation (94°C) followed by 30secs annealing (60°C) and 1 min extension (72°C), with a final extension at 72°C for 10mins. Agarose gel electrophoresis (2% m/v) at 100V constant voltage was carried out and gel images were taken using SynGene GeneSnap software, to visualise PCR products.

6.2.2 Western blots

6.2.2.1 Tissue homogenisation and protein quantitation

100-200mg frozen samples were ground to a fine powder using a mortar and pestle pre-cooled with liquid nitrogen. The tissue was transferred to a Potter-Elvejem homogeniser containing 2ml

ice-cold RIPA buffer (150mM sodium chloride, 1% NP-40, 1% (m/v) sodium deoxycholate, (0.1% m/v) SDS, 50mM Tris-HCl, 1mM EDTA, 1mM PMSF, 1mM general protease inhibitor cocktail (Sigma), pH 8.0). Samples were incubated on ice for 30mins with occasional stirring to improve protein solubilisation and extraction. Samples were then centrifuged at 10,000rpm at 4°C (EBA12R, Global Medical Instrument, USA), the supernatant was collected and the protein concentration in sample was measured.

Protein concentrations were measured using the Bradford assay (Bradford, 1976) using BSA (Sigma) as a standard. Briefly, BSA at a range of concentrations (0-10mg/ml) were measured on a 96-well plate reader in a plate reader set to 595nm, to produce a standard curve of absorption vs. protein concentration. This allowed estimation of the protein concentration of the diluted samples based on the equation of the standard curve. Samples were diluted with sample buffer (5% (v/v) glycerol, 0.5% (m/v) SDS, 3mM β-mercaptoethanol, 0.02% (m/v) bromophenol blue and Tris-HCl, pH6.8) so that all samples had equal protein content.

6.2.2.2 Gel preparation

eNOS size is 140kDa and nNOS is 160kDa, therefore a resolving gel (8% (m/v) acrylamide, 0.1% SDS, 8% sucrose, 0.25% APS, 0.25% TEMED, 0.4M Tris-HCl, pH 8.8) with a stacking gel (4% acrylamide, 0.05% SDS, 0.8% APS, 0.04% TEMED 0.13M Tris-HCl, 6.8) was prepared. Briefly, the resolving gel was poured into the cassette and allowed to polymerise for 50 mins and 0.1% SDS

was overlayed to prevent exposure to oxygen inhibiting polymerisation. When the gel had polymerised, the overlay was removed and the top of the gel was rinsed once with deionised water to remove any unpolymerised acrylamide- this was followed by washing with 4% stacking gel solution without APS and TEMED. The stacking gel solution (with APS and TEMED) was poured into the cassette with an appropriate sized comb, and allowed to polymerise.

6.2.2.3 Loading and electrophoresis of protein samples

The gel cassette was assembled in the electrophoresis tank, the comb removed, and the tank filled with running buffer (0.01% (m/v) SDS, 2M glycine, 250mM Tris base). Samples were heated at 90°C for 3 mins, to denature the secondary structure. Samples were then transferred to ice and loaded into wells (60-100µg). The gel was run at 100V until the bromophenol dye front reached the bottom of the gel. The gel was removed from the tank and the stacking gel was cut away and discarded. The resolving gel was then placed in transfer buffer (25mM Tris base, 192mM glycine, 20% (v/v) methanol) for 5 min.

6.2.2.4 Protein transfer

A polyvinylidene fluoride (PVDF) membrane, pore size 0.4µm (Millipore, Sigma) pre-soaked in methanol for 15 sec, followed by deionised water for 2 min, along with filter paper and fibre pads were soaked in transfer buffer. A gel 'sandwich' was prepared in which the fibre,

membrane, filter paper and gel were placed in the following order: fibre pad, filter paper, gel, PVDF membrane, filter paper, fibre pad. Air bubbles were removed and the gel sandwich was placed in a transfer module with transfer buffer, and the proteins were transferred from the gel to the adjacent membrane for 12 hours at constant amplitude (100mA). Successful transfer was determined using a Coomassie stain (0.2% (m/v) Coomassie in 11% (v/v) methanol, acetic acid and H_2O in equal proportions) to highlight the transfer of proteins out of the gel.

6.2.2.5 Antibody binding and developing

Once the transfer was complete, the membrane was blocked with 5% (m/v) milk in Tris buffered salt solution + Tween-20 for 4 hours at room temperature followed by incubating overnight in primary antibody in PBS (anti-eNOS AB16301, 1:2000, Chemicon, Temecula, CA, USA; anti-eNOS, Becton and Dickinson; or anti-nNOS (SC-648; 1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at various dilutions. After primary antibody incubation, the membrane was washed 3 x 10mins in TBS followed by 3 x 10 mins in PBS + 0.1% (v/v) Tween-20). The membrane was then incubated with secondary antibody (horseradish peroxidise-conjugated antibody, 1:2000) and washed 3X in TBS + 0.1% Tween. Membranes were developed using enhanced chemiluminescence destection. Briefly, membranes were drained of excess wash buffer and gel reagent (Roche Diagnostics) was layered onto the surface held on a flat sheet of glass, to ensure even coating. Excess gel reagent was drained and the membrane transferred to x-ray cassette.

In a darkroom, X-ray film was overlaid to expose the film to chemiluninescence. Exposure times were increased to give optimum contrast between developed bands and background.

6.2.3 Immunohistochemistry

To detect eNOS by immunohistochemistry and to compare its localisation to nNOS, white muscle sections (for preparation of sections, see chapter 2), were prepared by creating a hydrophobic barrier around each section with a wax pen (Vector laboratories), fixed with acetone (5 min), washed 3X5 mins in PBS and blocked for 30mins with bovine serum albumin (BSA) and normal goat serum (NGS) in PBS. Sections were stained with either rabbit polyclonal anti-eNOS (AB16301; 1:200; Chemicon, Temecula, CA, USA) or anti-nNOS (SC-648; 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies overnight at 4°C. Sections were washed (3 x5 min) with PBS before incubating for 1 hour at room temperature, with Alexa-594 goat anti-rabbit (Invitrogen). The slides were washed again (3 x5 min) in PBS and mounted with a mounting medium (Vector Laboratories) containing 4', 6 '-diamidino-2-phenylindole (DAPI) for identifying nuclei. The sections were visualised using a fluorescence microscope (Zeiss Axioscop) and CCD camera (Zeiss, MRc). Images were captured using Axiovision imaging software.

6.3 Results

6.3.1 Microsynteny

It was found that the eNOS gene in mice and human was flanked by KCNH2 (gene for voltage-operated K^+ channel, subfamily H) and ATG9B (anti-autophagy-related protein 9B), see Fig 6.1. These genes were sought in the zebrafish genome. In zebrafish, there was no eNOS gene near ATG9Band the KCNH2 gene was positioned next to SIC12A7A, which in humans and mouse is not located on the same chromosome as eNOS.

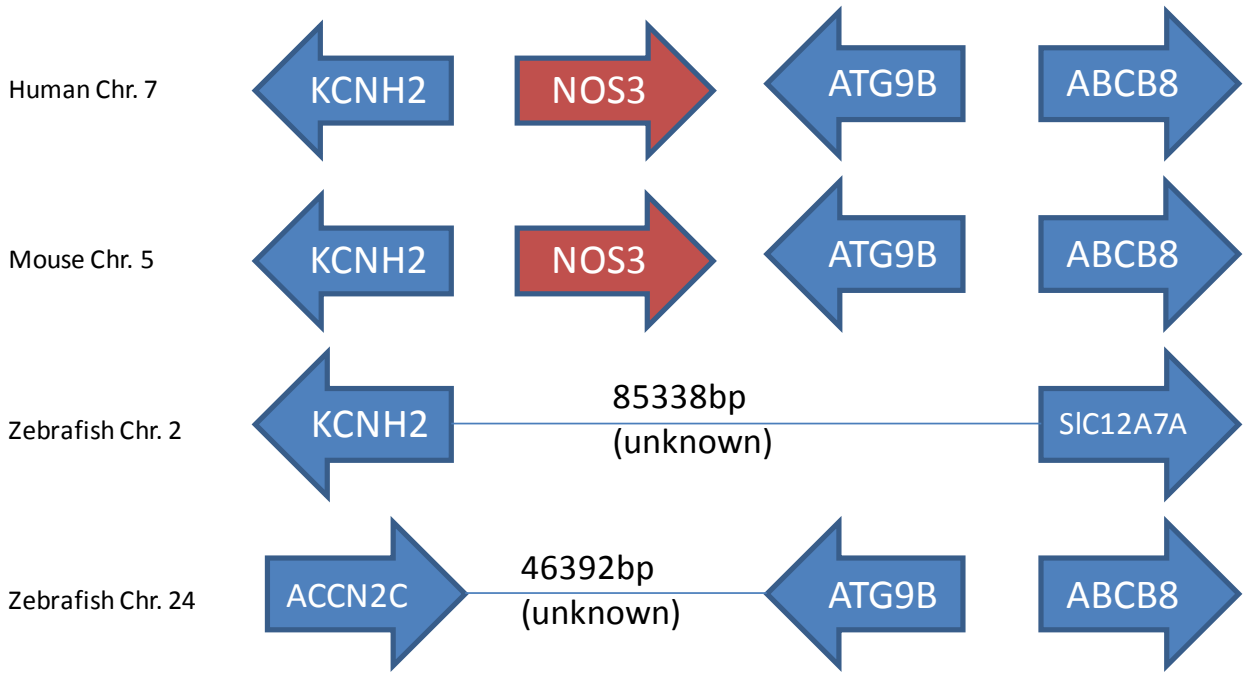


Fig 6.1 Comparison of eNOS genes and neighbouring gene loci in human, mouse and zebrafish genomes. Arrows indicate the direction of transcription. Information taken from Ensembl genome browser.

6.3.2 eNOS transcription

The search for submitted sequences for fish species on the NCBI pages (protein and nucleotide databases) and general primary literature search produced no data, so the gene was sought across all species in the Ensembl genome browser. This revealed there were 47 deposited sequences in 34 species. With the exceptions of the frog, *Xenopus tropicalis* and the lizard, *Anolis carolinensis*, all closely related sequences were from mammals. A maximum likelihood gene tree of eNOS orthologues was created (Fig. 6.2), which demonstrated that the eNOS nucleotide sequences from mammals shared greater identity than those from the frog and lizard. This apparently low identity with mammalian eNOS was investigated further as non-mammalian vertebrate sequences are likely to be of particular use in generating PCR products from fishes. The sequence from the turkey (*Meleagris gallopavo*) was not considered because it shared even lower identity with mammalian eNOS than the frog and lizard.

In other species, including the zebrafish, the most similar genes were those for NOS2a and NOS2b (not shown). The supposed orthologues in lizards, *Aronis Carolinensis*, and frogs, *Xenopus tropicalis* appeared relatively distant so the putative amino acid and nucleotide sequences for xenopus were examined more closely. Neighbour-joining distance trees showed that although the putative xenopus eNOS shared greatest identity with mammalian eNOS, and greater identity with human enOS than nNOS or iNOS (Fig. 6.4), it was not as similar to eNOS as perhaps expected for an orthologue. The maximum identity of xenopus eNOS with any

mammalian eNOS was 71% (as discovered by carrying out a BLAST search on the xenopus sequence). Mammalian nNOS shares 68% identity with eNOS, whereas eNOS sequences from other species share between 87-100% identity, so it was not possible to ascertain whether this sequence was an orthologue or just similar to eNOS. Despite its low identity compared to the mammalian sequences, the xenopus sequence were more closely related to eNOS than nNOS or iNOS, so the likelihood of inaccurate automated annotation by Ensembl genebuild was dismissed. However, owing to the low identity, the xenopus nucleotide sequence was not used directly to design primers. It was concluded that the most suitable primers were degenerate primers, based on the highly conserved amino acid sequences from mammals. Degenerate primers were used alongside commercially available primers for mouse and human tissues. PCR analysis showed eNOS was absent in all fish species studied, using degenerate and non-degenerate primers (Fig. 6.8). Degenerate primers are designed based on the alignment of known amino acid sequences from several species. Where there is high identity, it is assumed that that region is well conserved in that protein. As several codon triplets can code for one amino acid (degeneracy), the primers are designed such that those points where any or several of the bases could be present, the nucleotide at that point is substituted for a nucleotide that can pair with any of the bases expected. PCR carried out for nNOS (using degenerate primers) demonstrated that nNOS is present in all fishes (Fig. 6.8).

Gene	Forward primer	Backward primer	Product size (base pairs)
eNOS	5’-CAGGAGCCCGGCCCNCCNTGYGG-3'	5’-ACTCCCGCAGGTGGTANGTNCCN-3’	465
	5’-GAGCAGCGGCTGCARGARGTNG-3'	5’-CGAACCACTCCAGGGTNGGTTGY-3’	500
	5’-TGGCCGTGTGCATGGAYYTNGA-3'	5’-CGGTGTTGGAGGACTCYTTNCK-3’	753
	5’-TGGAGCGGGGVVAYATGTTYG-3’	5’-TACCTYRANCTGCTCCGGCCGCT-3’	165
	5’-CGTAGGTGATGGAGCCCAVYTCCA-3’	5’-CCGTGGGCCAGGARCCNGGNC-3’	280*
nNOS	5’-GCCAAGCACGCCTGGMGNAAY-3’	5’-GTACTTGATGTGGTTGCADATR-3’	210*
	5’-GCCAAGCACGCCTGGMGNAAY-3’	5’AGGAGGTGAACAARGARATHGA-3’	235

Table 6.1 List of degenerate primers used in order to optimise PCR and primer conditions *Primer pairs used in final PCR experiment, Fig. 6.7

