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COLD-INDUCED ANGIOGENESIS IN SEASONALLY ACCLIMATIZED RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Summary

Seasonal acclimatization of rainbow trout induced an inverse relationship between environmental temperature and the capillary:fibre ratio of slow locomotory muscle, which increased from 1.73±0.09 in the summer (18 °C) to 2.50±0.15 in the winter (4 °C). However, the rate of capillary growth (angiogenesis) was exceeded by that of fibre growth at low temperatures such that the extensive fibre hypertrophy found at 4 °C led to a decrease in capillary density, N_A(c,f), to 57 % of that found at 11 °C and 85 % of that at 18 °C. Cold-induced angiogenesis resulted in an expanded capillary bed of similar topology to the existing network, with capillary orientation deviating from that of muscle fibres by only 4–7 %. Capillary length density was maximal at 11 °C, J_V(c,f)=2421±239 mm^{-2}, which as previously described corresponds to the point when muscle blood flow is highest and the scope for aerobic swimming is greatest, reflecting an integrated response to optimize aerobic performance at intermediate temperatures. In contrast, ventricular N_A(c,f) parallels heart rate and hence was highest at 18 °C, while there was no seasonal variation in myocyte diameter. Although a systemic response to seasonal adjustments in humoral factors may occur, the data reported here suggest that angiogenesis is probably stimulated by different mechanical factors in these two muscles.

Key words: capillary supply, cold acclimatization, myocardium, skeletal muscle, rainbow trout, *Oncorhynchus mykiss.*

Introduction

The swimming musculature of fishes is usually divided into anatomically and functionally discrete regions of white (fast glycolytic) and red (slow oxidative) fibres. It is generally accepted that only the red muscle fibres are active at truly sustainable swimming speeds, i.e. those that may be maintained indefinitely without obvious signs of fatigue. Salmonids are often found in swiftly flowing water so that lengthy bouts of continuous swimming are required to maintain their station, during which much of the trunk is involved in power generation (sub-carangiform locomotion). Under these conditions, cardiovascular performance of trout held at 11 °C was adequate to supply oxygen and metabolic fuels to the maximally working muscle without any disturbance in blood chemistry (Wilson and Egginton, 1994). As part of our continuing investigations into the anatomical, metabolic and cardiovascular limits to aerobic exercise across the full temperature range of a relatively eurythermal fish species (Taylor *et al.* 1996; Cordiner and Egginton, 1997), this communication examines the possible role of the microcirculation in limiting sustained swimming.

There are many factors that may affect the rate of peripheral gas and/or metabolite exchange, including myoglobin concentration, haemoglobin oxygen-affinity and the level of mitochondrial respiration. In addition, the extent of the capillary supply represents a structural limit to aerobic muscle performance by setting an upper limit to the maximum blood flow and available exchange surface. Not surprisingly, therefore, the capillary supply to mammalian muscles has been correlated with metabolic fibre type, mitochondrial volume and oxygen consumption. Moreover, it shows an adaptive response to changes in these parameters (Hudlická *et al.* 1992). The few available data suggest that the capillary bed is unusually homogeneous in trunk muscles of fishes (Egginton *et al.* 1988) and that it scales with fibre size rather than phenotype (Egginton, 1992). However, relatively few studies have considered the response of the microcirculation to an altered environment in these animals.

In most vertebrates, there is an apparent increase in capillarisation following exposure to low temperatures (Hudlická *et al.* 1992), and this may be especially evident in fishes, where efficient branchial heat exchange leads to cellular temperatures within a fraction of a degree of that of the surrounding water. Given the seasonal excursion in environmental temperature, growth of capillaries (angiogenesis) may be expected to overcome the effect of impaired tissue perfusion in the cold caused by an increase in blood viscosity (Egginton, 1992). Indeed, Johnston (1982) showed a vigorous capillary proliferation in the red muscle of

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crucian carp *Carassius carassius* which had a numerical capillary to fibre ratio (C:F) of 2.2 at 28 °C, increasing to 4.8 when held at 2 °C for 8 weeks. However, the rise in C:F of cold-acclimated striped bass *Morone saxatilis* was countered by muscle fibre hypertrophy, resulting in a capillary density that was similar at 5 and 25 °C (Egginton and Sidell, 1989). Unfortunately, blood flow estimates are not available for either species, so we are unable to relate the potential supply capacity to functional demand.

