The Journal of Experimental Biology 206, 411-421

doi:10.1242/jeb.00088



# Muscle fine structure may maintain the function of oxidative fibres in haemoglobinless Antarctic fishes

K. M. O'Brien[1,*], C. Skilbeck[2], B. D. Sidell[1] and S. Egginton[2,†]

[1]*School of Marine Sciences, University of Maine, Orono, ME 04469, USA* and [2]*Department of Physiology, University of Birmingham, B15 2TT, UK*

*Present address: Department of Molecular, Cellular and Developmental Biology, University of Colorado at Boulder, Boulder, CO 80309, USA
†Author for correspondence (e-mail: s.egginton@bham.ac.uk)

*Accepted 21 October 2002*

**Summary**

**Muscle fine structure and metabolism were examined in four species of Antarctic fishes that vary in their expression of haemoglobin (Hb). To determine how locomotory pectoral muscles maintain function, metabolic capacity, capillary supply and fibre ultrastructure were examined in two nototheniid species that express Hb (*Notothenia coriiceps* and *Gobionotothen gibberifrons*) and two species of channichthyid icefish that lack Hb (*Chaenocephalus aceratus* and *Chionodraco rastrospinosus*). Surprisingly, icefish have higher densities of mitochondria than red-blooded species (*C. aceratus*, 53±3% of cell volume; *C. rastrospinosus*, 39±3%; *N. coriiceps*, 29±3%; *G. gibberifrons*, 25±1%). Despite higher mitochondrial densities the aerobic metabolic capacities per g wet mass, estimated from measurements of maximal activities of key metabolic enzymes, are lower in icefish compared to red-blooded species. This apparent incongruity can be explained by the significantly lower mitochondrial cristae surface area per unit mitochondrion volume in icefishes (*C. aceratus*, 20.8±1.6 $\mu m^{-1}$; *C. rastrospinosus*, 25.5±1.8 $\mu m^{-1}$) compared to red-blooded species (*N. coriiceps*, 33.6±3.0 $\mu m^{-1}$; *G. gibberifrons*, 37.7±3.6 $\mu m^{-1}$). Consequently, the cristae surface area per unit muscle mass is conserved at approximately 9 $m^2 g^{-1}$. Although high mitochondrial densities in icefish muscle do not enhance aerobic metabolic capacity, they may facilitate intracellular oxygen movement because oxygen is more soluble in lipid, including the hydrocarbon core of intracellular membrane systems, than in aqueous cytoplasm. This may be particularly vital in icefish, which have larger oxidative muscle fibres compared to red-blooded nototheniods (*C. aceratus*, 2932±428 $\mu m^2$; *C. rastrospinosus*, 9352±318 $\mu m^2$; *N. coriiceps*, 1843±312 $\mu m^2$; *G. gibberifrons*, 2103±194 $\mu m^2$). These large fibres contribute to a relatively low capillary density, which is partially compensated for in icefish by a high index of tortuosity in the capillary bed (*C. aceratus*=1.4, *N. coriiceps*=1.1).**

Key words: muscle, haemoglobin, Antarctic icefish, capillary supply, metabolic enzyme, lipid, mitochondria.

## Introduction

The fish fauna of Antarctic waters are dominated by the suborder Notothenioidei, both in species number and biomass (Eastman, 1993). Within this suborder there are seven families composed of 120 species, representing nearly 35% of the total number of fish species living in the Southern Ocean. Most notothenioids are demersal fishes and all use a labriform style of swimming powered by large pectoral fins, a ubiquitous form of locomotion presumably because the ancestral notothenioid stock used this swimming style (Eastman, 1993). In general, Antarctic fishes are not particularly active and swim at approximately 1–2 *SL* $s^{-1}$, while many species spend large amounts of time resting on pelvic and anal fins on the substrate (Montgomery and Macdonald, 1984; Archer and Johnston, 1989). Labriform sculling provides a high degree of maneuverability and may be the most efficient form of swimming at these slow speeds (Blake, 1979), possibly conferring a selective advantage at cold temperatures. Although notothenioids tend to have reduced axial muscles compared to temperate species, some do employ sub-carangiform swimming powered by glycolytic axial muscle at higher swimming speeds (4–5 *SL* $s^{-1}$) (Montgomery and Macdonald, 1984; Archer and Johnston, 1989). Notothenioids also have a small amount of oxidative myotomal muscle that provides direction during swimming (Archer and Johnston, 1989).

There are two types of labriform swimming: drag-based and lift-based. Pectoral fins move perpendicular to the flow during the power stroke of drag-based swimming, while in lift-based swimming, fins move up and down like bird wings (Lindsey, 1978). Notothenioids employ drag-based labriform locomotion, controlled by six muscles of the pectoral fin (Johnston, 1989). The power stroke is produced by the

pectoral adductor profundus, which constitutes approximately 3% of body mass. This muscle is composed of 80% oxidative fibres, which contain large densities of mitochondria and aerobically poised enzymes, and a rich capillary network (Walesby and Johnston, 1980; Johnston and Harrison, 1985; Dunn et al., 1989). Despite its high aerobic capacity and role in powering sustained swimming, pectoral muscle does not express the oxygen-binding protein myoglobin (Mb), which in other vertebrates both stores and facilitates the diffusion of oxygen within aerobic muscle (Wittenberg and Wittenberg, 1989). 25 species from five families of notothenioids have been examined and all lack Mb in the oxidative skeletal muscle of the pectoral girdle (Moylan and Sidell, 2000; and T. J. Moylen and B. D. Sidell, unpublished observation).

One family of Antarctic fishes, the Channichthyidae, lack both Mb in pectoral muscles and the circulating oxygen-binding protein haemoglobin (Hb). Lack of Hb gives channicthyids a translucent, ice-like appearance that inspired their common name of icefish. Despite having an oxygen carrying capacity nearly an order of magnitude lower than that of red-blooded notothenioids (Egginton, 1994), icefish have similar oxygen consumption rates and activity patterns to their red-blooded relatives of the family Nototheniidae (Ruud, 1954; Holeton, 1970).