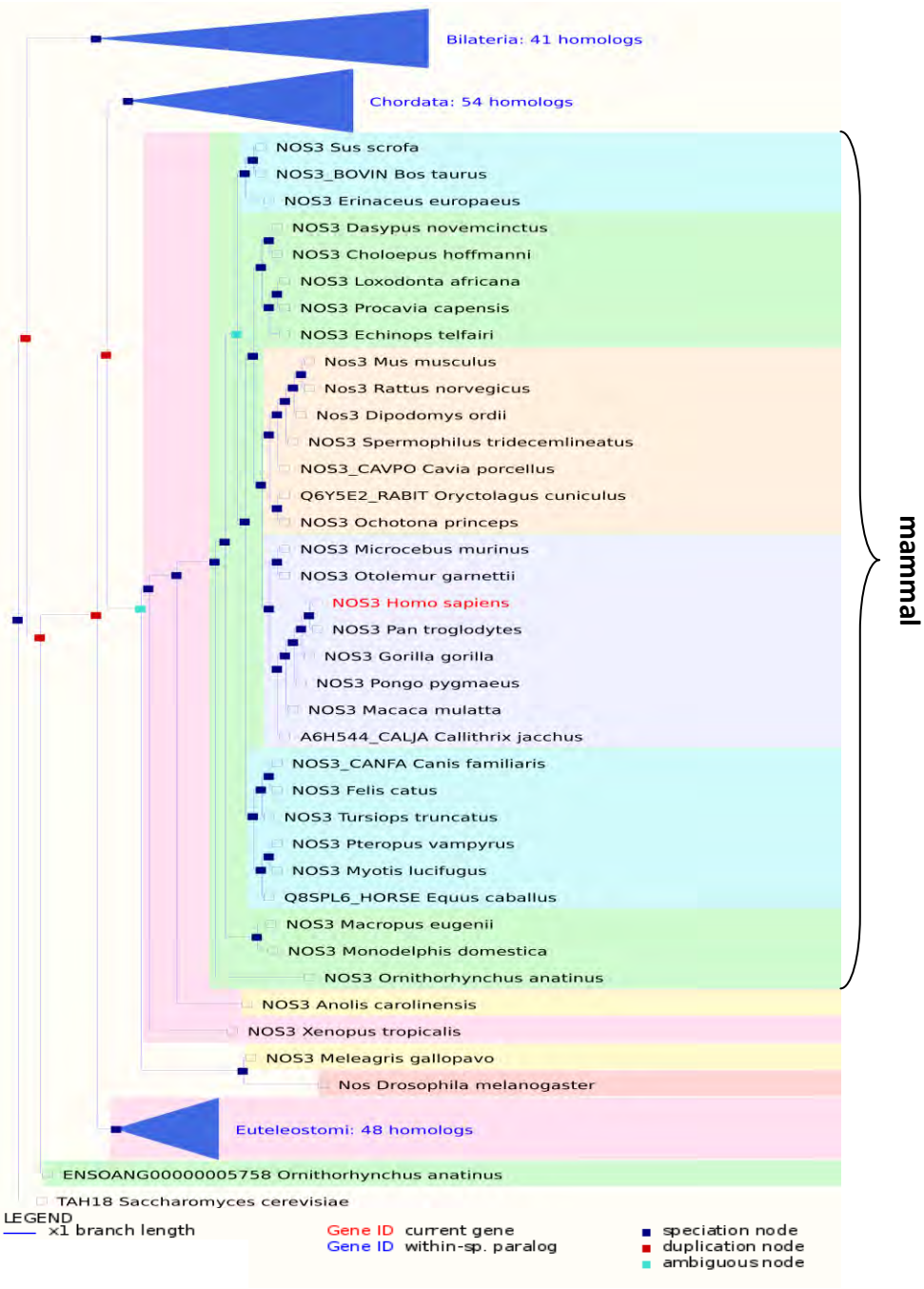


Figure 6.2 A maximum likelihood gene tree displaying distance between annotated eNOS (NOS3) gene orthologs in Ensembl. Note the lack of an eNOS orthologue in any fish species. There are NOS3 orthologs in the African clawed frog (*X.tropicalis*) and a lizard, the Carolina Anole (*A. carolinensis*) but the distance seen here suggests that these may have low identity to mammalian eNOS, therefore may be similar but not homologous to eNOS.

A

```
m.musculus              IVDHHAATASFMKHLENEQKARGGCPADWAWIVPPISGSLTPVFHQEMVNYFLSPAFRYQ 475
r.norvegicus            IVDHHAATASFMKHLENEQKARGGCPADWAWIVPPISGSLTPVFHQEMVNYFLSPAFRYQ 475
h.sapiens               IVDHHAATASFMKHLENEQKARGGCPADWAWIVPPISGSLTPVFHQEMVNYFLSPAFRYQ 476
p.troglodytes           IVDHHAATASFMKHLENEQKARGGCPADWAWIVPPISGSLTPVFHQEMVNYFLSPAFRYQ 379
m.mulatta               IVDHHAATASFMKHLENEQKARGGCPADWAWIVPPISGSLTPVFHQEMVNYFLSPAFRYQ 471
b.taurus                IVDHHAATVSFMKHLDNEQKARGGCPADWAWIVPPIYGSLPPVFHQEMVNYILSPAFRYQ 478
o.aries                 IVDHHAATVSFMKHLDNEQKARGGCPADWAWIVPPISGSLTPVFHQEMVNYILSPAFRYQ 478
s.scrofa                IVDHHAATASFMKHLENEQKARGGCPADWAWIVPPISGSLTPVFHQEMVNYVLSPAFRYQ 478
c.lupus_familiaris      IVDHHAATASFMKHLENEQKARGGCPADWAWIVPPISGSLTPVFHQEMVNYVLSPAFRYQ 478
x.tropicalis            IVDHHAATESFMKHMEHEIRQRGGCPADWVWIVPPTSASLTPVFHQEMANYYLSPSFLYQ 446
                        ******** *****:::* : ********.*****  .**.*******.** ***:* **

m.musculus              PDPWKGSAAKGAG--ITRKKTFKEVANAVKISASLMGTVMAKRVKATILYGSETGRAQSY 533
r.norvegicus            PDPWKGSAAKGTG--ITRKKTFKEVANAVKISASLMGTVMAKRVKATILYGSETGRAQSY 533
h.sapiens               PDPWKGSAAKGTG--ITRKKTFKEVANAVKISASLMGTVMAKRVKATILYGSETGRAQSY 534
p.troglodytes           PDPWKGSAAKGTG--ITRKKTFKEVANAVKISASLMGTVMAKRVKATILYGSETGRAQSY 437
m.mulatta               PDPWKGSAAKGTG--ITRKKTFKEVANAVKISASLMGTVMAKRVKATILYGSETGRAQSY 529
b.taurus                PDPWKGSATKGAG--ITRKKTFKEVANAVKISASLMGTLMAKRVKATILYASETGRAQSY 536
o.aries                 PDPWKGSATKGAG--ITRKKTFKEVANAVKISASLMGTLMAKRVKATILYASETGRAQSY 536
s.scrofa                PDPWKGSAAKGTG--IARKKTFKEVANAVKISASLMATVMPKRVKASILYASETVRAQSY 536
c.lupus_familiaris      TDPWKGSASKGAG--VTRKKTFKEVANAVKISASLMGTVMAKRVKATILYGSETGRAQSY 536
x.tropicalis            PDPWKHHVWKGSQSTITRRRTFKEVANAVKFSAKLMGKAMARRVKATILYATETGRSETY 506
                        .****  . **:   ::*::**********:**.**.. *.:****:***.:** *:::*

m.musculus              AQQLGRLFRKAFDPRVLCMDEYDVVSLEHEALVLVVTSTFGNGDPPENGESFAAALMEMS 593
r.norvegicus            AQQLGRLFRKAFDPRVLCMDEYDVVSLEHEALVLVVTSTFGNGDPPENGESFAAALMEMS 593
h.sapiens               AQQLGRLFRKAFDPRVLCMDEYDVVSLEHETLVLVVTSTFGNGDPPENGESFAAALMEMS 594
p.troglodytes           AQQLGRLFRKAFDPRVLCMDEYDVVSLEHETLVLVVTSTFGNGDPPENGESFAAALMEMS 497
m.mulatta               AQQLGRLFRKAFDPRVLCMDEYDVVSLEHETLVLVVTSTFGNGDPPENGESFAAALMEMS 589
b.taurus                AQQLGRLFRKAFDPRVLCMDEYDVVSLEHEALVLVVTSTFGNGDPPENGESFAAALMEMS 596
o.aries                 AQQLGRLFRKAFDPRVLCMDEYDVVSLEHEALVLVVTSTFGNGDPPENGESFAAALMEMS 596
s.scrofa                AQQLGRLFRKAFDPRVLCMDEYDVVSLEHETLVLVVTSTFGNGDPPENGESFAAALMEMS 596
c.lupus_familiaris      AQQLGRLFRKAFDPRVLCMDEYDVVSLEHETLVLVVTSTFGNGDPPENGESFAAALMEMS 596
x.tropicalis            AQKLCKIFSYAFDPKVICMDQYDIVNLEHETLVLVVTSTFGNGDPPENGEAFARELMEMT 566
                        **:* ::*  ****:*:***:**:*.****:********************:**  ****:

m.musculus              GPYNSSPRPEQHKSYKIRFNSVSCSDPLVSSWRRKRKESSNTDSAGALGTLRFCVFGLGS 653
r.norvegicus            GPYNSSPRPEQHKSYKIRFNSVSCSDPLVSSWRRKRKESSNTDSAGALGTLRFCVFGLGS 653
h.sapiens               GPYNSSPRPEQHKSYKIRFNSISCSDPLVSSWRRKRKESSNTDSAGALGTLRFCVFGLGS 654
p.troglodytes           GPYNSSPRPEQHKSYKIRFNSISCSDPLVSSRRR-GKESSNTDSAGALGTLRFCVFGLGS 556
m.mulatta               GPYNSSPRPEQHKSYKIRFNSISCSDPLVSSWRRKRKESSNTDSAGALGTLRFCVFGLGS 649
b.taurus                GPYNSSPRPEQHKSYKIRFNSVSCSDPLVSSWRRKRKESSNTDSAGALGTLRFCVFGLGS 656
o.aries                 GPYNSSPRPEQHKSYKIRFNSVSCSDPLVSSWRRKRKESSNTDSAGALGTLRFCVFGLGS 656
s.scrofa                GPYNGSPRPEQHRSYKIRFNSVSCSDPLVSSWRRKRKESSNTDSAGALGTLRFCVFGLGS 656
c.lupus_familiaris      GSYNSSPRPEQHKSYKIRFNSVSCSDPLVSSWRRKRKESRNTDSAGALGTLRFCVFGLGS 656
x.tropicalis            SPSTLALAPEQQKSYKIRFNSVSQGDQLVSSWKNKRKESSNTDSAGPLGTLRFSVFGLGS 626
                        .. . :  ***::********:* .* **** :.  *** ******.******.******

m.musculus              RAYPHFCAFARAVDTRLEELGGERLLQLGQGDELCGQEEAFRGWAQAAFQAACETFCVGE 713
r.norvegicus            RAYPHFCAFARAVDTRLEELGGERLLQLGQGDELCGQEEAFRGWAQAAFQAACETFCVGE 713
h.sapiens               RAYPHFCAFARAVDTRLEELGGERLLQLGQGDELCGQEEAFRGWAQAAFQAACETFCVGE 714
p.troglodytes           RAYPHFCAFARAVDTRLEELGGERLLQLGQGDELCGQEEAFRGWAQAAFQAACETFCVGE 616
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B

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h.sapiens               -GCTCCCCGCTAACCCAGCCCCCAGAGGGGCCCA--AGTTCCCTCGTGTGAAGAACTGGG 519
m.mulatta               -GCTCCCCGCTAACCCAGCCCCCGGAGGGGCCCA--AGTTCCCTCGTGTGAAGAACTGGG 532
p.troglodytes           -CCCATCCCTGCACCCCTCCTCCCTCTGGCCCCC--AACTCCCATCCC------------ 211
s.scrofa                AGCCCCACGCTGACCCGGCCCCCGGAGGGGCCCA--AGTTCCCTCGTGTGAAGAACTGGG 235
b.taurus                AGCCCCACGCTGACCCGGCCTCCCGGAGGGGCCCA--AGTTCCCTCGCGTGAAGAACTGGG 286
o.aries                 AGCCCCACGCTGACCCGGCCTCCGGAGGGGCCCA--AGTTCCCTCGCGTGAAGAACTGGG 257
c.lupus_familiaris      AGCCCCCCACTCACCCGGCCTCCAGATGGGCCCA--AGTTCCCTCGTGTGAAGAACTGGG 1496
m.musculus              AGCCCCCCGCTAACCCGGCCCCCAGACGGACCCA--GGTTTCCTCGAGTAAAGAATTGGG 239
r.norvegicus            AGCCCCCCGTTAACCCGGCCCCCAGACGGACCCA--AGTTTCCTCGAGTAAAGAACTGGG 226
x.tropicalis            GGGGCTCCACCAATGGGGGCCAAGCTCAGACCCAGAAATGTATTCGAGTCCGGAACTGGG 133
                                 *    *      *      * ***

h.sapiens               AGGTGGGGAGCATCACCTATGACACCCTCAGCGCCCAGGCGCAGCAGGATGGGCC-CTGC 578
m.mulatta               AGGTGGGGAGCATCGCCTATGACACCCTCAGCGCCCAGGCATCCCAAAGTGGTCCACTCC 592
p.troglodytes           ACCCCTGCACCCTGGCCTGTCCTGACCTTTGCACTCCCTCGACCCAGGATGGGCC-CTGC 270
s.scrofa                AGGTGGGGAGCATCACCTATGACACCCTGTGCGCCCAGTCCCAACAGGATGGGCC-CTGC 294
b.taurus                AGCTGGGGAGCATCACCTACGACACTCTGTGCGCGCAGTCCCAACAGGACGGGCC-CTGC 345
o.aries                 AGCTGGGGAGCATCACCTACGACACTCTGTGCGCGCAGTCCCAACAGGACGGGCC-CTGC 316
c.lupus_familiaris      AGGTGGGGAGCATCACCTATGACACCTTGAGTGCCCAGTCACAGCAGGATGGGCC-CTGC 1555
m.musculus              AAGTGGGCAGCATCACCTACGACACCCTCAGTGCCCAGGCTCAGCAGGATGGGCC-CTGT 298
r.norvegicus            AAGTGGGCAGCATCACCTACGATACCCTCAGTGCACAGGCTCAGCAGGATGGGCC-CTGT 285
x.tropicalis            AAAACAGAACCCTCTCTTACGATACCCTGAATGTGCAGACAGAGAAGGATGTTCC-CTGC 192
                        *     * * * *  * *          *       *   *     *    *  ** **

h.sapiens               ACCCCAAGA------CGCTGCCTGGGCTCCCTGGTATTTCCACGGAAACTACAGGGCCGG 632
m.mulatta               CTTCCCAGATCCCAACACCACATGGGCCCCTCCTGCCCTCCGCCAGTGCT-CACACATAG 651
p.troglodytes           ACCCCAAGA------CGCTGCCTGGGCTCCCTGGTATTTCCACGGAAACTACAGGGCCGG 324
s.scrofa                ACCCCCAGA------CGCTGCCTGGGCTCCCTGGTGTTGCCCCGGAAACTACAGTCCCGG 348
b.taurus                ACTCCCAGG------CGCTGCCTGGGCTCCCTGGTGTTGCCCCGGAAACTGCAGACCCGG 399
o.aries                 ACTCCCAGG------TGCTGCCTGGGCTCCCTGGTGTTGCCCCGGAAACTGCAGACCCGG 370
c.lupus_familiaris      ACCCCAAGA------CGCTGTCTAGGCTCTCTGGTATTTCCACGGAAACTACAGAGCCGG 1609
m.musculus              ACCTCAAGA------CGCTGCTTGGGATCCCTGGTGTTTCCAAGGAAGTTACAGAGCCGG 352
r.norvegicus            ACCCCAAGA------CGCTGCTTGGGATCCCTGGTATTTCCAAGGAAGTTACAGAGCCGG 339
x.tropicalis            A--CCAAGTCC----CGGTGCCTGGGTTCCGTTGTTTACCCCTCCCAGCTGACCTGCCCA 246
                            * **              * **  *          **        *

h.sapiens               CCCTCCCCCGGCCCCCCGGCCCCTGAGCAGCTGCTGAGTCAGGCCCGGGACTTCATCAAC 692
m.mulatta               CCTGGAGGAGGGCCTCCCTGTCCCACACAACT------TCCTGCTTGTCCCTTTGCCACC 705
p.troglodytes           CCCTCCCCCGGCCCCCCGGCCCCTGAGCAGCTGCTGAGTCAGGCCCGGGACTTCATCAAC 384
s.scrofa                CCCTCCCCCGGACCCCCACCCGCTGAGCAGCTGCTGAGCCAGGCCAGGGACTTCATCAAC 408
b.taurus                CCCTCCCCCGGGACCTCCACCCGCTGAGCAGCTGCTGAGCCAGGCCAGGGACTTCATCAAC 459
o.aries                 CCCTCCCCCGGGACCTCCACCCGCTGAGCAGCTGCTGAGCCAGGCCAGGGACTTCATCAAC 430
c.lupus_familiaris      CCCTCCCAGAACCCTGCACCCCCTGAGCAGCTGCTGAGCCAGGCCAGGGACTTCATCAGC 1669
m.musculus              CCCACCCAGGGCCCTTCACCCACTGAGCAGCTATTGGGTCAAGCCCGGGACTTCATCAAT 412
r.norvegicus            CCCACCCAGGGCCCTTCACCCACTGAGCAGCTATTGGGTCAAGCCCGGGACTTCATCAAT 399
x.tropicalis            TTACCAGAAGGACCAAAGAGCCCGGAGGAGCTGCTACCCAAAGCCAAGGATTTTATCAAC 306
                                     **        *     * **          **        **   **

h.sapiens               CAGTACTACAGCTCCATTAAGAGGAGCGGCTCCCAGGCCCACGAACAGCGGCTTCAAGAG 752
m.mulatta               CCTCTC-----CTCCCC-----AGGAGCGGCTCCCAGGCCCACGAACAGCGGCTTCAAGAG 756
p.troglodytes           CAGTACTACAGCTCCATTAAGAGGAGCGGCTCCCAGGCCCACGAACAGCGGCTTCAAGAG 444
```