Most studies of thermal acclimation have involved laboratory experiments with parallel groups of animals exposed to differing temperatures. These and other studies show that the fast muscle system is generally much less responsive to physiological challenges than is the slow muscle. We therefore followed changes in capillary supply to the aerobic slow swimming musculature and myocardium of trout in response to the natural shift in environmental temperature to determine whether this could explain the increased fatigue and/or decreased cardiac output at the seasonal extremes reported previously (Taylor *et al.* 1996). In addition to direct effects of altered temperature, this allows for indirect effects (e.g. activity level and food consumption) as well as hormonal adjustments to changing photoperiod.

Materials and methods

Rainbow trout [*Oncorhynchus mykiss* (Walbaum)] were obtained from Leadmill trout farm (Hathersage, Derbyshire) and allowed to recover in the laboratory for a few days at the appropriate temperatures of 18 °C (summer), 11 °C (autumn) or 4 °C (winter); body masses for the three groups were 755±44 (18 °C), 748±62 (11 °C) and 711±28 g (4 °C) (mean ± S.E.M., N=6 per group). Under tricaine methane sulphonate anaesthesia (MS222; 0.1 g l^{-1}), chronically indwelling cannulae were inserted into the dorsal aorta and exteriorised (Soivio *et al.* 1972). Fish were left to recover for 96 h for the vessel to make a pressure-tight seal around the cannula, then anaesthetised a second time. After being secured in a supine position, they were fixed by perfusion with a buffered glutaraldehyde/paraformaldehyde preparation containing 0.05 % sodium azide (Egginton and Cordiner, 1995), with the bulbus arteriosus cut to allow drainage. On the basis of previous reports, we initially infused 20 ml of buffer to wash out most of the blood, and then 40–60 ml of fixative until the trunk became rigid and the exudate was clear. Good preparations were evident from the pale appearance of the muscle after perfusion. More reliable results were obtained, however, by omitting the buffer washout and slowly infusing approximately 5 ml of fixative prior to cutting the bulbus arteriosus. This was particularly important for the preservation of cardiac capillaries and was adopted for all specimens used in the present study. *In situ* fixation was allowed to proceed for 2–3 h at approximately 10 °C, after which samples of red muscle from the hypaxial region of the lateral line triangle at the level of the dorsal fin were carefully dissected out to maintain resting sarcomere length. Salmonid ventricles possess both avascular spongy myocardium, which obtains its nutrition from the venous blood with which it is bathed, and the compact myocardium which has a well-developed vascular supply. Lateral strips of the compact ventricular myocardium were taken and trimmed into pieces with a cut face of approximately 1 mm×2 mm. Samples were stored overnight in fresh fixative at 4 °C then post-fixed in buffered OsO_4 for 1 h, dehydrated in a series of alcohols and embedded in Epon resin. Six blocks per fish were prepared for both muscle types, and one was chosen at random for analysis.