We sought to understand how the aerobic pectoral locomotory muscles maintain function despite the lack of facilitated transport by Hb in icefish. We chose species for this study that are evolutionarily closely related, and all lack Mb expression; they only differ in their expression of Hb. We examined differences in ultrastructure and capillarity of the pectoral adductor muscle between two benthic/epibenthic nototheniids (*Notothenia coriiceps* and *Gobionotothen gibberifrons*) and two benthic/epibenthic channichthyids (*Chaenocephalus aceratus* and *Chionodraco rastrospinosus*). We also estimated key enzyme activities within these four species to determine if loss of oxygen-binding proteins has reduced oxidative metabolic capacity.

## Materials and methods

### *Animals*

The nototheniids *Notothenia coriiceps* Richardson (mass 629±66 g, length 37±3.8 cm) and *Gobionotothen gibberifrons* Lönnberg (480±33 g, 35±0.8 cm), and the channichthyids *Chaenocephalus aceratu*s Lönnberg (852±75 g, 88±7.8 cm) and *Chionodraco rastrospinosus* Dewitt and Hureau (557±24 g, 40±0.5 cm), were caught by trammel net fished from small boats supporting the British Antarctic Survey base at Signy Island (64°43′S, 45°26′W) or Otter trawl deployed from the *R/V Polar Duke* in Dallman Bay in the vicinity of Astrolabe Needle (64°10′S, 62°35′W). Animals were maintained in tanks with circulating seawater at 0±0.5°C until samples were collected. Prior to dissection and collection of samples, fish were killed by a stunning blow to the head, followed by spinal transection.

### *Tissue preparation for microscopy*

The oxidative pectoral muscle, m. adductor profundus, was fixed while pinned at resting length to strengthened cork strips (*N. coriiceps* and *C. aceratus*), or while attached to the underlying cartilage (*C. rastrospinosus*). Tissue was fixed in an ice-cold buffered glutaraldehyde/paraformaldehyde solution containing 0.05% sodium azide (Egginton and Cordiner, 1994) or a solution of 3% glutaraldehyde, 0.1 mol $l^{-1}$ sodium cacodylate, 0.11 mol $l^{-1}$ sucrose and 2 mmol $l^{-1}$ $CaCl_2$, pH 7.4, of similar osmolality. Samples were then trimmed into blocks with a cut face of approx. 1 $mm^2$ and post-fixed in buffered 1% $OsO_4$ for 1–2 h. Blocks were cut from the outer edge of the tissue, where the fixative had penetrated well, and care was taken to avoid areas that appeared damaged. Samples were subsequently dehydrated and embedded in epoxy resin (Araldite) under vacuum. 2–4 blocks were prepared from each individual (5–6 individuals per species), and one was chosen at random for subsequent analysis. In addition to the present measurements of capillary size and cristae surface density, indices of capillarity and tissue ultrastructure for *G. gibberifrons* were taken from Londraville and Sidell (1990a,b).

### *Light microscopy*

Semi-thin (0.5–1.5 μm) sections were stained with 1% Toluidine Blue to orient the blocks for true transverse (TS) or longitudinal (LS) sections of muscle fibres. Parameters of capillary supply were calculated from samples viewed at a magnification of ×500 (*N. coriiceps*, *C. aceratus*) or ×400 (*C. rastrospinosus*) using an unbiased sampling rule (the 'forbidden line' procedure; Egginton, 1990). Mean fibre area, $\bar{A}_f$, capillary to fibre ratio, C:F, and numerical density of capillaries, $N_A$(c,f), were calculated from digitized images using in-house software or SigmaScan (Jandel). Mean fibre area was calculated from measurements of a minimum of 20 fibres per section; C:F and $N_A$(c,f) were measured using a square-lattice test pattern with an interval spacing equal to 25–37.5 μm (sample area of 0.051–0.079 $mm^2$). The test pattern was place on a digitising tablet and viewed, along with the sections through a light microscope fitted with a drawing tube. Between two and four non-overlapping regions were sampled from each section. Capillary tortuosity, $c$(K,0), i.e. the deviation from an anisotropic orientation of capillaries with respect to the long axis of muscle fibres, was estimated from the ratio of unbiased capillary counts in LS and TS, assuming a spherical-normal distribution (Egginton, 1990). We also calculated the capillary length density, $J$v(c,f), which is a measure of the length of capillaries per unit volume of tissue and accounts for the degree of tortuosity in the capillary bed, estimated as $c$(K,0) × $N_A$(c,f). The maximum diffusion distance, also known as Krogh's radius ($Kr$), is related to capillary length density [$J$v(c,f)] as: $Kr=1/[\pi J\text{v(c,f)}]^{1/2}$ (Weibel, 1984). Capillary volume and surface densities were calculated from the product of $J$v(c,f) and capillary cross sectional area and perimeter, respectively.

### *Electron microscopy*

Ultrathin (approx. 80 nm) sections were double-stained with 2% uranyl acetate followed by 0.5% lead citrate, or methanoic uranyl acetate (30%) and aqueous lead tartrate (2%). Electron micrographs were taken at an accelerating voltage of 60 kV using the systematic area-weighted subsampling method (Cruz-Orive and Weibel, 1981). 10–12 micrographs per block were analysed at a final magnification of ×8500–15 750. Surface area of the capillary, $\bar{a}$(c), and the boundary length of the capillary, $\bar{b}$(c), were measured by projection onto a digitising tablet. 5–20 capillaries were measured from each individual. The volume density (volume of cellular components per unit volume of fibre) of mitochondria, *V*v(mit,f), and intracellular lipid droplets, *V*v(lip,f), were quantified using point-counting and a square lattice test grid with a spacing (*d*) equal to 0.8–1.65 μm on the projected image (Weibel, 1979).