Figure 6.3 Representative snapshots of eNOS **A** amino acid and **B** nucleotide sequence alignment carried out in ClustalW. Positions that are identical in all five sequences are indicated with an asterisk (*), Strongly conserved positions are indicated with double dots (:) and weakly conserved are indicated by a single dot (.). Alignment of the xenopus eNOS amino acid sequences reveals that the submitted sequences have high identity with mammalian eNOS but less than the mammalian sequences have with each other. Nucleotide alignment shows that the sequence is not very well conserved.

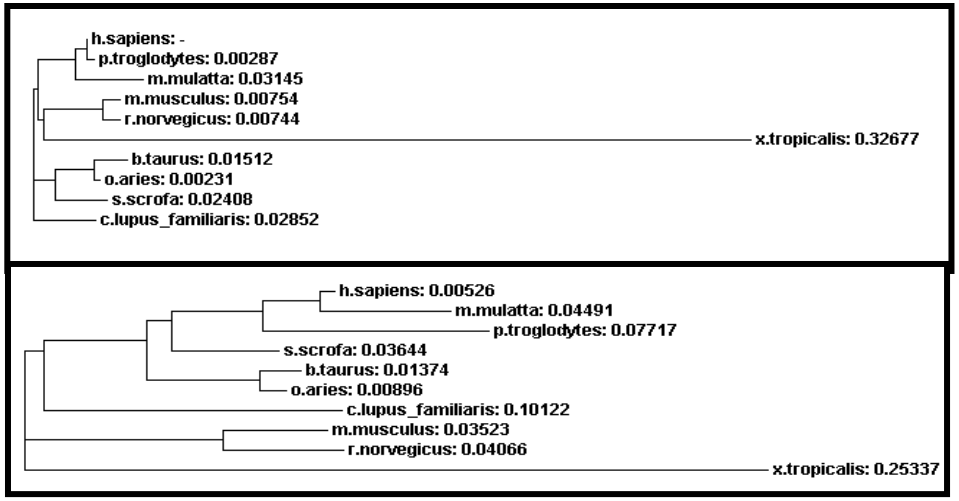

Figure 6.4 Phylogram showing the distance of the xenopus amino acid (top) and nucleotide (bottom) sequences, from sequences from mammals.The phylogram was created using a neighbour-joining method in ClustalW. The number on each branch is the fraction of mismatches between sequences, and the branch lengths demonstrate inferred evolutionary distance. Note that the trees do not overlap because of the degeneracy of the amino acid sequences. Note that the trees do not overlap because of the degeneracy of the amino acid sequences.

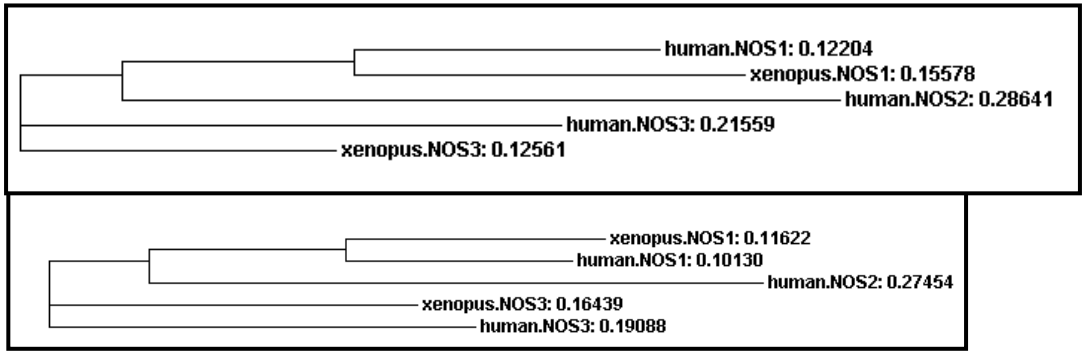

Figure 6.5 Phylogram showing that the putative xenopus amino acid (top) and nucleotide (bottom) sequence shares greater identity with mammalian eNOS (NOS3) than nNOS (NOS1) or iNOS (NOS2). The phylogram was created using a neighbour-joining method in ClustalW. The number on each branch show the fraction of mismatches in aligned sequences, and the branch lengths demonstrate inferred evolutionary distance based on the difference between sequences. Note that the trees do not overlap because of the degeneracy of the amino acid sequences.

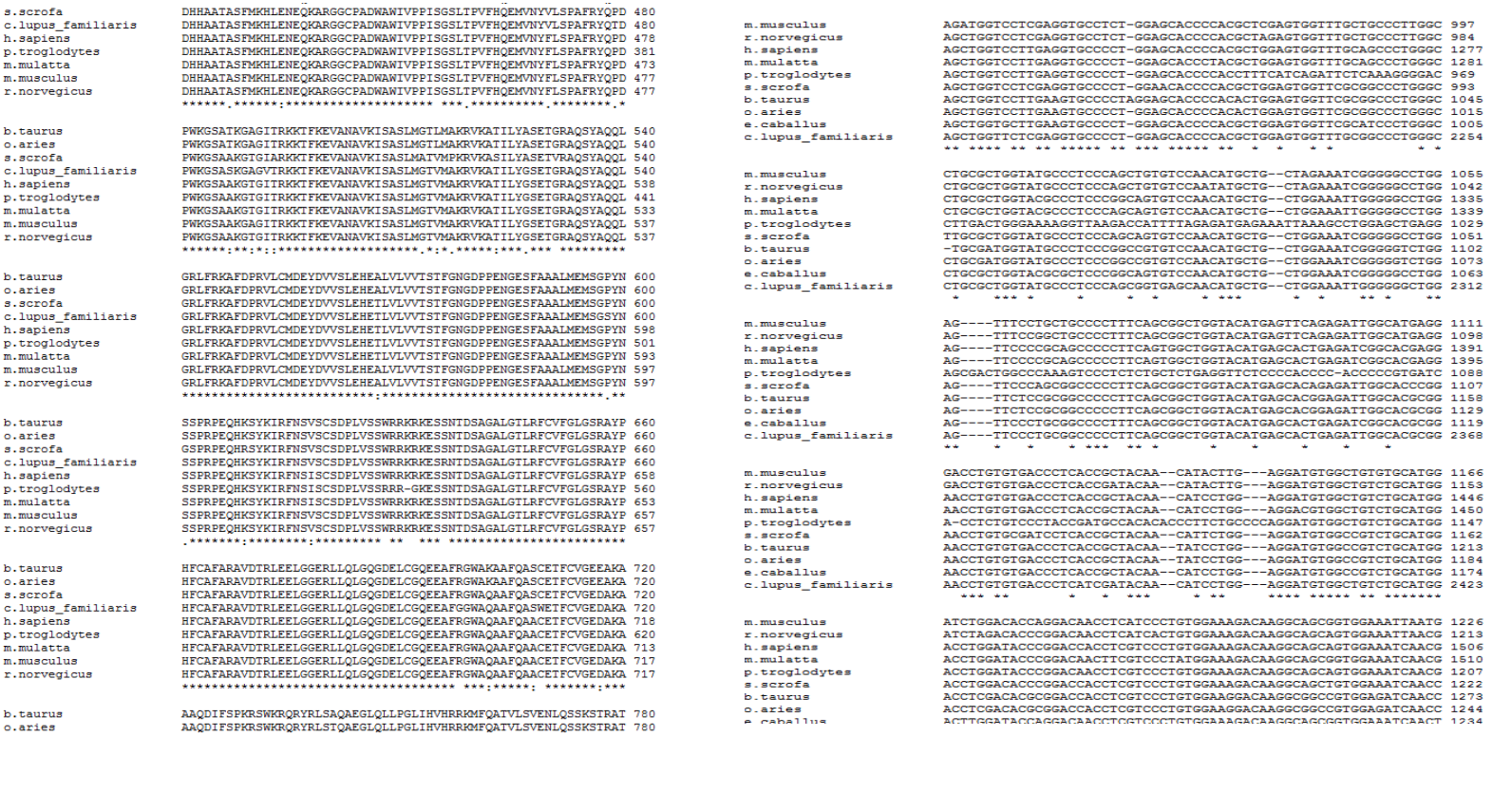

Figure 6.6 Representative snapshots of eNOS amino acid (left) and nucleotide sequences (right) alignments generated in ClustalW. Positions that are identical in all aligned sequences are indicated with an asterisk (*), Strongly conserved positions are indicated with double dots (:) and weakly conserved are indicated by a single dot (.). As expected, aminoacid sequences are well conserved, but nucleotide sequences are not so degenerate primers are required for PCR in species where the eNOS nucleotide sequence is unavailable.

Figure 6.7 Selection of primers so they did not overlap with regions conserved in all three NOS isoforms. Positions that are identical in all aligned sequences (generated in ClustalW) are indicated with an asterisk (*), Strongly conserved positions are indicated with double dots (:) and weakly conserved are indicated by a single dot (.).The black boxes demonstrate examples of regions that were excluded from primers.

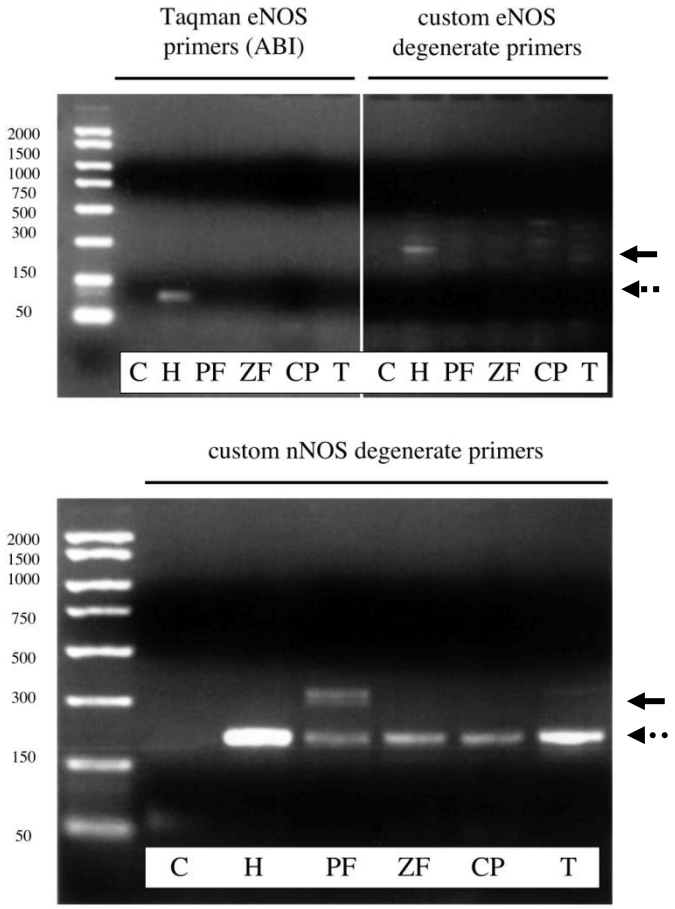

Figure 6.8 PCR amplicons from 35 cycles using Taqman eNOS primers, custom-made eNOS degenerate primers, and custom-made nNOS degenerate primers. C= control (water), H= human umbilical vein endothelial cells, PF= pufferfish liver, ZF= zebrafish liver, CP= carp red muscle, T= trout red muscle. Top panel: Dotted arrow points to the expected size of the PCR amplicon using the Taqman eNOS primer. Solid arrow points to the expected size of the amplicons using the degenerate eNOS primer. Bottom panel: Dotted arrow represents the expected size for the amplicon using the nNOS degenerate primer. Solid arrow points to an unidentified amplicon.

6.3.1 Western blots

Western blotting using the polyclonal eNOS antibody previously used in the literature (McNeill & Perry, 2005; Mustafa & Agnisola, 1998) demonstrated that in rat liver-where eNOS protein (Shah *et al.*, 1997) and mRNA (Zhang *et al.*, 1999) has previously been detected - and in the zebrafish, this antibody was unable to detect eNOS protein. In rat liver, this antibody detects a protein at a low molecular weight (MW; ~ 60kDa), which is clearly not eNOS. In the trout, it appears to detect a protein that is at the same molecular weight as eNOS, as previously demonstrated in trout by McNeill *et al* (2006). However, it also detects another protein at a lower MW (~ 100 kDa), with the lower MW band intensity being far greater. In carp tissue, the eNOS antibody binds to many proteins, some of which are of similar size to eNOS (~ 150 kDa), but most of which are in the range of (~52kDa – 100kDa). This suggests that the antibody that has been used to identify eNOS in the fish in the current literature binds non-specifically to many proteins, and in carp muscle in particular there are several proteins that contain the epitope to which the antibody binds. The fact that it does not bind to eNOS in rat liver further demonstrates its lack of specificity to eNOS protein. A monoclonal eNOS antibody that binds to eNOS in rat liver, with no evidence of binding non-specifically to other proteins, does not bind to any of the fish tissues (Fig. 6.8).