Semi-thin (0.5 μm) transverse sections (TS) stained with Toluidine Blue were used to quantify the capillary supply (Egginton and Johnston, 1983). For skeletal muscle, estimates were made of mean fibre area, a(f), numerical capillary to fibre ratio, C:F, and capillary density, N_A(c,f), by means of a stereological counting procedure (Egginton, 1990). In addition, N_A(c,f) estimates were also made from longitudinal sections (LS) of the same blocks (rotated by 90 °) in order to calculate the degree of tortuosity, or deviation from an anisotropic orientation with respect to the longitudinal axis of muscle fibres, and hence derive the capillary length density, J_V(c,f), i.e. the length of capillaries per unit volume of muscle fibres. Briefly, the length density of structures is related to the numerical density obtained from tissue sections, i.e. $J_V \propto N_A(\theta)$, where N_A is the capillary count per unit area of tissue and θ is the angle (in radians) between the axis of anisotropy and an orthogonal projection from the plane of sectioning. For perfect anisotropy (orientation) $J_V=N_A$, while for perfect isotropy (randomness) $J_V=2\times N_A$. Using the assumption of a spherical–normal distribution, the constant of proportionality, $c(K,0)$, may be derived from the concentration parameter, K. K is conveniently estimated from the ratio of numerical densities in TS and LS, i.e. $N_A(0)/N_A(\pi/2)$ (Mathieu *et al.* 1983). The length density can then be calculated from any transverse section as $J_V(c,f)=c(K,0)\times N_A(c,f)$. Because of the large variability in orientation of cardiac muscle fibres, samples were taken along the lateral medial ventricular axis, and only N_A(c,f) was determined. Myocyte width (minor axis) was measured, rather than area, to avoid dimensional problems associated with imprecise orientation.

Statistical analysis

Single-factor analysis of variance (ANOVA) was used for comparison of values, with Scheffe's multiple-range F-test to estimate significance between groups. Values are mean ± S.E.M., N=6 fish per temperature.

Results

The oxidative locomotory muscle of teleost fishes has a mitochondrial content and capillary supply similar to that found in mammalian myocardium. Trout red muscle at all three temperatures reflects this highly aerobic appearance, with extensive intracellular lipid deposits and numerous capillaries being prominent features (Fig. 1). The capillary supply to trout slow muscle changed with season in two ways. The capillary