Cristae surface density (surface area of inner-mitochondrial membranes per volume of mitochondria), *S*v(cs,mit), was estimated from 10–12 micrographs taken at ×36 000–39 000, as described previously (Egginton and Sidell, 1989; O'Brien and Sidell, 2000). The field of view was chosen using the systematic-area-weighted-quadrats subsampling method (Cruz-Orive and Weibel, 1981), and the mitochondria most clearly viewed in cross-section were photographed. *S*v(cs,mit) was calculated using the line-intercept method with a square lattice test pattern overlaid on top of projected micrographs at a final magnification of ×72 000 (*d*=0.10 μm; *N. coriiceps* and *C. aceratus*) or ×91,000 (*d*=0.11 μm; *C. rastrospinosus*). In addition, *S*v(cs,mit) from *G. gibberifrons* was analyzed from material previously collected by Londraville and Sidell (1990a), using the same sampling method, with sections re-stained for best clarity and photographic prints of mitochondria (×81 000; *d*=0.12 μm). There was no significant difference between measurements of *S*v(cs,mit) made on projected micrographs and printed micrographs of mitochondria from *C. rastrospinosus* (O'Brien and Sidell, 2000). Mean free sarcoplasmic spacing was also calculated as $\lambda_a=[4(1–Vv)/Sv]$ (Egginton et al., 1988). Mitochondrial cristae surface densities per gram of tissue were calculated using a value of 1.055 g $cm^3$ for the density of muscle (Webb, 1990).

### *Enzyme analysis*

Measurements of maximal activities of phosphofructokinase (PFK), carnitine palmitoyltransferase I (CPT-I) and cytochrome oxidase (CO) require fresh tissue and were performed shortly after tissue dissections. Tissues used for measuring the maximal activities of citrate synthase (CS), β-hydroxyacyl-CoA-dehydrogenase (HOAD), pyruvate kinase (PK) and lactate dehydrogenase (LDH) were quickly frozen in liquid nitrogen, and stored at –70°C until assays were completed. Maximal activity of hexokinase (HK) measured on both fresh and frozen tissues after control experiments determined that this enzyme was stable to freezing. Measurements were made using a Perkin-Elmer Lambda 6 spectrophotometer equipped with a six-carriage cuvette carrier chilled with a circulating and refrigerated water bath. Assays were performed in triplicate at 1±0.5°C.

Tissue was homogenized in a 10% w/v ice-cold buffer (40 mmol $l^{-1}$ Hepes, 1 mmol $l^{-1}$ EDTA, 2 mmol $l^{-1}$ $MgCl_2$, pH 7.8 at 1°C), except for tissues used in CO and CPT-I assays (see below). Dithiothreitol (DTT) was added to a final concentration of 2 mmol $l^{-1}$ in buffer for PFK, LDH and HK assays. Tissue was first minced on a chilled stage, then homogenized with two 10–15 s bursts of a Tekmar Tissuemizer, keeping the homogenate on ice between bursts. Homogenisation was completed by hand using a Tenbroeck ground glass homogeniser.

Background enzyme activities were measured in the absence of initiating substrate and subtracted from total activity in the presence of substrate. Maximal activities were determined by measuring the rate of oxidation or reduction of pyridine nucleotides at 340 nm for 5 min, except when noted differently. Details of assay conditions are described below.

#### *Phosphofructokinase (PFK, EC 2.7.1.11)*

The assay used was modified from that described by Opie and Newsholme (1967) and Read et al. (1977). The final reaction mixture contained 7 mmol $l^{-1}$ $MgCl_2$, 200 mmol $l^{-1}$ KCl, 1 mmol $l^{-1}$ KCN, 2 mmol $l^{-1}$ AMP, 0.15 mmol $l^{-1}$ NADH, 2 mmol $l^{-1}$ ATP, 4 mmol $l^{-1}$ fructose 6-phosphate (F6P), 2 U $ml^{-1}$ aldolase, 10 U $ml^{-1}$ triosephosphate isomerase, 2 U $ml^{-1}$ glycerol-3-phosphate dehydrogenase, 75 mmol $l^{-1}$ triethanolamine, pH 8.4 at 1°C. Reactions were initiated by addition of a mixture of ATP and F6P.

#### *Lactate dehydrogenase (LDH, EC 1.1.1.27)*

Procedure for this assay was that described by Hansen and Sidell (1983). The final reaction mixture contained 5 mmol $l^{-1}$ pyruvate, 0.15 mmol $l^{-1}$ NADH, 1 mmol $l^{-1}$ KCN, 50 mmol $l^{-1}$ imidazole, pH 7.7 at 1°C. Reactions were initiated by addition of pyruvate.

#### *Pyruvate kinase (PK, EC 2.7.1.40)*

This assay was conducted as described by Hansen and Sidell (1983). The final reaction mixture contained 150 mmol $l^{-1}$ KCl, 1 mmol $l^{-1}$ KCN, 10 mmol $l^{-1}$ $MgSO_4$, 0.15 mmol $l^{-1}$ NADH, 5 mmol $l^{-1}$ ADP, 2.5 mmol $l^{-1}$ phosphoenol pyruvate (PEP), 10 U $ml^{-1}$ LDH, 50 mmol $l^{-1}$ imidazole, pH 7.1 at 1°C. Reactions were initiated by addition of PEP.

#### *3-hydroxyacyl CoA dehydrogenase (HOAD, EC 1.1.1.35)*

The methods for this assay were first described by Beenakkers et al. (1967) and subsequently modified by Hansen and Sidell (1983). The final reaction mixture contained 1 mmol $l^{-1}$ EDTA, 1 mmol $l^{-1}$ KCN, 0.15 mmol $l^{-1}$ NADH, 0.1 mmol $l^{-1}$ acetoacetyl CoA, 50 mmol $l^{-1}$ imidazole, pH 7.7 at 1°C. Reactions were initiated by addition of acetoacetyl CoA.