The nNOS polyclonal antibody that detected a protein in rat brain tissue, which was a lower molecular weight than expected (expected size 160 kDa, product size: ~140kDa) were unable to

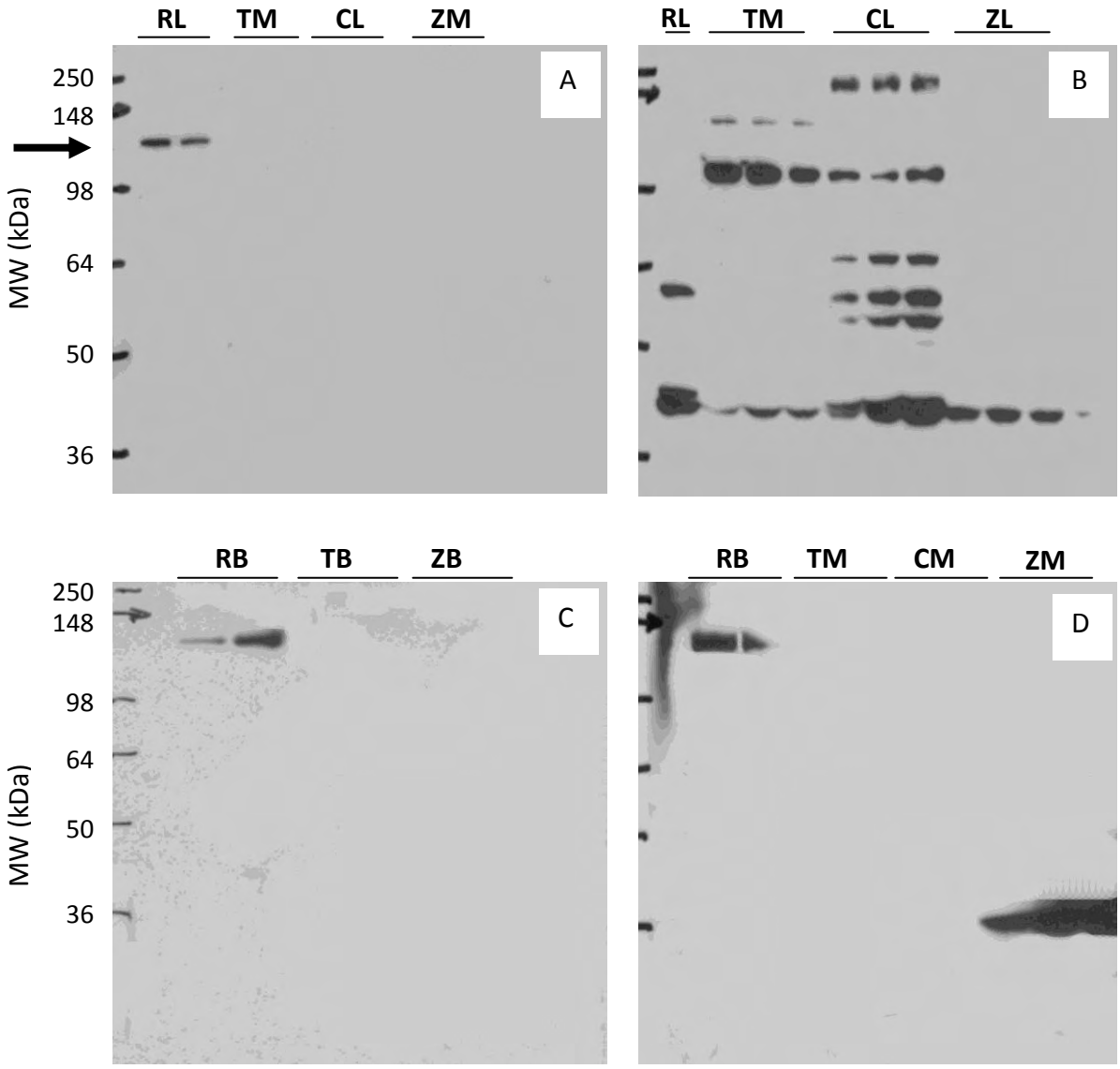

Fig 6.9 Western blots demonstrating monoclonal (A) and polyclonal (B) eNOS antibody binding and polyclonal (C and D) in rat liver (RL) and brain (RB), trout red muscle (TM) and brain (TB), carp liver (CL) and red muscle (CM), zebrafish liver (ZL), head (ZB) and muscle (ZM),

detect any protein in the muscle or brain of any of the fishes. Binding to a lower molecular weight protein than expected using this antibody has previously been reported in the literature, and it was suggested that this antibody was non-selective because the band was also detected in nNOS$^{-/-}$ mice (Lacza *et al.*, 2003). This may explain the lack of binding seen in fish, where the nNOS gene has been sequenced, and evidence for its existence is convincing.

6.3.1 Immunohistochemistry

Immunohistochemistry using the polyclonal eNOS and nNOS antibodies, using identical protocols and secondary antibodies, so that the only variable was the primary antibody, demonstrated that the eNOS antibodies produced high background staining to many proteins whereas the nNOS antibody bound very specifically to regions surrounding the muscle fibres. This correlates with the single band shown in the western blot, although it has not been clarified which protein the antibody is binding to. There was some evidence for nNOS staining within the muscle fibres (Fig 6.9B, top right corner). Parallel staining of eNOS with alkaline phosphatase to demonstrate co-localisation of eNOS with capillaries, revealed that the eNOS antibody did not co-localise with the alkaline phosphatase stain (see Fig 6.9C&D) i.e. IHC demonstrated that the antibody bound to regions that did not have capillaries, and also often did not bind to regions that had capillaries. This supports the findings from the western blots that polyclonal antibodies and it should not be assumed that one of these proteins is eNOS.

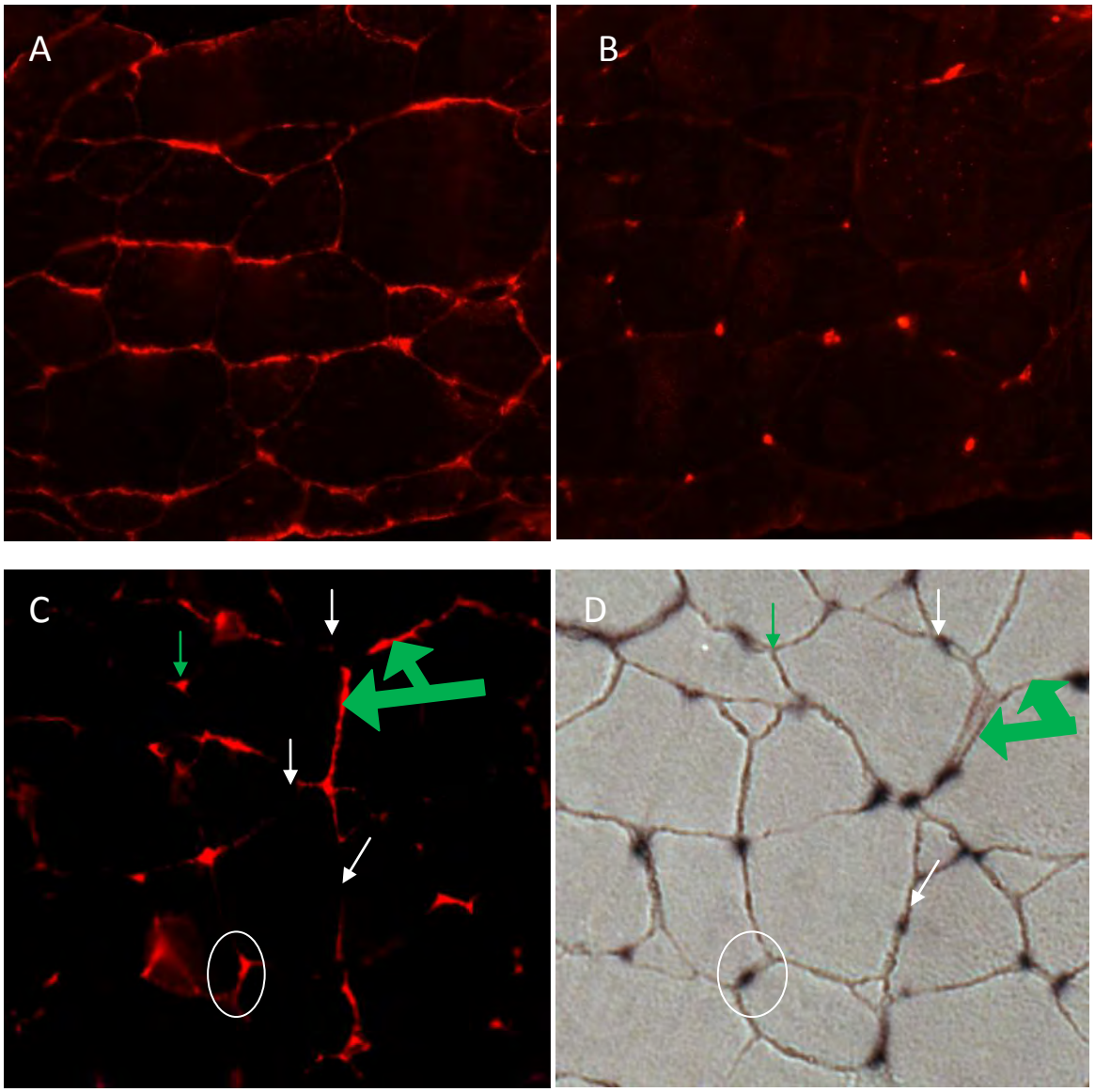

Figure 6.10 Immunohistochemistry in parallel trout glycolytic muscle sections comparing polyclonal eNOS staining (A) with polyclonal nNOS staining (B) and polyclonal eNOS staining (C) with alkaline phosphatase staining for capillaries (D). eNOS IHC produces high background whereas nNOS staining is distinct with little background. Green arrows: eNOS staining where there is no alkaline phosphatase (AP) staining; white arrows: no eNOS staining where there is AP staining; white circle: eNOS and AP stains are adjacent but not overlayed.

previously used to demonstrate eNOS in fish species bind non-selectively to several proteins.

6.4 Discussion

The NOS genes of mammals have been well characterised at the molecular, pharmacological and to some extent, functional levels. Nitric oxide synthase activity in fishes has been characterised primarily using non-specific antagonists that are used for mammalian NOS subtypes. The purpose of this chapter was to identify whether use of mammalian antibodies and reliance on mammalian nucleotide sequences was suitable for use in studies of eNOS activity in fish species. Furthermore, the growing evidence for a lack of eNOS in fishes was investigated.

Comparison of mammalian amino acid sequences demonstrated high homology but some regions of the nucleotide sequences were not as well-conserved as may have been expected, due to amino acid degeneracy. Creation of phylograms demonstrated that mammalian eNOS nucleotide sequences shared greater identity than those from frog and lizards. There were no deposited amino acid or nucleotide eNOS sequences for fishes. The frog and lizard sequences shared greater identity with mammalian eNOS than iNOS or nNOS, but still far less identity with eNOS than mammalian sequences shared with each other. This suggests that there may be

some intermediate NOS subtype that exists in lower vertebrate, and the investigation of this may be useful to understand the evolutionary basis for NOS existence.

At the molecular level, amino acid sequences of eNOS are very well conserved between mammals. However, the middlemost regions of all mammalian NOS subtypes are also very well conserved demonstrating a need for targeting antibodies to either the C or N-termini of these amino acid sequences. Designing of eNOS primers must also be carried out using the N and C-termini.

Microsynteny search performed for the eNOS gene in humans and mouse compared with fishes revealed that the genes KCNH2 and ATG9B that flank are not in the same locations in fish. KCNH2 in fishes lies next to the gene for an astrocyte protein which is not related to eNOS in mammals. Moreover, the AGT9B gene (which is antisense for eNOS and inhibits its overexpression) does not appear to exist in fishes, and it may be the case given that if the eNOS gene does not exist, the existence of its antisense gene would be extraneous.

PCR amplification using mouse primers and degenerate primers showed no evidence for the existence of eNOS in pufferfish, zebrafish, carp and trout, whereas it existed in HUVEC. However, nNOS was abundant in all four species. The lack of eNOS mRNA could have been due to extremely low abundance in these tissue types but as the amplification was carried out over

35 cycles, which is the upper recommended limit for PCR amplification of 50ng RNA, as used in this study (Qiagen, Technical Information, Guidelines for determining the number of PCR cycles, http://www.qiagen.com/resources/info/guidelines_for_pcr.aspx#4), this seems unlikely. It is also possible that the lack of mRNA transcript is low due to the lack of a stimulus for eNOS transcription in some of the fishes (particularly the zebrafish and pufferfish, which were not cold-acclimated therefore did not experience increased blood viscosity), whereas in HUVEC, the physical force of pipetting imposes shear stress on the cells directly, which could cause eNOS transcription (Ziegler *et al.*, 1998). As such, isolation of endothelial cells from each fish species may have been a more reliable method of measurement of eNOS transcription so that all cells were exposed to similar shear stress levels immediately prior to RNA extraction.

In the current study, there was a lack of evidence for eNOS protein in fishes. Western blotting and IHC demonstrated the non-specificity of the eNOS antibodies previously used in the literature, using rat liver as a positive control as eNOS is abundant in liver. The polyclonal eNOS antibody that bound to a protein of approximately the same MW as eNOS in trout liver, did not bind to the rat liver and the binding in trout was of low intensity in an organ which is highly vascular. Although this may suggest that eNOS exists in trout but below the detection limit, the lack of eNOS mRNA and the fact that it does not co-localise with capillaries suggests that this is unlikely. The antibody binds to several other proteins in the carp, so it is unclear what the antibody is binding to. The lack of a smearing effect suggests that binding in several places is not

due to protein degradation and that the polyclonal nature of the antibody is the cause of the excessive binding. The monoclonal eNOS antibody used here is specific to the rat liver. To determine that this is in fact true binding to eNOS protein, the western blots could be repeated to compare binding in wild-type and $eNOS^{-/-}$ mice.

The evidence suggests that in fish species, eNOS may not exist. However, nNOS is abundant and the actions of NO demonstrated in previous studies (Mustafa & Agnisola, 1998; Tota *et al.*, 2005), may be attributed to nNOS-derived NO. Given that prostaglandins are synthesised in the fish endothelium (Evans & Gunderson, 1998) this may play a more major role in the control of vascular tone and may in fact be the EDRF in fishes.

6.4.1 Conclusions

The role of NO in the control of vascular tone has been extensively studied but the alternative mechanisms of control of vascular tone in species which have intrinsically low vascular tone may be exploited to identify alternative ways of reducing high blood pressure, without targeting a ubiquitous molecule like NO. Here, it has been demonstrated that previous studies demonstrating eNOS protein in fishes may have been misleading due to the antibody selected, and that eNOS mRNA is not present in model and non-model fish species.