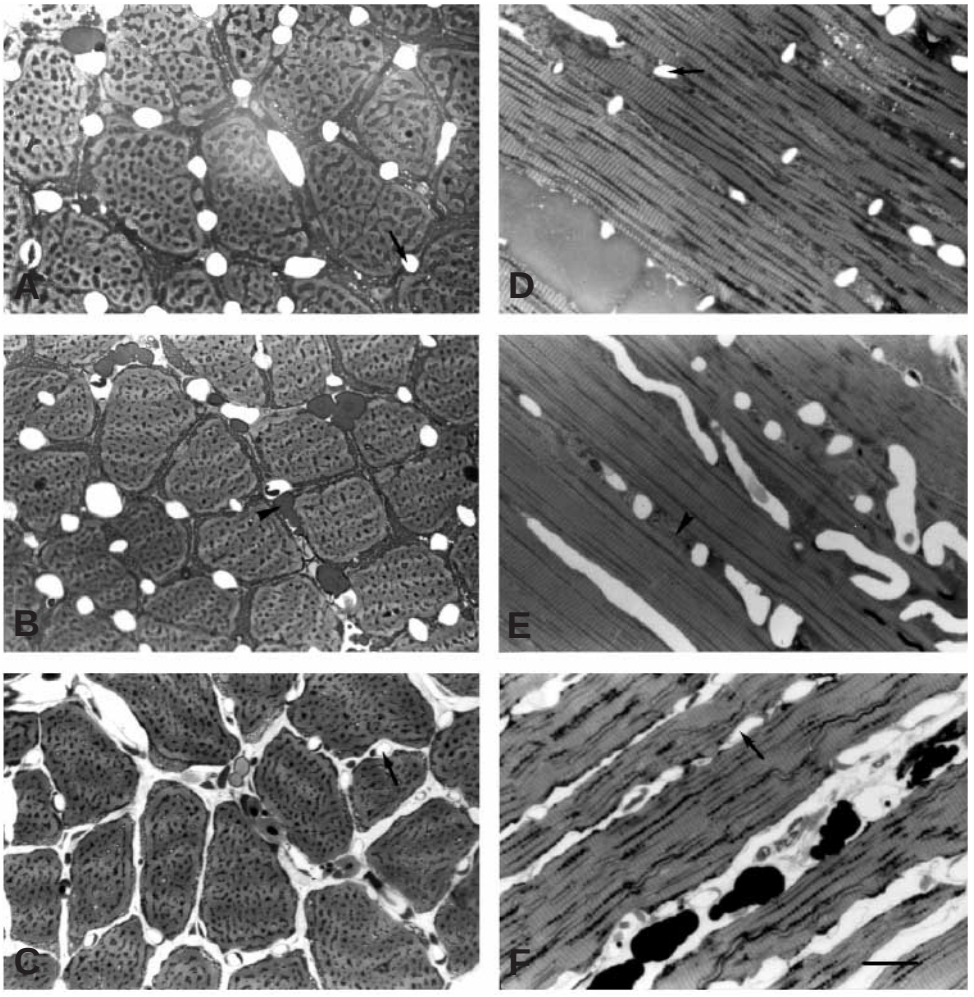

Fig. 1. Photomicrographs of semithin resin-embedded sections from slow muscle of rainbow trout acclimatized to (A,D) 4 °C, (B,E) 11 °C and (C,F) 18 °C viewed in transverse section (TS) (A,B,C) and longitudinal section (LS) (D,E,F). Note the high density of mitochondria (dots inside fibres), lipid (arrowheads) and capillaries (arrows) in TS and the modest numbers of lateral capillary profiles in LS. Scale bar, 26 μm.

to fibre ratio, C:F, was inversely related to environmental temperature (ANOVA, P<0.001), rising from 1.73±0.09 at 18 °C to 2.50±0.15 at 4 °C (Fig. 2). Cold-induced angiogenesis was accompanied by a non-linear fibre hypertrophy which was only apparent in the coldest group (949±91 μm^2 at 11 °C compared with 1539±69 μm^2 at 4 °C), so that capillary density, N_A(c,f), was significantly higher in the mid range of temperatures (2328±229 mm^{-2} at 11 °C) than for either extreme (ANOVA, P<0.001). Although some increase in slow muscle mass may be attributed to hyperplasia, which would lead to a decrease in C:F, this was not a major factor as the distribution of fibre size was not markedly different among groups (Fig. 1).

The transport capacity of the microcirculation depends not only on the density of capillaries in muscle cross sections, but also on the additional length available for diffusive exchange by means of their sinuous arrangement along the fibre axis, and branching of new capillaries (Fig. 1). The underlying growth process results in a modest increase in the concentration parameter, K, at 11 °C, which suggests that the tortuosity of the capillary bed is lowest at the point where N_A(c,f) is highest. This indicates that newly formed capillaries have a preferential orientation along the muscle fibre axis, rather than forming lateral branches or anastomoses. However, the non-linear relationship between K and the constant of proportionality, $c(K,0)$, means that there is little net effect on the capillary length density, J_V(c,f), the magnitude of which, therefore, also decreases in the order 11 °C>18 °C>4 °C (Table 1). However, the increased mass of slow muscle during the winter (Taylor *et al.* 1996) suggests that the total capillary length was similar at both 4 °C and 11 °C.

Although ventricular mass is also significantly increased in the cold (Taylor *et al.* 1996), there was no change in myocyte diameter, which averaged 19–21 μm (Table 2). In contrast to skeletal muscle, therefore, myocardial N_A(c,f) was

Table 1. *Calculation of capillary anisotropy in trout slow muscle at different seasonal temperatures*

	4 °C	11 °C	18 °C
K	3.47±0.48	4.57±0.40	3.28±0.29*
$c(K,0)$	1.06	1.04	1.07
J_V(c,f) (mm^{-2})	1723±76.3*	2421±239	2009±94.4

Values are means ± S.E.M., N=6.

*Significantly different from values at 11 °C (P<0.05).

K, concentration parameter; $c(K,0)$, proportionality constant; $J_V = N_A \times c(K,0)$, where N_A is the capillary count per unit area of tissue.