#### *Citrate synthase (CS, EC 4.1.3.7)*

For this assay, we used the protocol described by Srere et al. (1963), with minor changes. The final reaction mixture

Table 1. *Maximal activity of metabolic enzymes from pectoral adductor muscle of Antarctic fishes*

| | *N. coriiceps* | *G. gibberifrons* | *C. aceratus* | *C. rastrospinosus* |
|---|---|---|---|---|
| CO | ND | 28.38±2.06$^{A}$ | 14.82±0.96$^{B}$ | 14.53±1.28$^{B}$ |
| CS | 25.83±1.28$^{B}$ | 21.02±1.50$^{A}$ | 15.38±0.56$^{C}$ | 18.13±1.24$^{C}$ |
| HK | 0 | 0.20±0.04$^{A}$ | 0.28±0.03$^{A}$ | 0.37±0.03$^{B}$ |
| PFK | 1.06±0.06$^{A}$ | 1.48±0.10* | 1.45±0.04$^{B}$ | 0.91±0.06$^{A}$ |
| LDH | 29.87±1.08$^{B}$ | 14.07±0.52$^{A}$ | 32.73±1.66$^{B}$ | 25.27±1.99$^{C}$ |
| PK | 17.43±0.38$^{B}$ | 14.42±0.75$^{A}$ | 13.04±1.03$^{A}$ | 9.85±1.36$^{C}$ |
| CPT-I | 84.52±2.23$^{B}$ | 107.00±5.55$^{A}$ | 55.42±2.56$^{C}$ | 74.41±5.11$^{B}$ |
| HOAD | 2.51±0.11$^{A,B}$ | 2.28±0.07$^{A,C}$ | 2.03±0.07$^{C}$ | 2.70±0.17$^{B}$ |

CO, cytochrome oxidase; CS, citrate synthase; HK, hexokinase; PFK, phosphofructokinase; LDH, lactate dehydrogenase; PK, pyruvate kinase; CPT-I, carnitine palmitoyltransferase I; HOAD, β-hydroxyacyl-CoA-dehydrogenase.

Enzyme activities are expressed as μmol product formed $min^{-1}$ $g^{-1}$ wet mass (U), except for CPT-I (mU).

Values are means ± S.E.M.

Letters designate significant differences among species ($P<0.05$). ND, not determined.

*Crockett and Sidell (1990).

contained 0.25 mmol $l^{-1}$ 5,5′-dithiobis-(2-nitrobenzoic) acid (DTNB), 0.4 mmol $l^{-1}$ acetyl CoA, 0.5 mmol $l^{-1}$ oxaloacetate, 75 mmol $l^{-1}$ Tris-HCl, pH 8.2 at 1°C. Reaction was initiated by addition of oxaloacetate. Progress of the reaction was monitored by following production of the reduced anion of DTNB at 412 nm.

*Hexokinase (HK, EC 2.7.1.1)*

We used the methods described by Zammit and Newsholme (1976) for this assay. The final reaction mixture contained 7.5 mmol $l^{-1}$ $MgCl_2$, 0.8 mmol $l^{-1}$ EDTA, 1.5 mmol $l^{-1}$ KCl, 0.4 mmol $l^{-1}$ NADP, 2.5 mmol $l^{-1}$ ATP, 10.0 mmol $l^{-1}$ creatine $PO_4$, 1.0 mmol $l^{-1}$ α-D-glucose, 0.9 U $ml^{-1}$ creatine phosphokinase, 0.7 U $ml^{-1}$ glucose-6-phosphate dehydrogenase, 75 mmol $l^{-1}$ Tris-HCl, pH 7.6 at 1°C. Reactions were initiated by the addition of glucose.

*Cytochrome oxidase (CO, EC 1.9.3.1)*

The method of Wharton and Tzagoloff (1967) was used to measure activity. Tissue was homogenized in 50 mmol $l^{-1}$ $K_2HPO_4/KH_2PO_4$, 0.05% Triton X-100, pH 7.5. The assay medium consisted of 10 mmol $l^{-1}$ $K_2HPO_4/KH_2PO_4$, 0.65% (w/v) reduced ($Fe^{2+}$) cytochrome *c* and 0.93 mmol $l^{-1}$ $K_3Fe(CN)_6$. Reaction was initiated by the addition of enzyme. Maximal activities were measured by following the oxidation of reduced cytochrome *c* at 550 nm.

*Carnitine palmitoyltransferase-I (CPT-I, EC 2.3.1.21)*

Maximal activities of CPT-I were measured in isolated mitochondria (Rodnick and Sidell, 1994). Tissue was homogenised in 10% w/v of ice-cold 40 mmol $l^{-1}$ Hepes, 10 mmol $l^{-1}$ EDTA, 5 mmol $l^{-1}$ $MgCl_2$, 150 mmol $l^{-1}$ KCl, 35 mmol $l^{-1}$ sucrose and 0.5% BSA, pH 7.27 at 1°C, using a Duall ground glass homogeniser. A sample of crude homogenate was reserved for measuring total CPT activity. Homogenate was centrifuged at 270 ***g*** for 10 min. Supernatant was collected and recentrifuged at 270 ***g***. Supernatant was collected and centrifuged at 15,000 ***g*** for 20 min. The mitochondrial pellet was gently resuspended in homogenisation buffer (–BSA) and centrifuged at 15,000 ***g*** for 20 min. The pellet was gently resuspended in homogenization buffer lacking BSA to a final concentration of approximately 5 μg protein $\mu l^{-1}$. A sample of the mitochondrial suspension was frozen at –70°C for later protein determination using the bicinchoninic acid method (Smith et al., 1985).

The final assay medium consisted of 1.0 mmol $l^{-1}$ EGTA, 220 mmol $l^{-1}$ sucrose, 40 mmol $l^{-1}$ KCl, 0.13% BSA, 0.1 mmol $l^{-1}$ DTNB, 40 μmol $l^{-1}$ palmitoleoyl-CoA, 1 mmol $l^{-1}$ carnitine, 20 mmol $l^{-1}$ Hepes, pH 8.0 at 1°C. Activity was simultaneously measured in six cuvettes. Malonyl-CoA, a known inhibitor of CPT-I, was added to three of the six cuvettes to a final concentration of 10 μmol $l^{-1}$. Reactions were initiated by the addition of carnitine. Maximum activity was measured by following the production of the reduced anion of DTNB at 412 nm. Maximal activities of CPT-I were estimated as the fraction of total activity inhibited in the presence of malonyl-CoA.