6.4.2 Future experiments

To validate the studies on eNOS transcription on fish endothelium, extraction of mRNA from isolated fish endothelial cells may produce a higher signal:noise ratio, so whether the cause of the apparent lack of eNOS is due to the relatively low abundance of endothelium in tissue, rather than the absence of eNOS, could be determined. Alternatively, PCR carried out on laser microdissected capillaries, visualised by histochemistry for dissection (Milkiewicz & Haas, 2005), may increase the signal. The possible existence of an eNOS-like protein in fishes (i.e. similar, but not identical in structure to eNOS but having an identical function), for example that which appears to exist in frogs and lizards, could be investigated. The generation of PCR primers based solely on the proposed frog eNOS sequence on the Ensembl database may reveal that it or a similar isoform exists in fish. If such a sequence exists in fishes, it would be possible to generate peptides based on these sequences, in order to generate antibodies against the protein. IHC with these antibodies would then allow localisation of the proposed eNOS protein in fish tissues. In order to validate the IHC experiments in the current study, IHC on mouse tissue with the same eNOS antibodies, with parallel staining with lectin to visualise capillaries, needs to be carried out.

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Chapter 7 General Discussion

7.1 Conclusions

Angiogenesis can broadly be divided into two categories: pathological- that which occurs in disease states such as tumour growth and diabetic retinopathy- or physiological- that which takes place under 'normal' conditions i.e. during exercise, muscle growth and during the menstrual cycle. Both physiological and pathological angiogenesis can be triggered by mechanical and metabolic cues. In the current study, the focus was on physiological angiogenesis, primarily as a result of mechanical stimulation.

It has previously been observed that chronic cold exposure (cold acclimation) can bring about angiogenesis in the skeletal mucle of non-hibernating endotherms such as rats and ectotherms such as fishes. In the case of endotherms, the increased metabolic demand associated with maintenance of core body temperature for example by shivering (increases muscle activity) which causes fibre hypertrophy, increasing mean oxygen diffusion distance, means that an angiogenic response may be expected in order to maintain an adequate oxygen supply to muscle. Increased muscle activity and fibre hypertrophy are also mechanical cues for angiogenesis. However, in ectotherms where body temperature follows that of the environmental temperature and where there is greater plasticity of muscle metabolic enzymes to cold-acclimation (Johnston & Temple, 2002) in addition to the increased oxygen available for

extraction in cooled water, and the onset of torpor in fishes, it seems unlikely that increased metabolic demand is the cause of cold-induced angiogenesis. Therefore, it was hypothesised that there were two mechanical triggers of cold-induced angiogenesis in fishes A) increased shear stress due to increased viscosity of cooled blood B) increased abluminal strain due to muscle fibre hypertrophy.

It was hypothesised that increased blood viscosity would lead to elevated shear stress on the arteriolar and capillary endothelium. In response to elevated shear stress, the arteriolar endothelium releases NO which increases vessel diameter to reduce shear stress. The consequence of this on the capillary endothelium, which does not dilate as it lacks smooth muscle, is increased blood flow in capillaries, further augmenting the shear stress on the endothelium. This maintains the stimulus for NO release in capillaries, in turn providing a prolonged stimulus for VEGF synthesis, which brings about angiogenesis, and promotes endothelial cell proliferation and migration (Ando *et al.*, 1987).

Fibre hypertrophy caused by cold acclimation causes strain on the interstitium and endothelium lining the capillary wall. As a result of the strain, the endothelium loses its contact inhibition and matrix metalloproteinases from the interstitium are activated to cause digestion of the extracellular matrix and basement membrane to permit migration of endothelial cells.

Angiogenesis can be divided into 3 'types'- splitting and intussusception, which occur intraluminally, and sprouting, which takes place abluminally. The source of stimuli for intraluminal splitting and intussusceptions is the capillary lumen and the source for abluminal sprouting is the extracellular matrix and the host tissue. As such, it was hypothesised that elevated shear stress caused by increased blood viscosity would cause splitting angiogenesis and fibre hypertrophy would bring about sprouting angiogenesis and that cold-induced angiogenesis is a product of both mechanisms. It was also hypothesised that if viscous blood caused shear stress-induced angiogenesis, then reduced viscosity in warm blood, would cause capillary rarefaction, as shear stress inhibits endothelial cell apoptotis (Dimmeler *et al.*, 1996).

To test the shear stress hypothesis, the effects of elevated and reduced shear stress were investigated by attempting to reduce arteriolar blood flow by blocking the synthesis of two archetypal vasodilators, NO and prostaglandin and increasing blood flow by chronic vasodilatation by α_1-adrenoceptor blockade. This was carried out in warm and cold-acclimated animals where blood viscosity, therefore shear stress was expected to be increased and reduced, respectively. In this study, capillary to fibre ratio (C:F) and capillary density (CD) were measured to obtain two different measurements of capillarity. Capillarity was measured in trout using alkaline phosphatase staining, but capillary visualisation using immunohistochemistry was unsuccessful in common carp. Mean fibre area was measured to assess whether fibre hypertrophy had taken place. A direct measurement of hyperplasia or atrophy was not possible

because in order to distinguish whether a reduction in mean fibre size is due to hyperplasia or muscle atrophy, the total mass of that fibre size was required and the low number of animals used in this study did not permit such an investigation. The alternative method of measuring hyperplasia was to stain for proliferating cell nuclear antigen (PCNA) but in red muscle, PCNA staining was unclear due to the small inter-fibre and inter-capillary distances, even at the highest magnification used in this lab (x40), so it was only possible in white muscle. To identify whether splitting or sprouting angiogenesis takes place in cold-induced angiogenesis, capillary-associated proliferating cells were quantified using a mammalian PCNA antibody. The quantification of capillary-associated VEGF protein by immunohistochemistry using mammalian antibodies was also used as an index of angiogenesis.

In this study, cold acclimation did not induce angiogenesis and warm acclimation did not cause capillary regression. Cold acclimation caused a shift towards an increase in capillarity in the first batch of summer animals but not in winter animals. It is probable that trout pre-exposed to low temperature had reached their angiogenic potential, and therefore were not as susceptible to the effects of cold as summer trout. Interestingly, cold acclimation did not elicit capillary growth in a second batch of summer animals, suggesting a batch-dependency of effects or that temperature is a permissive stimulus for angiogenesis but other factors such as proper feeding, time and perhaps stress levels impact acclimation. There may have been due to an intrinsic resistance to angiogenesis, or because as a group, these trout were stressed, and high cortisol

levels suppressed VEGF synthesis and endothelial cell migration (Jaggers *et al.*, 1996). Although statistical analysis showed no significance, in those experiments in which some capillarity growth was seen, these changes, though minor, may be of biological significance in reality as there was an increase in VEGF and PCNA staining in white muscle where angiogenesis was not observed. PCNA staining demonstrated cell proliferation in the immediate vicinity of capillaries, but the identity of the cell type was not clarified in this study. If the proliferating cells were endothelial cells, this would be indicative of sprouting angiogenesis, which could be a result of increased metabolic demand or abluminal mechanical strain.

The effects of treatment with chronic vasoconstrictors did not parallel one another and chronic vasodilatation did not cause angiogenesis unlike that which has previously been demonstrated in mice (Williams *et al.*, 2006a). NOS blockade brought about capillary rarefaction (although statistical analysis for significance was not possible because tne number of replicates was 2) in the oxidative muscle of cold acclimated summer animals but had no effect on either the glycolytic muscle of the same animals or the oxidative muscle of winter animals. Surprisingly, NOS blockade brought about a consistent but non-significant, increase in capillarity at intermediate and high temperatures, suggesting a temperature-dependency of NO activity. The origin of the NO activity was sought, both in terms of the localisation and identity of the NOS isoform that synthesises NO in trout and in terms of the systemic effect NOS blockade may be having on the trout at different temperatures to cause conflicting responses. The effects of NOS

blockade on heart rate at high temperature were investigated in one animal (due to failures in the technique used), and this caused a transient tachycardia followed by a prolonged bradycardia. As bradycardia brings about decreased cardiac output therefore decreased blood flow in trout (Taylor *et al.*, 1996), it is unlikely that the effects of NOS blockade on HR at high temperature influence angiogenesis. It is also possible that temperature directly influences NO or NOS synthesis and degradation. Like other substances such as cytochrome C (Sidell, 1977) in fishes, at high temperatures, both NO synthesis and degradation is likely to be high so NOS blockade would inhibit NO synthesis and have no effect on degradation, reducing total NO. This should lead to a reduction in the angiogenic stimulus, when in fact the opposite seemed to be occurring. At low temperatures, degradation rates are expected to be low but as rates of synthesis are also expected to be low, the synthesis of NO should be inhibited entirely, again causing an attenuation of the angiogenic stimulus. Therefore, neither the direct effects of NO on the vasculature, nor its effects on cardiac function explain its possible temperature-dependency. As such, further investigation into whether NO directly affects cell migration and VEGF synthesis differentially at different temperatures, is required. Attempts to investigate NOS presence and activity directly, were unsuccessful. The citrulline assay, a commonly used enzyme assay was unreliable and produced false positive results, and a NO probe, which measures real-time NO synthesis could not be used in homogenised tissue.

Given that there was some evidence for a lack of eNOS mRNA and protein in this study, in zebrafish, pufferfish, trout or carp, and no available literature describing the existence of an eNOS gene in the genome of any fishes, it is unlikely that any NO present in the vasculature acting on the endothelium, is eNOS-derived. This is consistent with recent literature suggesting that nNOS-derived NO may be the primary contributor to its effects on the vasculature in non-mammalian vertebrates such as frogs (Jennings & Donald, 2010) and fish (Jennings *et al.*, 2008). In the current study, immunohistochemistry clearly demonstrated that mammalian nNOS antibody cross-reacts in fish, although its binding in Western blots has not been demonstrated, and whether it is binding to nNOS has not been clarified, which is required because its binding at a lower MW than expected may suggest that it is not binding to nNOS, as has been suggested by others (Lacza *et al.*, 2003). However, this is a common problem encountered with antibodies where those antibodies that are successful for Western blotting are not always successful in IHC and *vice versa*. This is at least partly because IHC depends on binding to an epitope which is still in its tertiary state, whereas Western blotting is carried out on denatured proteins in their primary conformation. The evidence for the absence of an eNOS in fishes is growing (Evans & Gunderson, 1998; Jennings *et al.*, 2008; Olson & Villa, 1991). Antibodies previously used to demonstrate the presence of eNOS protein in fishes (McNeill & Perry, 2005), in our hands, showed no co-localisation to capillaries as demonstrated by alkaline phosphatase staining. This suggests that in fishes, other proteins that carry the same epitope that this antibody has been raised against exist, or the polyclonal nature of this antibody causes high levels of non-selective

staining. Previous studies on carp and Antarctic fishes such as *Notothenia corriceps*, in this lab, support the idea that an NO-dependent response does not exist in fishes (see **Publications**).

Microarray analysis of global gene expression in cold and warm-acclimation in carp did not highlight any important mediators of angiogenesis. This was partly because of poor hybridisation of the target cDNA to the microarray probes. This showed that although the interwoven loop design may be useful to reduce the statistical error associated with multiple t-tests that would have been needed if all samples were compared to a control sample, the success of the interwoven loop design is wholly dependent on all microarrays and spots being of high quality and perfect hybridisation, which was not the case in the present study.

However, acclimation of fish to a common temperature in their experimental tanks for several weeks prior to temperature change, and changing the temperature in one direction only, brings about a response from a stable start-point as indicated by Principal Component Analysis, which showed that when animals were stabilised, their genetic profile remained fairly stable for at least a month providing no changes were imposed. Thus, any response seen after temperature acclimation should predominantly be a result of the temperature change rather than tank stress. Microarray analysis also demonstrated that transcriptional responses were time and temperature-dependent and that the greatest transcriptional responses took place in warm-acclimated animals. Some of these responses were primarily related with apoptosis, immune

regulation and proteolysis, which may be expected in heat stress but the predominantly differentially expressed genes were linked with general biological and metabolic processes.

7.2 Future experiments

That temperature-dependent capillarity was inconsistent between animals and groups of animals in this study, suggests that a protocol for creating uniformity in animal selection needs to be attempted in the future. This could include calculating the condition factor of the animals before and after the experimental procedure to minimise the possible effects of size and inadequate or excessive feeding and growth and monitoring fishes individually. In addition, levels of inflammatory mediators or stress-response hormones could be assayed in tissue from these animals and those animals with high levels of these could be excluded from the study, in order that physiological angiogenesis can be measured. Administering drugs using alternative methods such as mini-osmotic pumps may reduce some of the variability of drug intake within a group, but the temperature-dependency of drug infusion from the pumps could still affect the outcome between groups.

To address the influences of heart rate on temperature-related capillarity, the use of dataloggers for continuous monitoring of heart rate in unrestrained animals may be useful. This would allow the effects of acute and chronic temperature changes to be monitored and muscle samples taken immediately after acute temperature changes and following temperature

acclimation would allow transcriptional and functional changes to be monitored so that the time-course of adaptive changes to temperature can be identified.

Future microarray analyses into cold-induced angiogenesis in carp require that a parallel assessment of capillarity from muscles of identical origin is carried out. This would clarify whether changes in endothelial gene transcription influences capillary growth.

Appendix A Information on all animals used for acclimation experiments

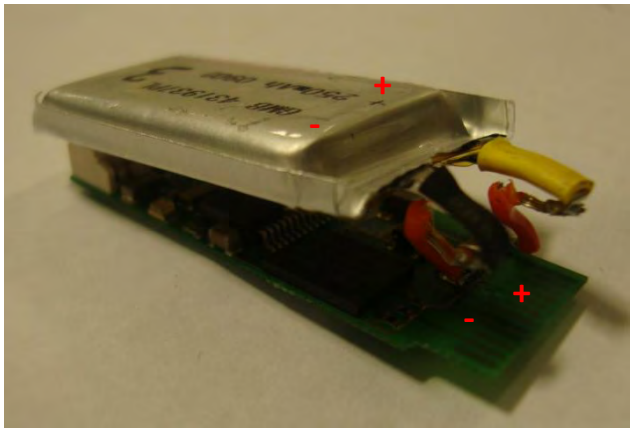
EXPERIMENT	CONDITIONS	NUMBER AT START	NUMBER AT END (week)
LNNA cold acclimation	11°C	6	2
	11°C + LNNA	6	3
	4°C	18	3 (1), 2 (4), 3 (8)
	4°C + LNNA	6	2
LNNA warm acclimation	11°C	6	4
	11°C + LNNA	6	2
	18°C	18	5 (1), 3 (4), 1 (8)
	18°C + LNNA	6	3
Prazosin cold acclimation	11°C	7	6
	11°C + prazosin	7	4
	4°C	21	6 (1), 6 (4), 3 (8)
	4°C + prazosin	7	3
Prazosin warm acclimation	11°C	7	6
	11°C + prazosin	7	4
	18°C	21	6 (1), 4 (4), 3 (8)
	18°C + prazosin	7	5
Indomethacin cold acclimation	11°C	6	6
	11°C + indomethacin	6	5
	4°C	12	3 (1), 3 (4)
	4°C + indomethacin	6	3

Appendix B Datalogger preparation

Standard operating procedure

All loggers were programmed with the same software (which is named LoNoise512-1 with serial number 1.21, visible when using the z_CONFIG.EXE program). The sample rate was 512 samples per second and the deep sleep mode was disabled.