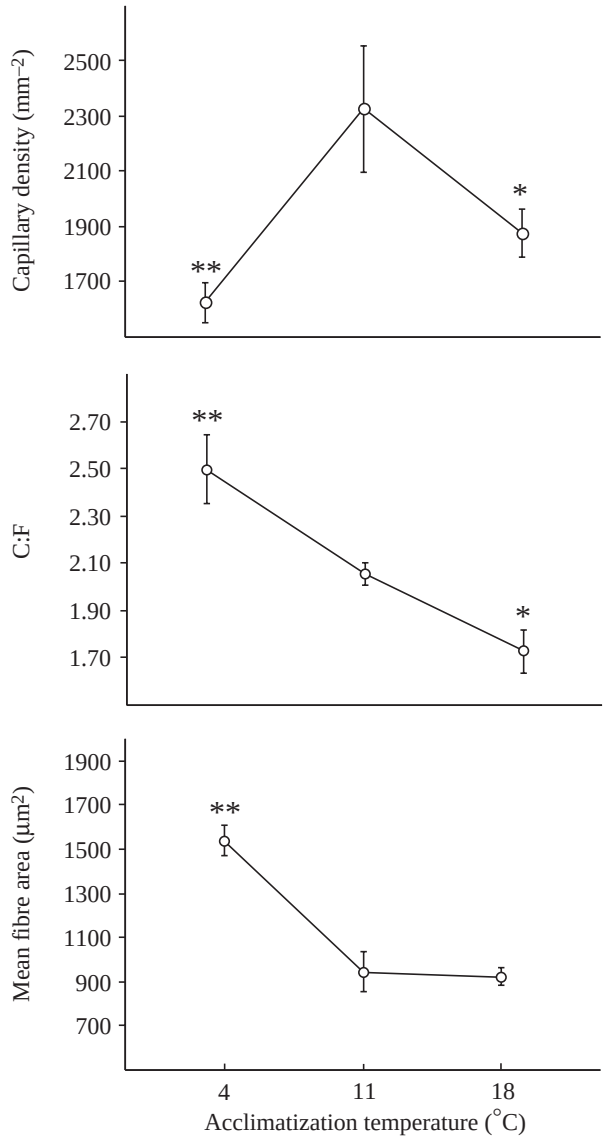

Fig. 2. Capillary density, capillary to fibre ratio (C:F) and mean fibre area of rainbow trout slow muscle sampled over the seasonal range of environmental temperature. Values are mean ± S.E.M., N=6 fish per temperature. Asterisks denote values significantly different from values at 11 °C (*P<0.05; **P<0.01).

significantly higher in summer than in either autumn or winter animals, being 2178 mm^{-2} for 18 °C trout, 1194 mm^{-2} for 11 °C trout and 1371 mm^{-2} for 18 °C trout (ANOVA, P<0.001).

Discussion

Slow muscle C:F shows a clear, inverse relationship with environmental temperature in seasonally acclimatized trout. Capillary supply usually reflects fibre size, such that during development the increase in diameter of fibres appears to stimulate angiogenesis and, hence, to increase C:F, although as the rate of fibre growth often exceeds that of capillaries their density tends to decrease (Egginton, 1990). Fish are unusual in that both hyperplasia and hypertrophy play an important role in postnatal growth of skeletal muscle. Clearly, any difference in the relative contribution of these processes at different temperatures may have a significant effect; e.g. a lack of hyperplasia at 4 °C would lead to a greater C:F than would otherwise be the case. However, the histological profile (Fig. 1) suggests that both mechanisms underlie the increase in slow muscle mass reported earlier (Taylor *et al.* 1996), as was also found in striped bass (Egginton and Sidell, 1989). This graded angiogenic response may reflect a number of changes accompanying seasonal alterations in water temperature, such as growth and activity. The humoral changes that accompany the parallel decrease in photoperiod which may stimulate metabolism and, hence, might be expected to drive an increased capillary supply (e.g. plasma levels of growth hormone, thyroid hormones and catecholamines) tend to be depressed with reduced day length or temperature (Prosser *et al.* 1991; McCormick *et al.* 1995; Senthilkumaran and Joy, 1995). In contrast, the capillary endothelium is exposed to an elevated luminal shear stress at low temperatures, because of the greater blood viscosity and erythrocyte rigidity, which are known to stimulate the growth of capillaries in mammalian skeletal muscle (Hudlická *et al.* 1992). Interestingly, the angiogenic stimulus is inadequate to compensate for fibre hypertrophy in winter fish, which leads to a reduction in capillary density and, hence, to a reduced physical capacity to accommodate an increased blood flow during activity.