*Statistical analysis*

Data were analyzed using factoral ANOVA, with a *post-hoc* Fisher's least-significant-difference test used to assess comparisons among the four species.

## Results

*Metabolic capacities*

Overall, metabolic capacities were not strikingly different among the four species. However, maximal activities of CO and/or CS, two enzymes considered to be good measures of aerobic metabolic capacity, were greater in pectoral muscles of red-blooded species (*N. coriiceps* and *G. gibberifrons*) compared to the icefish (*C. aceratus* and *C. rastrospinosus*) (Table 1).

PFK catalyzes one of the non-equilibrium reactions in

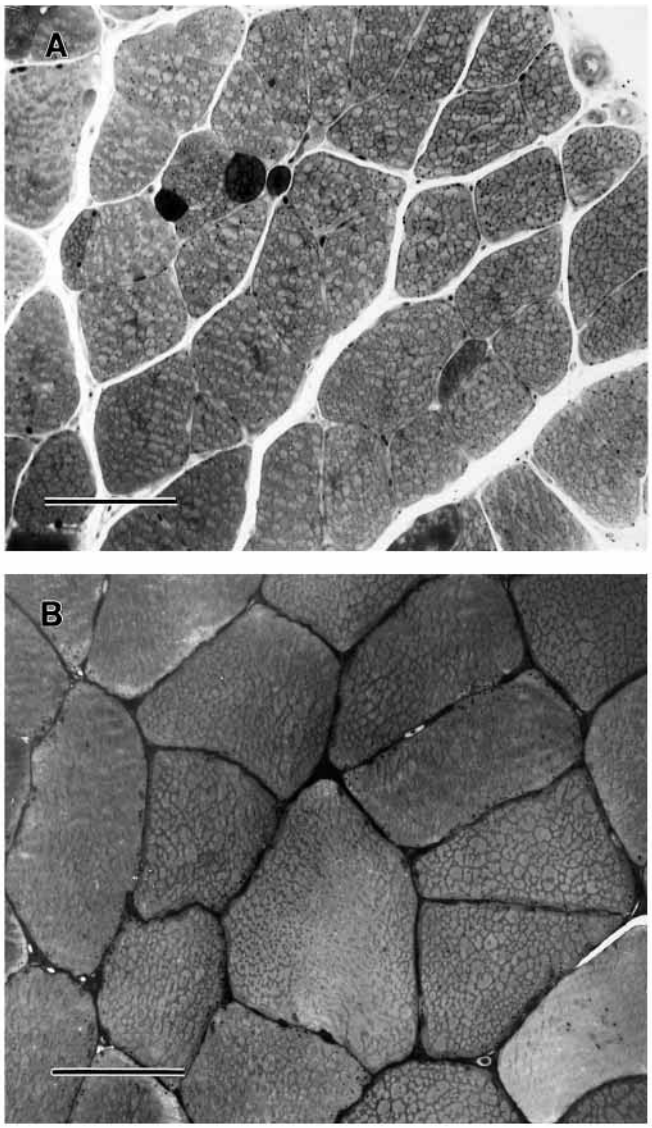


Fig. 1. Semi-thin sections from (A) *G. gibberifrons* and (B) *C. rastrospinosus* pectoral adductor muscle, illustrating the striking difference in fibre cross-sectional area between notothenioids and channichthyids. Scale bars, 100 μm.

glycolysis and measurement of its maximal activity is a good indicator of anaerobic metabolic potential (Crabtree and Newsholme, 1972a). Maximal activities of LDH and PK are also frequently used as indices of anaerobic metabolic capacity. Although the activities of each enzyme differ between red and white-blooded fishes, when all three enzymes are considered together, there is no clear pattern of differences in anaerobic potential between fishes that express Hb and those lacking the protein. Activity of HK, an enzyme generally indicative of capacity for aerobic oxidation of glucose, was relatively low and not significantly different among all species examined, and thus does not correlate with the presence or absence of circulating haemoglobin.

Maximal activity of CPT-I is a good index of potential for β-oxidation of fatty acids (Crabtree and Newsholme, 1972b), and activities were generally higher in red-blooded species compared to icefish. Although there was no significant difference between *N. coriiceps* and *C. rastrospinosus* ($P$=0.10), there were differences among both notothenioids and both channichthyids. In particular, activity of CPT-I was greater in *C. rastrospinosus* compared to *C. aceratus* ($P$=0.004). Despite the substantially greater densities of intracellular lipid droplets in red-blooded notothenioids compared to hemoglobinless channichthyids (cf. Table 3), no compelling parallels were observed in activities of either of the enzymatic markers of β-oxidation capacity, HOAD and CPT-I (Table 1).

### *Capillary and fibre dimensions*

The mean cross-sectional area of oxidative fibres, $\bar{A}_f$, were larger in icefish compared to red-blooded notothenioids (Fig. 1), and fibres from *C. rastrospinosus* were significantly larger than those from *C. aceratus* (Table 2). The capillary-to-fibre ratio (C:F) of all fishes is around one for the notothenioids, which is characteristic of sluggish fishes, but is higher in the channichthyids, and greatest in pectoral muscle from *C. rastrospinosus*. The combination of $\bar{A}_f$ and C:F results in a variable capillary density, $N_A$(c,f), among species, being lowest in *C. rastrospinosus* as a result of its exceptionally large fibre size (Table 2).