Boards need to be powered using a lithium polymer battery for configuring, recording and downloading data. The lithium batteries are attached like so:



Datalogger with a lithium polymer battery attached. This image demonstrates the correct placement and orientation of the battery.

To configure loggers

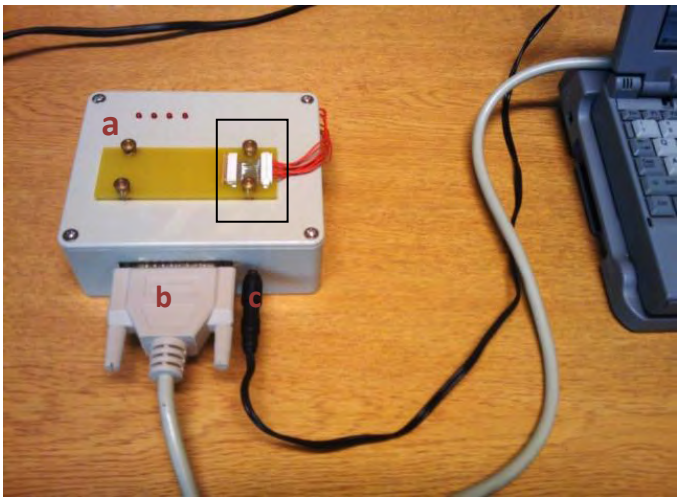


Image demonstrating how to connect the interface box to the Libretto computer and a power supply. Materials required: Libretto computer with a parallel port and correct folder "new 64 logger 2009". Interface box (a) with parallel cable (b) and mains adaptor (c)

Protocol:

1. Insert the datalogger into the interface (marked with a black rectangle) on the interface box, with the **gold contacts facing down**.

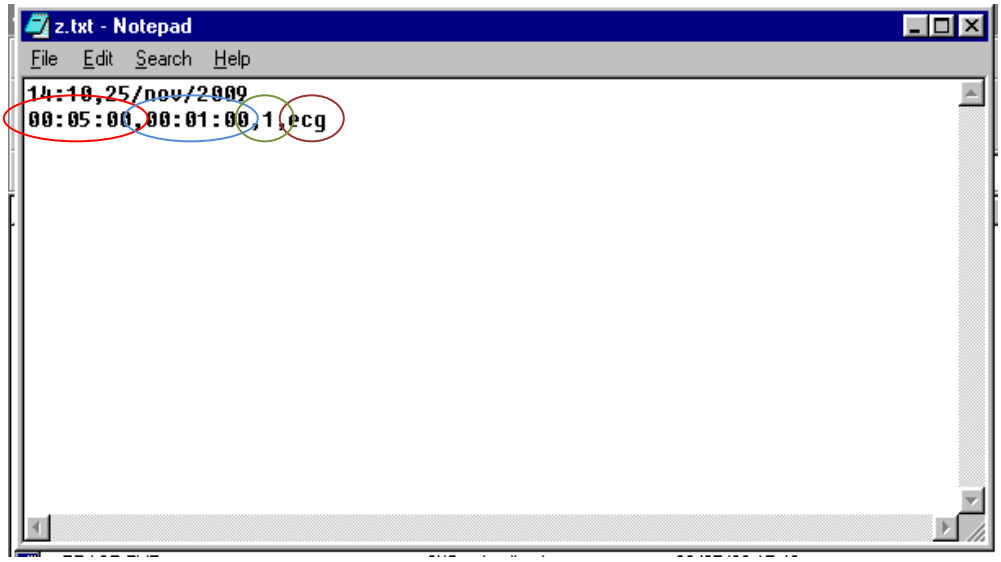
2. Open the folder 'new 64 logger 2009'. For this logger, the programs needed are:
    i. z.txt
    ii. z_CLOCK.exe
    iii. z_CONFIG.exe
    iv. z_ERASE.exe
    v. z_GETDATA.exe
    vi. z_SERNUM.exe

vii. z_UNWRAP.exe

viii. z_ViewECG(Robins Delphi program).exe

3. Open z_CLOCK.exe. It will display what the time and date is on the computer. If this is incorrect, correct it by double clicking on the time display (bottom right hand corner of the desktop), as you would a normal PC.

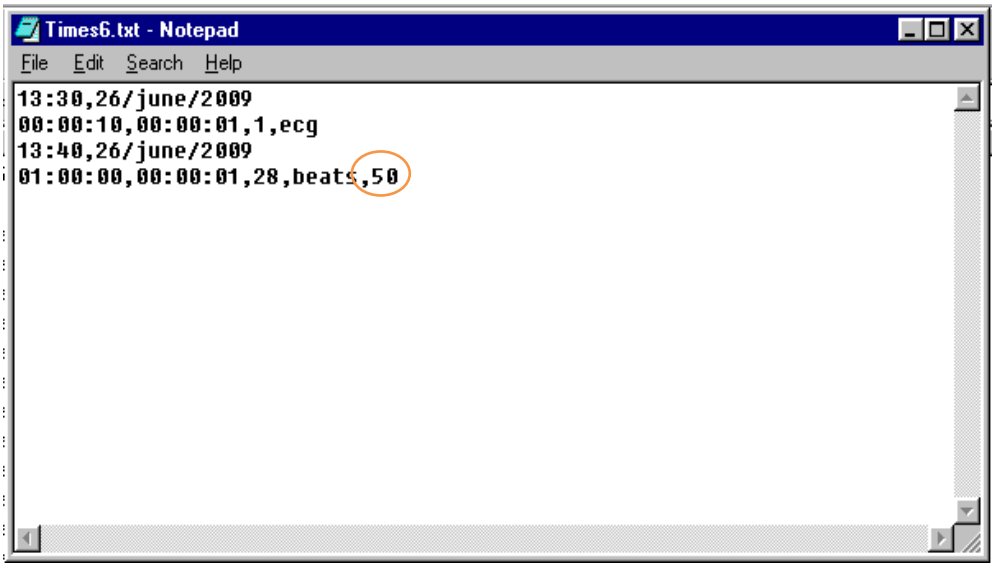
4. Open z.txt:



This is a text file that can be edited. Change the time and date to when you would like the logger to start recording. Also, indicate how long the logger should record for in every cycle (red circle); how long to take a break for before next cycle (blue circle); how many cycles to do (olive circle); what to record (brown circle). So, in this example, the logger is set to start recording at 14:10,

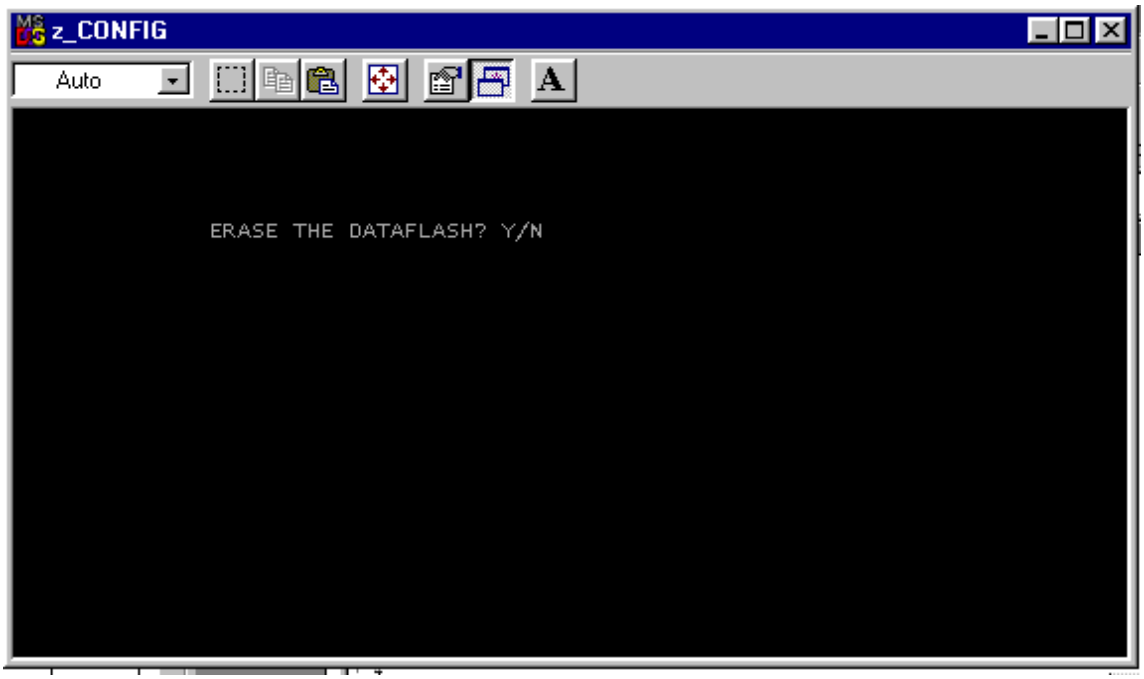
on the 25th of November 2009. The logger will record ECG signals for 5 minutes and pause for 1 minute, for 1 cycle only.

If you would like to take two sets of recordings, instructions can be written as below:

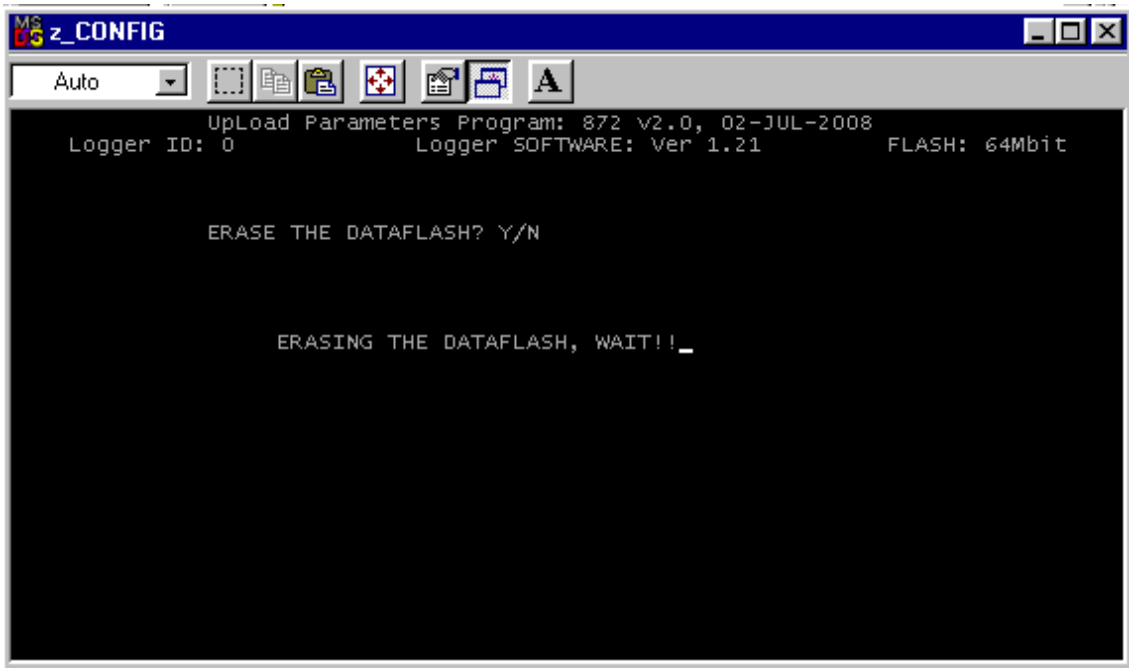


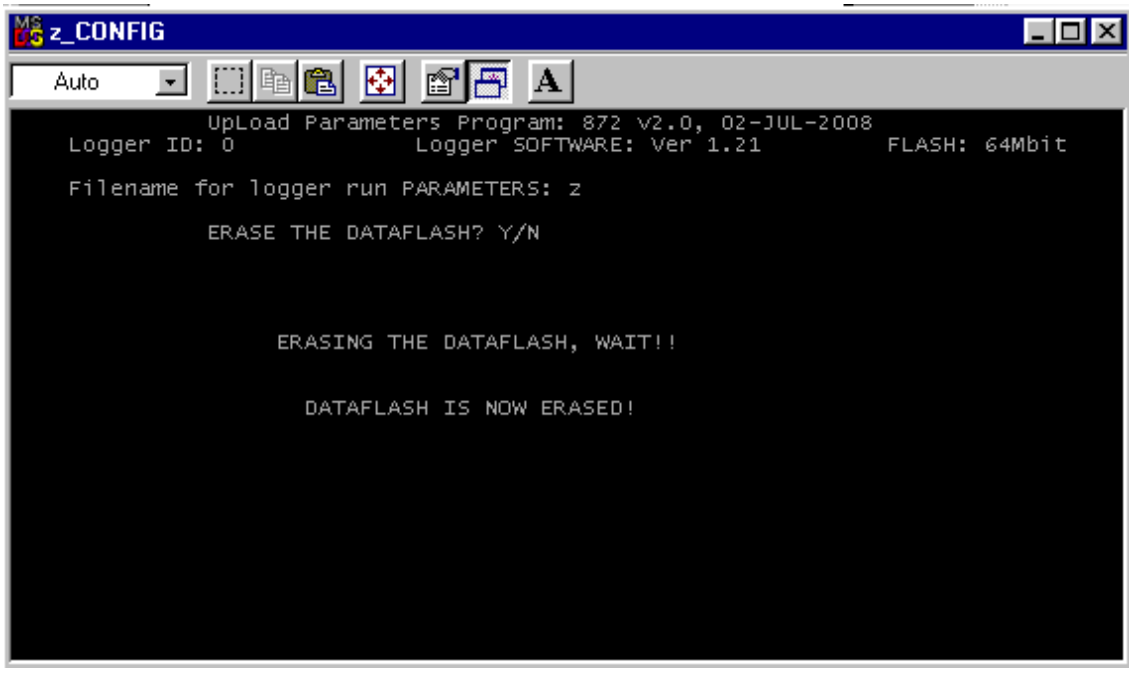
In this example one recording starts at 13:30 on 26th June 2009; 10 seconds of ECG recordings are taken with a 1 second break, for one cycle. The second recording starts at 13:40 on the same day, beats are recorded for 1 hour, with 1 second breaks between cycles. 28 cycles in total are recorded. The orange circle indicates that the beats are pooled together so each signal represents 50 beats (this is useful for species with higher heartrates- saves memory space)

5. Upload z.txt information the logger by opening the z_CONFIG.exe program:



Enter ‘Y’. The display will read, ‘erasing the dataflash, wait!!’ followed by ‘dataflash is now erased!’ and finally the prompt for entering the ‘filename for logger run parameters’ will appear. At this point, enter ‘z’. This will upload the time and recording data you set in the file ‘z.txt ‘in step 4.


z_CONFIG
Auto
UpLoad Parameters Program: 872 v2.0, 02-JUL-2008
Logger ID: 0
Logger SOFTWARE: Ver 1.21
FLASH: 64Mbit
ERASE THE DATAFLASH? Y/N
ERASING THE DATAFLASH, WAIT!!