Table 2. *Composition of the compact myocardium in trout sampled at different seasonal temperatures*

	4 °C	11 °C	18 °C
Myocyte diameter (μm)	20.1±1.07	18.8±0.81	20.9±0.91
Capillary density (mm^{-2})	1371±36*	1194±178*	2178±105

Values are means ± S.E.M., N=6.
*Significantly different from values at 18 °C (P<0.001).

The potential for sustained aerobic locomotion will be determined, in part, by the ability of the cardiac pump to drive convective transport and by the efficiency of oxygen delivery to working muscle, as determined by the size of the capillary bed. These two factors may be related. For example, no adaptation in blood rheology was found that would compensate for impaired transport at low temperatures (Lecklin *et al.* 1995), suggesting that excessively high cardiac afterload resulting from increased blood viscosity is avoided by decreasing peripheral resistance in the cold. Although vascular proliferation is an obvious candidate, and an increased capillary supply has the additional advantage of maintaining oxygen transport to working muscle when diffusivity is impaired (Hoofd and Egginton, 1997), any increase in structure probably involves a large investment of energy which needs to be balanced against the cyclical nature of the demand. In addition, regulation of vascular tone in the coeliacomesenteric circulation can potentially compensate for changes in systemic

vascular resistance caused by temperature changes or exercise (Randall and Daxboeck, 1982; Thorarensen *et al.* 1993). More importantly, the efficiency of vascular perfusion will be limited by the reduced scope for cardiac work in the cold (Taylor *et al.* 1996). It may then not be possible to compensate effectively for fibre hypertrophy by greater expansion of the capillary supply to skeletal muscle while also avoiding the hypotension that would result from underperfusion of the capillary bed. At high temperatures, when the viscosity of biological fluids is reduced and the intracellular diffusivity of oxygen is increased, the local capillary density is less critical than in the cold (Hoofd and Egginton, 1997), leading to a reduced pressure for vessel growth. This leads to the interesting phenomenon of initial cooling (18–11 °C) initiating angiogenesis (increase in C:F) that results in an increase in effective capillary supply (increased capillary density). Although further capillary growth is found between 11 and 4 °C, the mechanical stimulus is actually reduced in line with decreasing cardiac output and muscle blood flow (Taylor *et al.* 1996). Hence, the size of the capillary bed is maintained, rather than increased, during the winter. It would be of interest to determine whether a similar phenomenon occurs in cyprinids, where two-point acclimation experiments have revealed a tremendous increase in C:F in response to cold exposure (Johnston, 1982).