Table 2. *Fibre size and capillary dimensions of pectoral adductor muscle from four species of Antarctic fishes*

| | *N. coriiceps* | *G. gibberifrons* | *C. aceratus* | *C. rastrospinosus* |
|---|---|---|---|---|
| $\bar{A}_f$ (μm$^2$) | 1843±312$^A$ | 2103±194$^{A,B,*}$ | 2932±428$^B$ | 9352±318$^C$ |
| $\bar{a}$(c) (μm$^2$) | 30.5±1.2$^A$ | 35.1±1.2$^B$ | 48.3±1.8$^C$ | 26.2±3.0$^D$ |
| $\bar{b}$(c) (μm) | 19.6±0.3$^A$ | 22.4±0.4$^B$ | 24.6±0.6$^C$ | 22.9±1.48$^{B,C}$ |
| C:F | 1.17±0.11$^A$ | 0.91±0.08$^{A,*}$ | 1.49±0.24$^{A,B}$ | 2.65±0.22$^B$ |
| $N_A$(c,f) (mm$^{-2}$) | 819±77$^A$ | 438±9$^{B,*}$ | 621±58$^C$ | 257±18$^D$ |
| $Jv$(c,f) (mm$^{-2}$) | 1057±114$^A$ | 696±34$^B$ | 880±73$^A$ | 367±25$^C$ |

$\bar{A}_f$, cross-sectional area of fibres; $\bar{a}$(c), cross-sectional area of capillaries; $\bar{b}$(c), boundary length of capillaries; C:F, capillary to fibre ratio; $N_A$(c,f), numerical density of capillaries per fibre cross sectional area; $Jv$(c,f); capillary length per fibre volume, assuming $c$(K,0)=1.11 and 1.44 for notothenioids and channichthyids, respectively.

Values are means ± S.E.M.

Letters designate significant differences among species ($P$<0.05).

*Londraville and Sidell (1990a,b).

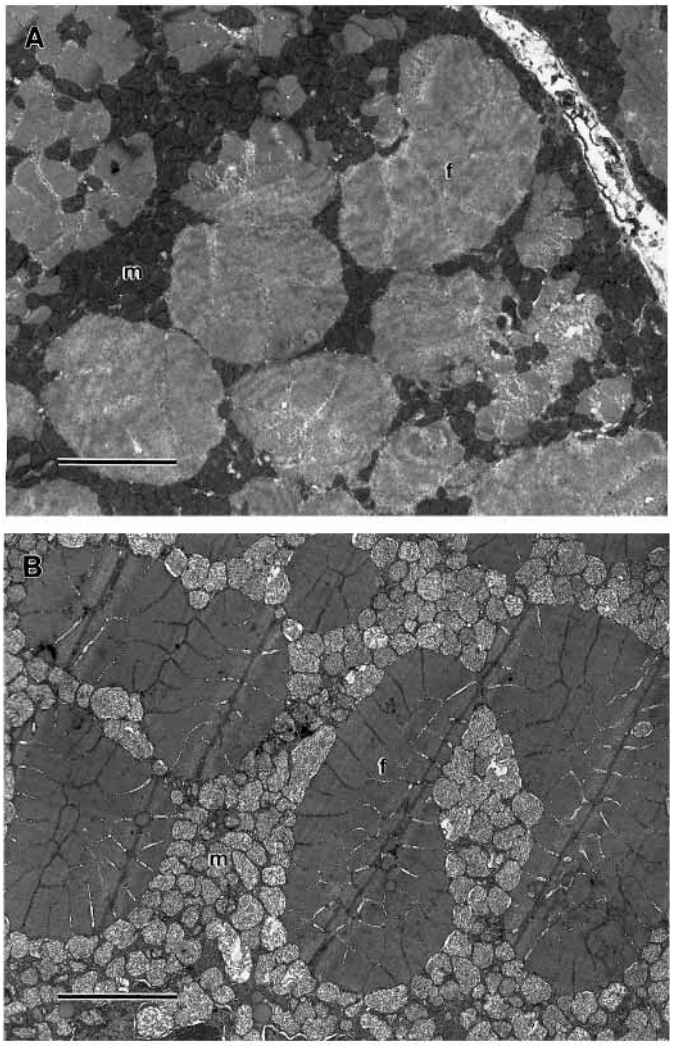


Fig. 2. Ultra-thin sections from (A) *G. gibberifrons* and (B) *C. rastrospinosus* pectoral adductor muscle, illustrating the differences in mitochondrial packing between notothenioids and channichthyids. m, mitochondria; f, myofibrils. Scale bars, 5 μm.

*C. aceratus* had capillaries with large surface areas due to the particularly large bore, which would minimize the pressure against which the heart must work to pump a large volume of blood through the circulatory system. It is surprising, therefore, that individual capillaries are smaller in the other icefish, *C. rastrospinosus*, compared to both *C. aceratus* and the red-blooded notothenioids (Table 2). Few capillaries were viewed in which the major axis measured less than 15% longer than the minor axis in notothenioids, a criterion frequently used for selecting capillaries for measuring cross sectional area, *a*(c) (Mathieu-Costello et al., 1992), suggesting a low degree of anisotropy with respect to the fibre long axis. The capillary bed was indeed more tortuous in icefish [tortuosity index, *c*(K,0)=1.44 compared to red-blooded species, *c*(K,0)=1.11], but the wide range in both fibre size and capillary supply resulted in measurements of capillary length density, *J*v(c,f), that showed no clear pattern among species (Table 2). Values for capillary volume and surface densities (data not shown) were dominated by the effect of *J*v(c,f), and varied in a similar manner among species. Consequently, Krogh's radius *Kr* was also not consistently different between the notothenioids and channichthyids: *N. coriiceps Kr*=17.4±1.02 μm; *G. gibberifrons Kr*=21.4±0.51 μm; *C. aceratus Kr*=19.0±0.79 μm; *C. rastrospinosus Kr*=29.4±1.07 μm ($P$<0.05 among, but not between notothenioids and channichthyids).

### *Fibre ultrastructure*

Mitochondria occupy a larger proportion of cell volume in oxidative muscle from the haemoglobinless channichthyids than in red-blooded notothenioids (Fig. 2), but with a 14% difference between the icefish *C. aceratus* and *C. rastrospinosus* (Table 3). The surface area of inner mitochondrial membranes per unit volume mitochondria (cristae surface density) was similar between *C. aceratus* and *C. rastrospinosus*, and values for both icefish were lower than in *N. coriiceps* and *G. gibberifrons* (Table 3). Little variation was seen in the volume density of intracellular lipid droplets within the two groups, but *V*v(lip,f) was lower in icefish than in the red-blooded species.