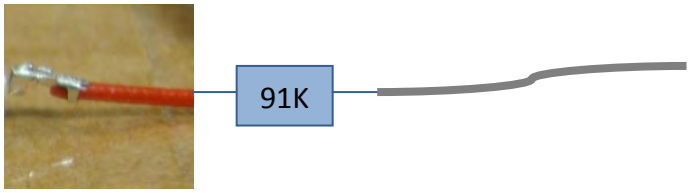

z_CONFIG
Auto
UpLoad Parameters Program: 872 v2.0, 02-JUL-2008
Logger ID: 0
Logger SOFTWARE: Ver 1.21
FLASH: 64Mbit
Filename for logger run PARAMETERS: z
ERASE THE DATAFLASH? Y/N
ERASING THE DATAFLASH, WAIT!!
DATAFLASH IS NOW ERASED!

The screen will then confirm the information uploaded and ask you to confirm that you would like to upload the data to the logger. Enter 'Y'.

The datalogger is now ready to record. Remove from the interface and the orange LED light should flash every second for 1 minute; after this it will flash once every minute.

Packaging the datalogger

Attach a Teflon coated stainless steel wires (grey) to wires that are compatible with receptacle housing (red), with a 91K resistor in series.



Attach a second Teflon coated wire to a wire without a resistor in series. The Teflon-coated wires must be 10cm long so there are no extraneous bits of wire that may catch.

Insert the metal tips of the two wires into the male receptacle housing in this orientation:

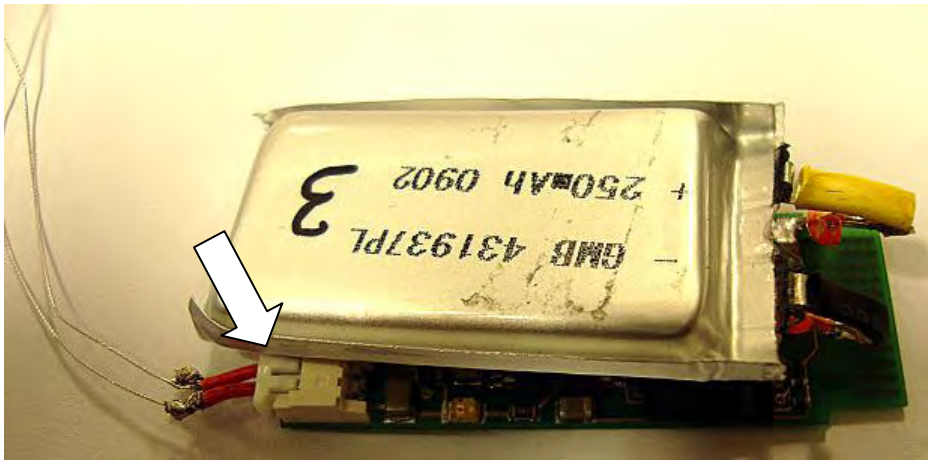
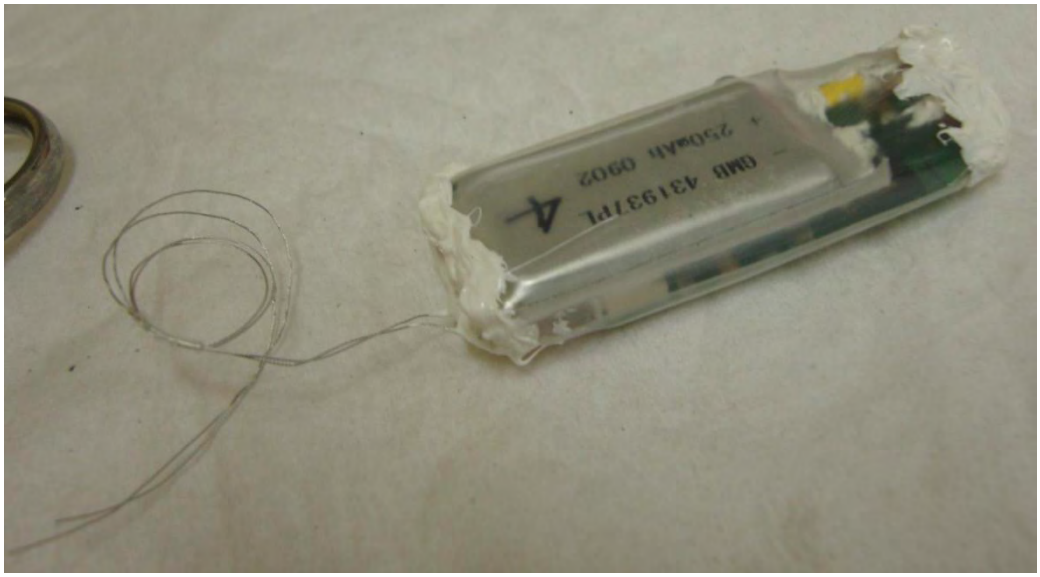


Image demonstrating where the male receptacle housing is to be inserted (white arrow)

Lock the male receptacle housing into the female housing on the rear of the board.

Place the logger with the batteries in heat shrink tubing (diameter 18m, shrink ratio 3:1) so that only the stainless steel wires are out of the tubing and heat shrink. To avoid heating the Teflon-coated wire, place a piece of aluminium foil over the wire to shield it. Seal the ends with Downing Corning and allow it to cure overnight. Apply a second coating after it has cured and allow that to cure also.



This logger has been sealed with Dow Corning and is ready for attachment to the fish

The logger is now ready for attachment to the fish and it may be placed internally or attached externally.

Attachment of datalogger to fish

Fish were lightly anaesthetised by exposure to MS222 (100mg/L) until righting reflex was lost, at which point the fish were transferred to an operating table with a water pump attached to a tube that was used to irrigate the gill with water. The water used to irrigate the gills contained 10mg/L MS22 and was cooled with a chiller block and continuously aerated. The fish were propped up using wet sponges to avoid drying and thermal stress (room temperature, 20°C). Dataloggers were attached internally (common carp) and externally (rainbow trout).

Intraperitoneal implantation of datalogger

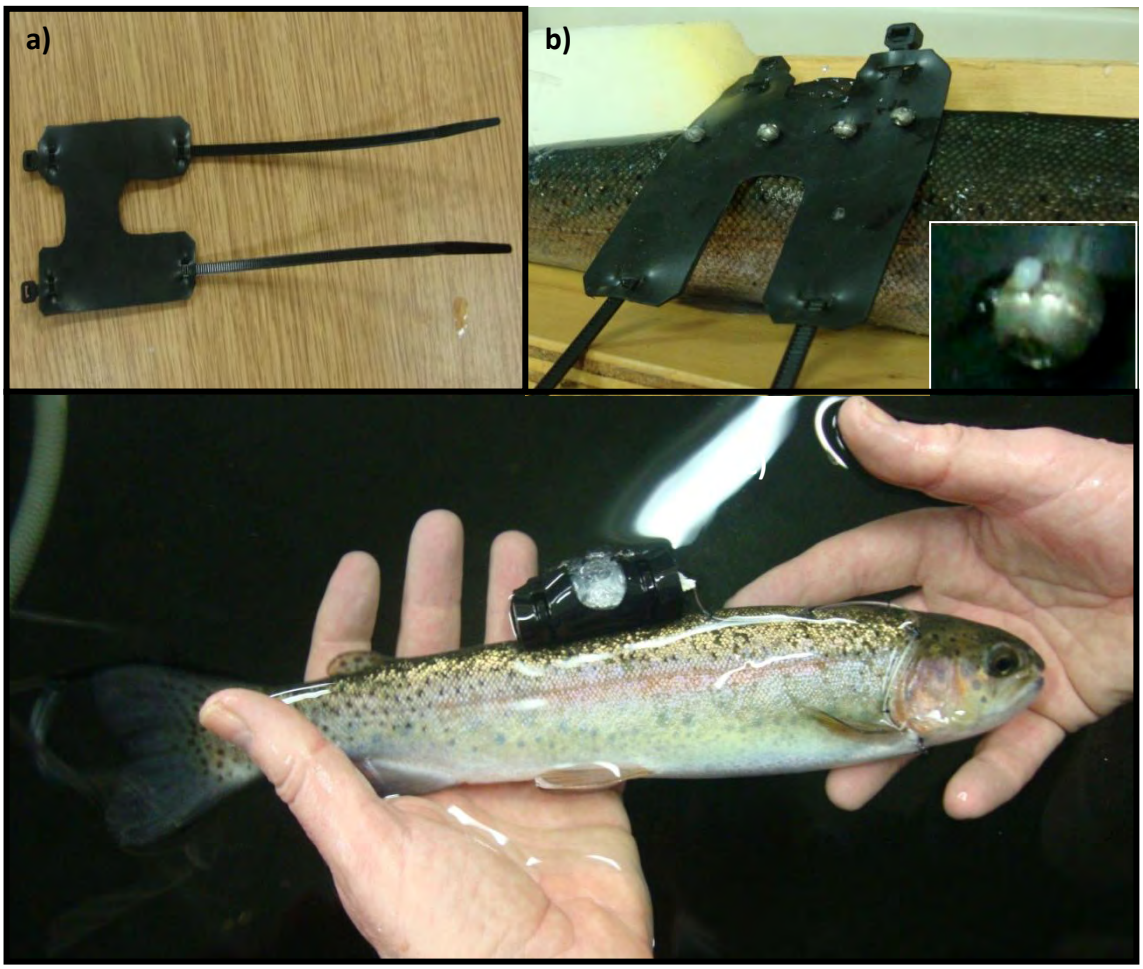
An incision large enough for the datalogger is to be made and the logger placed into the peritoneal cavity. In order to make the incision, the scales were removed around the selected area. The tip of one electrode (stainless steel wires) was hooked onto the pericardial sac close to the heart, using a 19G needle, and the other elsewhere as a reference. The incision was stitched using a curved needle and 3-0 suture.

Insertion of datalogger into the peritoneal cavity of the common carp

External attachment of datalogger

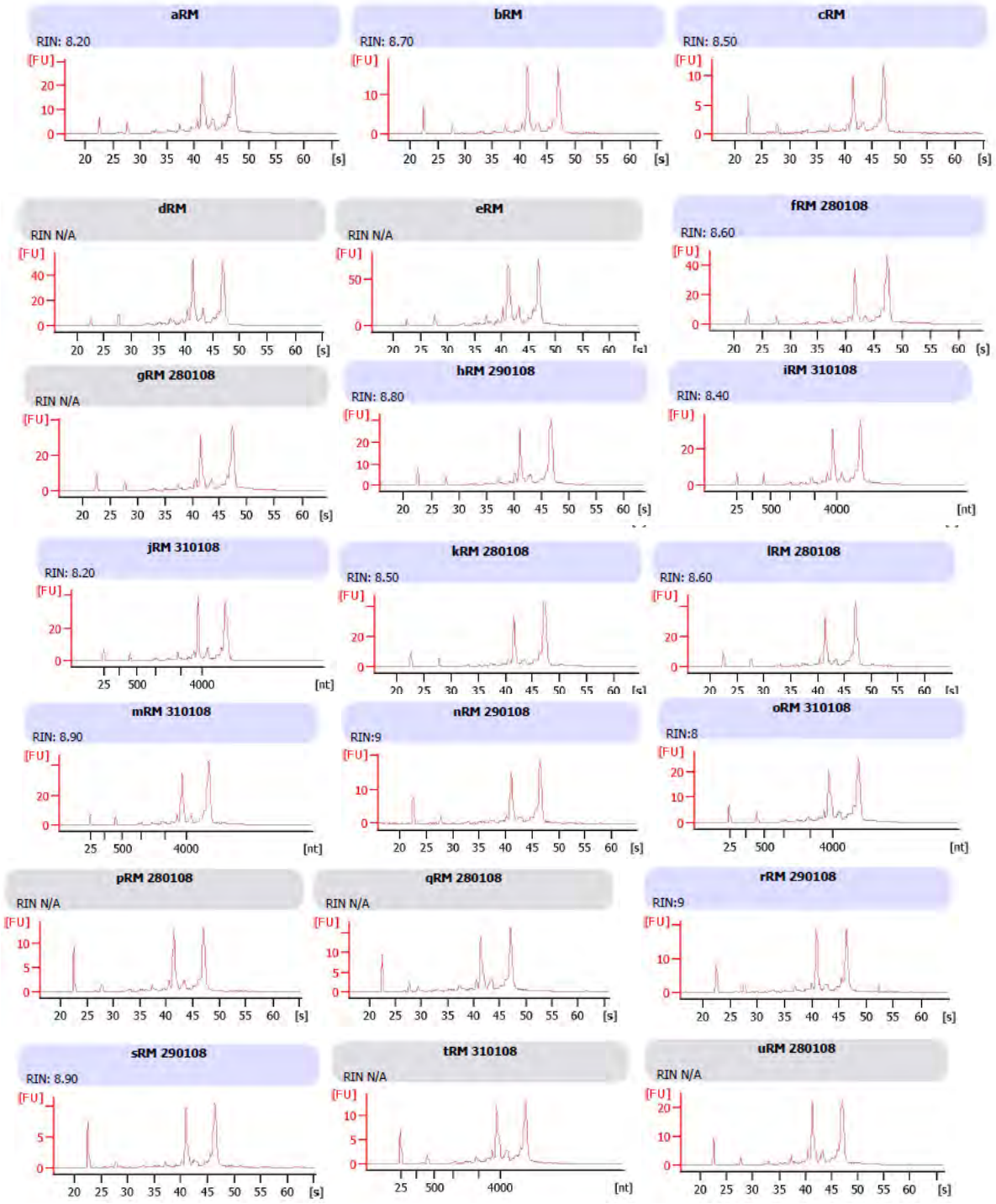
A saddle composed of a rubber sheet and cable ties (figure below) was attached to the muscle on the dorsal side of the fish using a Motex® tagging gun. The tags were cut and secured lead by crimping lead shot balls around them. Finally, the tips of the tags were heated using a cigarette lighter to secure the leadshot (inset).