Salmonids are routinely active fishes, maintaining station in rivers or actively migrating, which requires a highly aerobic red musculature that is well supplied with capillaries; for example, in Australian salmon *Arripis trutta*, C:F=1.9 (Mosse, 1979), which is similar to that for rainbow trout at 11 °C (C:F=2.1). The greatest reported aerobic capacity in fish red muscle is found in tuna *Katsuwonus pelamis*, where the average number of capillaries around a fibre is almost five (Mathieu-Costello *et al.* 1996), compared with little more than three in brook trout *Salvelinus fontinalis* (Johnston and Moon, 1980). In addition to simply packing more capillaries next to fibres, tuna also make use of a combination of scaling and topology to maximise their effective capillary supply. Although C:F is similar for tuna (1.6) and rainbow trout at 11 °C, red fibres of tuna are only half the size and hence N_A(c,f) is almost doubled, at 3391 mm^{-2} (Mathieu-Costello *et al.* 1996). The contributions to total capillary length of tortuosity and branching in rainbow trout are slightly higher than that found previously in the less active conger eel *Conger conger*, $c(K,0)$=1.01 (Egginton and Johnston, 1983), but lower than that of the more aerobic tuna, $c(K,0)$=1.44 (Mathieu-Costello *et al.* 1996). The efficacy of potential oxygen supply to maximal demand can then be estimated as the length of capillaries supplying a given volume of mitochondria. This is 10.4 km ml^{-1} for rainbow trout at 11 °C (this study) and 14.5 km ml^{-1} for tuna (Mathieu-Costello *et al.* 1996), compared with only 2.8 km ml^{-1} for the conger eel (Egginton and Johnston, 1983). In contrast, there are only modest differences in tissue demand for oxygen as reflected in mitochondrial volume densities of 22.7 % for eel, 23.3 % for trout and 28.5 % for tuna. Interestingly, seasonal acclimatization minimised the differences among groups, with 18 °C- and 4 °C-acclimatized rainbow trout having values of capillary length/mitochondrial volume only 23 and 9 % less than those for 11 °C trout (8 and 9.5 km ml^{-1}), respectively. If peripheral oxygen supply were the only factor determining the level of aerobic activity, a similar range in muscle performance ought then to be seen between seasons. However, there was a 40–50 % reduction in sustainable swimming speed and an 80–90 % lower blood flow during exercise at 18 ° and 4 °C (Taylor *et al.* 1996). Although the contribution of the resistance vessels (arterioles) to impaired performance is unknown, central cardiovascular mechanisms must play a key role in limiting such activity at both seasonal extremes of temperature.

The lack of a similar angiogenic response to cold in cardiac muscle suggests that the angiogenic stimulus is tissue-dependent. Cold-induced cardiac hypertrophy in trout (Taylor *et al.* 1996) probably occurs in response to the greater afterload imposed by increased blood viscosity, and primarily reflects growth of the spongy rather than the compact myocardium during the winter months (Farrell *et al.* 1988). So, unlike skeletal muscle, the coronary microcirculation supplies myocytes of a similar size at all times of the year and is not responding to temperature changes in a scale-dependent manner. Cardiac performance was impaired at both 4 and 18 °C (Taylor *et al.* 1996) and while the low N_A(c,f), and hence coronary blood flow, may be limiting in winter, other temperature-dependent effects probably explain the poor cardiac performance at high temperatures. In particular, the high values for N_A(c,f) in summer fish suggest that it is utilisation, rather than supply, of oxygen that is compromised. Indeed, Poupa *et al.* (1974) found no difference in activity of cytochrome oxidase in compact myocardium following warm acclimation, which would provide an inadequate metabolic flux at the higher temperature, while it was actually lower in the spongy myocardium. The stimulus for myocardial angiogenesis at high temperatures may also reflect an increased luminal shear stress, as the reduced efficacy of adrenergic vasoconstriction of the coronary circulation in these animals (Farrell, 1987) would result in a sustained hyperaemia. In addition, an elevated contraction frequency in mammalian myocardium leads to the local release of adenosine, which is both a potent vasodilator and an endothelial cell mitogen (Hudlická *et al.* 1992). Coupled with the increased mechanical deformation associated with an increased heart rate, this is likely to provide a powerful angiogenic stimulus in the hearts of summer-acclimatized trout.

We conclude that the capillary supply is limiting for blood flow in slow skeletal muscle and hence that seasonal swimming performance is directly related to N_A(c,f). This is not so for the myocardium since depressed cardiac output at high temperatures occurs when capillary density is greatest.

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