The potential consequence of differences in mitochondrial volume density, *V*v(mit,f), and cristae surface density, *S*v(cs,mit), was estimated by calculating mitochondrial cristae surface density per gram of tissue *S*v(cs,m). Results show that complimentary differences in *V*v(mit,f) and *S*v(cs,mit) result in values of *S*v(cs,m) that are nearly equivalent among all species (Table 3). Although *S*v(cs,m) is usually correlated with

Table 3. *Ultrastructural characteristics of pectoral adductor muscle fibres from four species of Antarctic fishes*

| | *N. coriiceps* | *G. gibberifrons* | *C. aceratus* | *C. rastrospinosus* |
|---|---|---|---|---|
| *V*v(mit,f) | 0.287±0.029$^{A}$ | 0.249±0.007$^{A,*}$ | 0.525±0.025$^{B}$ | 0.390±0.030$^{C}$ |
| *V*v(lip,f) | 0.013±0.004$^{A}$ | 0.012±0.004$^{A,*}$ | 0.001±0.001$^{B}$ | 0.004±0.003$^{B}$ |
| *S*v(cs,mit) (μm$^{-1}$) | 33.60±3.02$^{A}$ | 37.68±3.62$^{A}$ | 20.79±1.60$^{B}$ | 25.47±1.81$^{B}$ |
| *S*v(cs,m) (m$^{2}$ g$^{-1}$) | 9.14±0.88$^{A}$ | 8.89±0.85$^{B}$ | 10.91±0.65$^{B}$ | 9.08±0.53$^{A,B}$ |

*V*v(mit,f), volume density of mitochondria; *V*v(lip,f), volume density of lipid droplets; *S*v(cs,mit), cristae surface density; *S*v(cs,m), cristae surface density per g muscle.

Values are means ± S.E.M.

Letters designate significant differences among species ($P$<0.05).

*Londraville and Sidell (1990a).

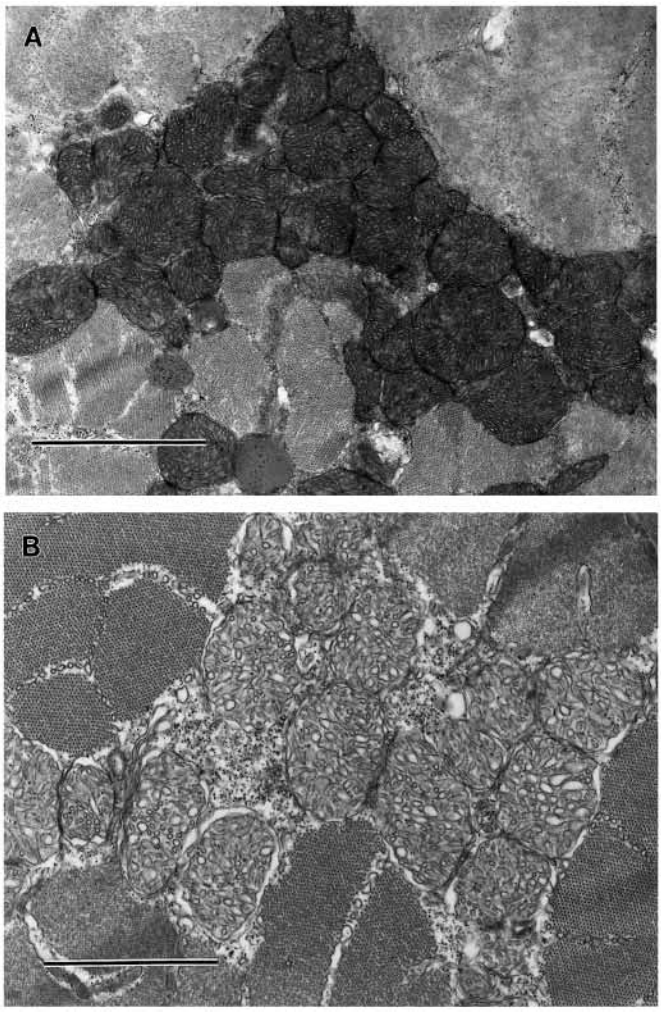


Fig. 3. Ultra-thin sections from (A) *G. gibberifrons* and (B) *C. rastrospinosus* pectoral adductor muscle, showing the greater mitochondrial cristae density in notothenids than channichthyids. Scale bars, 2 μm.

maximal activity of cytochrome oxidase (CO), this is not the case in Antarctic fishes. The specific CO activity per g tissue is greater in *G. gibberifrons* compared to the two icefish (Table 1), but the specific cristae surface area is equal to or less than that of icefish (Fig. 3, Table 3). These data indicate that CO and other components of the electron transport chain may be more densely packed within the mitochondrial cristae of *G. gibberifrons* compared to channichthyids. The greater packing of electron transport proteins may not be reflected in the ratio of cristae surface area to capillary surface area (Fig. 4) as this parameter is heavily influenced by the differences in capillary density, $N_A$(c,f).

## Discussion

We observed differences in the composition of pectoral adductor muscles in Antarctic fishes that correlated with the presence or absence of haemoglobin. Capillary length density, $J_V$(c,f), is considered the best descriptor of blood volume available for exchange of oxygen and metabolites between capillaries and muscle (Hoppeler et al., 1981a). In skeletal muscles there is a positive correlation between capillary density (supply) and mitochondrial density (demand) for mammals (Hoppeler et al., 1981a; Hoppeler and Billeter, 1991) and most fishes (Egginton and Sidell, 1989). This relationship is not observed in the pectoral *adductor profundus* of Antarctic fishes. Although capillary length density varies among the four species studied, with no clear pattern of differences between red and white blooded fishes, the ratio of capillary length density to mitochondrial volume density is consistently higher in red-blooded species than the haemoglobinless fishes (*N. coriiceps*, 36.44; *G. gibberifrons*, 26.52; *C. aceratus*, 16.60; *C. rastrospinosus*, 9.97). Despite this apparent mismatch in supply and demand, icefish have activity levels and oxygen consumption rates similar to red-blooded Antarctic species (Holeton, 1970). By examining cellular structure in icefish muscle we may not only explain this apparent mismatch and but also gain insight into how oxygen is adequately supplied to muscle mitochondria in these unusual fishes.