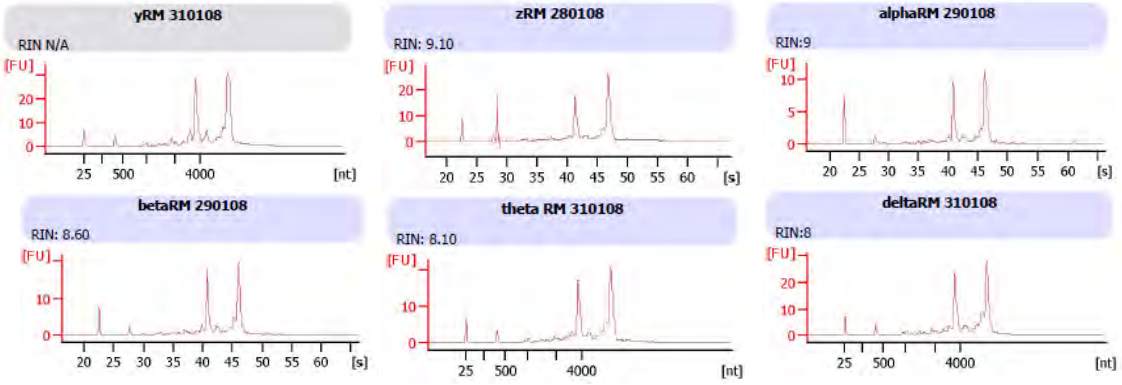
The logger was then wrapped in bubblewrap (purchased from local stationers) for buoyancy and secured to the saddle with the cable ties. The cable tie was then cut to size. The fish were then placed in a holding tank and were closely observed until they regained their righting reflex.

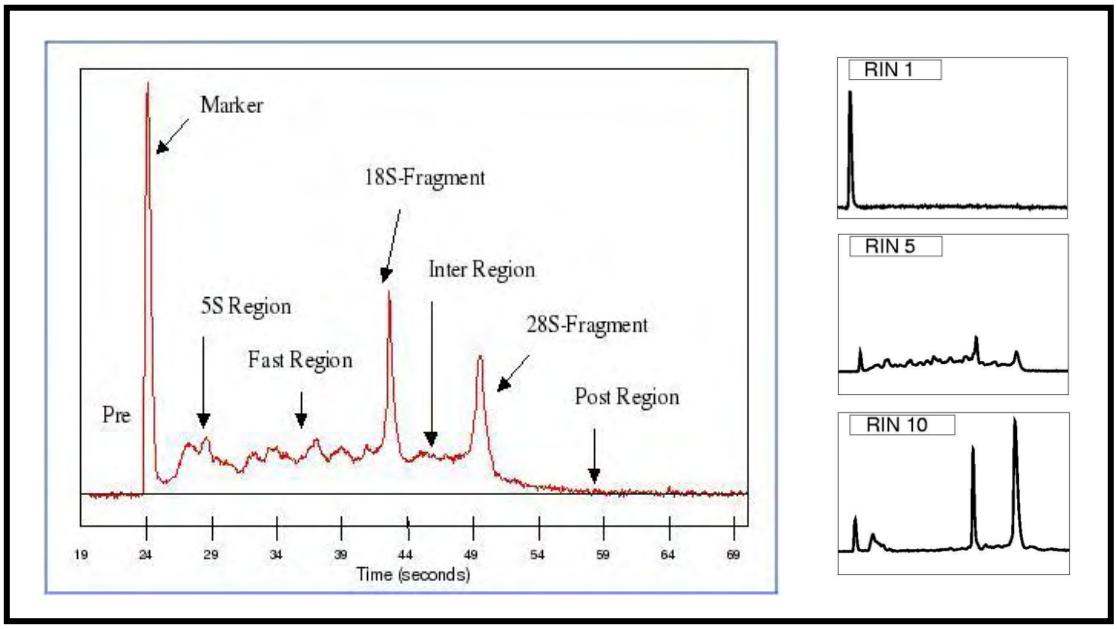


Attachment of datalogger to the dorsal fin of the rainbow trout

Appendix C RNA Quality and Integrity







Traces from the Agilent Bioanalyzer, demonstrating the RNA integrity of each sample used in the current study. Where the RNA integrity number (RIN) is displayed, it can be seen that all RNA samples are highly intact (RIN values 8-9) therefore suitable for microarrays. Where the RIN is not displayed, sample integrity was assessed using guidelines set by Schroeder *et al* (2006). A description of the electropherogram and a brief explanation of the expected output associated with selected RIN values are displayed in the black box (reproduced from Schroeder *et al.*, 2006). The electropherograms show that all 30 samples are almost identical in integrity.

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Publications

Non-technical summary: Themed Meeting: Cardiac & Respiratory Physiology (in press, *Physiology News*)

A 3-day themed meeting on Cardiac & Respiratory Physiology was held in Birmingham in September 2010, with a focused symposium on hypoxaemia ('Coping with hypoxaemia: strategies and solutions'). The meeting was divided into five sessions (O_2 sensing mechanisms, blood flow regulation, lessons from comparative studies, intermittent hypoxia, and chronic hypoxia) that gave an insight into research on the strategies for coping with hypoxia in a wide range of animals, through the mechanisms of hypoxia-sensing, to hypoxaemia-associated diseases. Some personal highlights are given here.

The first day focused on cellular responses associated with exposure to hypoxia, predominantly hypoxic pulmonary vasoconstriction (HPV) and oxygen-sensing by the carotid body. The different hypotheses for oxygen sensing were presented: whether the primary mechanism of carotid body activation was through oxygen-sensitive voltage-gated K^+ channels (for example the $K_V4.3$ subtype) (T. Perez Garca, Universidad de Valladolid, Spain), mitochondrial metabolism (Jeremy Ward and Keith Buckler, KCL and Oxford) or AMP kinase activation (Mark Evans, Edinburgh) were discussed in detail. This debate continued through to the Early Career Scientists' Session on the last day of the meeting although no firm conclusion was reached, even after the stimulating (and often heated) discussion by Stephen Archer (Chicago), and J. Ward on whether the 'redox hypothesis' or the 'mitochondrial hypothesis' was correct. It was agreed, however, that more consistency between the PO_2 levels used in experiments was needed to explain some of the differences seen between labs, and the physiological relevance of oxygen tensions used to mimic chronic hypoxia was questioned. The roles of these mechanisms in fetal growth restriction (FGR), which is also caused by hypoxia, were discussed and Uzo Sampson (Manchester) showed that although the causes of HPV and FGR may be similar, the mechanisms, especially with regards to K_V channels, are not.

The comparative physiology session on the second day began with a fascinating talk by Bill Milsom (British Columbia) on adaptations to hypoxia in a bird that migrates at high altitude, the Bar-headed goose. These adaptations include their more effective ventilation which involves slow and deep breathing, producing less dead space in the lungs. In these geese, oxygen availability and utility is enhanced by their high affinity haemoglobin, greater muscle capillarity and increased mitochondria compared with other species. Tobias Wang (Aarhus) followed with his talk on how reptiles cope with hypoxia by mixing oxygenated and deoxygenated blood in two-chamber hearts, a real feat of flow control by variable resistances. Goran Nilsson (Oslo) described the mechanisms of survival under anoxia by crucian carp, which initially change their gill structure in response to hypoxia to increase the surface area for oxygen diffusion. High

glycogen stores in muscle, and the ability to convert excess lactate produced by anaerobic respiration into ethanol, which is released into the environment then supports long-term survival in anoxia. Carp also increase the production of the endogenous tranquiliser, GABA, which reduces their activity and preserves energy. These clever mechanisms of survival in hypoxic conditions have hopefully inspired biomedical scientists to try and transfer these mechanisms for manipulation in humans at high altitude, although survival on a similar regime of 'drugs and alcohol' may be questionable!

The afternoon's talks were varied, from students' talks on hypoxia-induced reduced glucose sensitivity in the isolated whole carotid body, to the mechanisms of intermittent hypoxia-induced sleep apnoea, and the ability of hypoxia to cause respiratory muscle weakness as a result of increased muscle plasticity. The day ended with a talk by Janice Marshall (Birmingham) on local mediators of acute & chronic hypoxia in muscle, with particular reference to adenosine metabolism and its contribution to endothelium dysfunction, leading to cardiovascular disease in adult life. High altitude was the subject of the day, and at the Society Dinner that evening held at the National Sea Life Centre in Birmingham, Chris Imray (Coventry) gave an excellent after-dinner presentation on his experiences carrying out hypoxia research on Mount Everest. He described the extremely difficult, emotional but ultimately successful experiences his team encountered on their journey. If only they were Bar-headed geese...

The final session of the meeting began with a talk by Andy Cossins (Liverpool) challenging current ideas on myoglobin as an exclusively muscle protein, as it also exists in brain and in the vasculature of fishes. Asif Ahmed (Edinburgh) described the protective effects of haemoxygenase in pre-eclampsia (another disease associated with low oxygen content), and the possibility of a novel use for the anti-cholesterol drug simvastatin in clinical therapy for hypoxaemic diseases.

The two plenary lectures were by Peter Barnes (Imperial) who presented non-antibiotic macrolides or combination therapy of theophylline and steroids, as an alternative to steroid-only treatment in steroid-resistant diseases such as COPD and in asthmatic smokers, where oxidative stress caused by cigarette smoke promotes excessive inflammatory protein synthesis, and Nanduri Prabhakar who discussed possible carotid-body mediated mechanisms whereby the intermittent hypoxia that occurs in sleep apnoeic patients might give rise to a plastic change in chemoreceptor function leading to elevated blood pressure.

The talks were interspersed with poster sessions and coffee breaks that allowed one-on-one discussions between early career and established scientists. Particularly useful was the Point-Counterpoint session held at the end of the meeting, which demonstrated the need to challenge one another's ideas to further our knowledge and understanding. Well-deserved

poster prizes were awarded to Melissa Brereton (Manchester) and Andrew Holmes (Birmingham). All in all, a thoroughly enjoyable meeting, in a fantastic canalside venue in central Birmingham - with good catering too!

Meeting abstract: HOW UBIQUITOUS IS NITRIC OXIDE VASOACTIVITY? (British Microcirculation Society, 2010)

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The role of endothelial nitric oxide synthase (eNOS)-derived nitric oxide (NO) for vasodilatation and/or angiogenesis in humans is equivocal. A lack of eNOS-specific inhibitor suitable for *in vivo* use means it is difficult to ascertain the importance of this NOS isoform *in vivo* in animals apart from mice, where gene ablation has shown that eNOS, but not neuronal (nNOS), is essential in the regulation of vascular tone, control of blood pressure [1] and angiogenesis [2]. However, whether this phenomenon exists in vertebrates other than mice is unclear. Indeed, there is no direct evidence that eNOS exists in some fish species including rainbow trout, common carp and zebrafish. Although non-specific NOS blockade with L-NNA inhibits angiogenesis in rainbow trout (unpublished) and reduces blood flow in zebrafish [3], whether the eNOS isoform is responsible has not been established. Further, isolated carp vessels do not dilate in the presence of sodium nitroprusside (SNP) or exogenous cyclic guanidine monophosphate (cGMP), suggesting that the vascular NO-cGMP pathway does not exist in this species. In addition, there are no sequences in the literature for an eNOS gene in any fish species, and PCR performed on three fish species produces differing products. However, blood pressure in fishes is lower than in mammals, suggesting potent vasodilator activity exists. These observations suggest that the role of eNOS in blood flow and angiogenesis may not be the same in all vertebrates. *Supported by NERC*

[1] Shesely (1996)*PNAS* 93:13176
[2] Williams (2006)*J Physiol* 570:445
[3] Fritsche (2000) *Am J Physiol* 279:2200

Meeting abstract: ENDOTHELIAL RESPONSES TO COLD ACCLIMATION IN COMMON CARP: GENE EXPRESSION PROFILING IN OXIDATIVE SKELETAL MUSCLE

F.Syeda, L.Olohan, F Falciani, A Cossins, S. Egginton (British Microcirculation Society, 2009)

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In mammals, angiogenesis, the formation of new capillaries from a pre-existing capillary network may result from a mismatch in metabolic supply and demand. However, it has also been observed in oxidative (red) skeletal muscle of rainbow trout and common carp acclimated to low temperatures. As metabolism is reduced but oxygen dissolved in water increases at lower temperatures, oxygen supply is unlikely to be limiting. We are investigating the cause of this counterintuitive increase in capillarity, and the mechanisms involved. The metabolic profile in common carp, Cyprinus carpio, has previously been shown to change with acclimation temperature in several tissues including white (glycolytic) muscle (Gracey et al, 2004) but the genetic response in red muscle, where more angiogenesis takes place, has not yet been investigated.
Common carp were acclimated to the optimal temperature for carp, 15□C, for 4 weeks. At 4 weeks, the fish were either cooled (5□C) or warmed (25□C) over a day. Tissue samples were taken at 1 and 8 weeks after temperature change. Responses in the oxidative skeletal muscle were assessed using a carp cDNA microarray composed of 22,661 probes. A total of 226 differentially expressed genes have been identified showing time and/or temperature-dependent gene expression regulation. We are currently carrying out functional annotation of these genes.

Meeting abstract: COLD-INDUCED ANGIOGENESIS IN FISH SKELETAL MUSCLE IS INHIBITED BY NITRIC OXIDE SYNTHASE BLOCKADE (Society for Experimental Biology, 2008)

F.Syeda, D. Hauton, S. Egginton.

Centre for Cardiovascular Sciences, University of Birmingham, UK

Angiogenesis, the formation of new capillaries from a pre-existing capillary network, may occur in at least two ways: longitudinal division of capillaries is induced by luminal stimuli, while capillary sprouting requires perivascular stimuli.

In mammals, angiogenesis may result from a mismatch in metabolic supply and demand. However, it has also been observed in oxidative (red) skeletal muscle of fishes acclimated to low temperatures. As metabolism is reduced but oxygen dissolved in water increases at lower temperatures, oxygen supply is unlikely to be limiting. We are investigating the cause of this counterintuitive increase in capillarity, and the mechanisms involved. Rainbow trout (140-260g) were acclimated to cold over a period of 8 weeks, where cooled blood will become more viscous, imposing a greater shear stress on vessel walls. As fish are non-model species, the development of a practical and robust method for capillary identification was required, with alkaline phosphatase being the most practical. This study showed that angiogenesis occurs in cold-acclimated trout; the capillary to fibre (C:F) ratio reduced when the environmental temperature was reduced from 11°C (1.0±0.04; n=3) to 4°C (1.4±0.02; n=3). Reducing shear stress by nitric oxide synthase (NOS) blockade with L-NNA significantly reduced C:F to below control values (0.8±0.02; n=3, $P<0.05$), so NOS inhibition may induce capillary regression. This suggests that elevated shear stress induces angiogenesis, is important in maintaining vessel integrity, and that metabolic and mechanical stimuli of angiogenesis may not always be coupled. *Supported by NERC.*

List of publications

Syeda, F., Falciani, F., Cossins, A., Egginton, S. (2009) Endothelial responses to cold acclimation in common carp: gene expression profiling in oxidative skeletal muscle. *Microcirculation*, 16: 444-486

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