### *Mitochondrial ultrastructure*

Cristae surface densities, i.e. surface area per unit volume of mitochondrion, are nearly equivalent ($40\,m^2\,cm^{-3}$) among different mammals and muscle types (Hoppeler et al., 1981b; Schwerzmann et al., 1989). As a result, mitochondrial

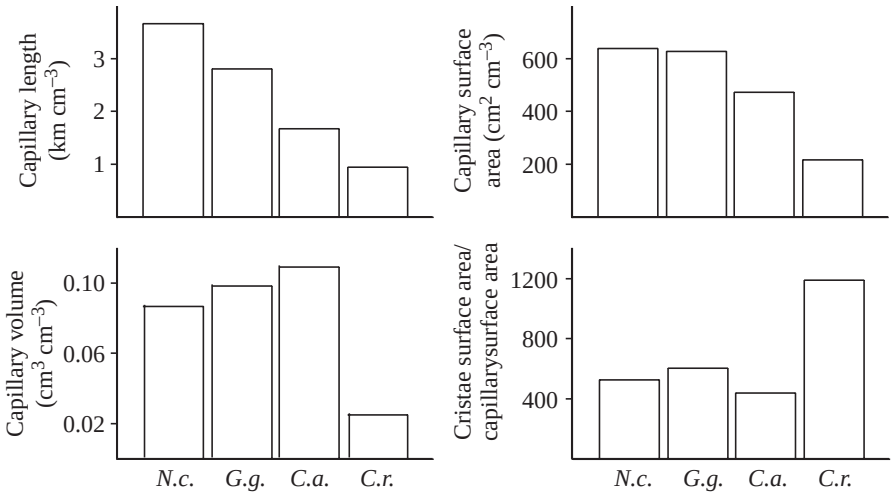


Fig. 4. Calculated ultrastructural and capillary dimensions per $cm^3$ mitochondria of pectoral adductor muscle from Antarctic fishes. *N.c.*, *Notothenia coriiceps; G.g.*, *Gobionotothen gibberifrons; C.a.*, *Chaenocephalus aceratus*; *C.r.*, *Chionodraco rastrospinosus.* Data for *G. gibberifrons* are taken from the present paper and Londraville and Sidell (1990a).

volume densities can be correlated reliably with oxygen consumption rates in mammalian muscle (Hoppeler and Lindstedt, 1985). In contrast, cristae surface densities vary among Antarctic fish species and are lower in icefishes compared to red-blooded fishes in both skeletal and cardiac muscle (Archer and Johnston, 1991; Johnston et al., 1998; O'Brien and Sidell, 2000). Thus, higher $V_V$(mit,f) in icefish muscle may not require correspondingly high densities of capillaries because each mitochondrion has a lower capacity for oxidative metabolism than those from red-blooded species. As a consequence, both cristae density and mitochondrial density must be considered in order to characterize capillary supply requirements of Antarctic fish muscles.

We can normalise for differences in mitochondrial ultrastructure among species by examining the capillary surface area supplying a given unit of mitochondrial inner membrane. The ratio between capillary surface density and cristae surface density is approximately 1:200 in several types of oxidative muscles measured in 13 mammalian species (Hoppeler and Billeter, 1991). This value is much higher than that in the Antarctic fish so far examined: *N. coriiceps* (1:527), *G. gibberifrons* (1:602), *C. aceratus* (1:434) and *C. rastrospinosus* (1:1182) in the present study, and previously (*C. aceratus*, 1:799; Archer and Johnston, 1991). Values calculated for a temperate zone teleost *Morone saxatilis* (1:432; Egginton and Sidell, 1989), are similar to those for the most sluggish species, but lower than for the more active notothenioids. These observations seem counterintuitive. One would expect an equivalent or perhaps even greater capillary surface area per surface area of cristae in icefishes, because their blood has a lower oxygen carrying capacity than that of red-blooded fishes.

Cristae surface densities alone, however, may not be a good measure of oxidative capacity in Antarctic fishes. When two sub-Antarctic species were compared, *Paranotothenia magellanica* had higher mitochondrial cristae surface densities ($45.1\pm4.7\,\mu m^{-1}$) than *Eleginops maclovinus* ($39.2\pm3.9\,\mu m^{-1}$), yet isolated mitochondria from both species had similar rates of oxygen consumption (Johnston et al., 1998). These differences may result from differences in lipid composition of the inner-mitochondrial membranes, catalytic activities of electron transport proteins, or densities of electron transport chain elements.

Maximal activities of cytochrome oxidase (CO) per gram of muscle were greater in red-blooded nototheniids than in icefish, yet the specific surface area of cristae was nearly equivalent. Together, these data indicate that CO and other elements of the electron transport chain may be less densely packed within the inner-mitochondrial membranes of icefish than in red-blooded species, and that lower cristae surface densities per mitochondrion in icefish were compensated by higher volume densities of mitochondria. Similar trends have been observed in cardiac muscle of Antarctic species (O'Brien and Sidell, 2000). Overall, the low density of electron transport proteins within cristae of icefish pectoral muscle suggests that a correspondingly low capillary surface area per unit inner-mitochondrial membrane may match rates of oxygen utilization. This may be a feature of cold-adapted species, e.g. striped bass acclimated to low temperature (5°C) had a lower ratio of capillary:cristae surface densities (1:810) than did individuals acclimated to 25°C (1:432; Egginton and Sidell, 1989).

### *Fibre ultrastructure may maintain $O_2$ flux*

A high density of lipid-rich subcellular structures may help compensate for low capillarity in icefish muscle and may contribute to maintaining oxygen diffusion to mitochondria. Oxygen is more than four times more soluble in non-polar solvents than in water (Battino et al., 1968). The higher solubility of oxygen in lipid than aqueous cytoplasm has led several investigators to hypothesize that intracellular lipid may be an important storage site for oxygen, and a low-resistance pathway for oxygen diffusion (Longmuir, 1980; Ellsworth and Pittman, 1984; Sidell, 1998). For example, the extent of intracellular lipid was inversely correlated with capillary supply in cyprinids (Sänger, 1999), and oxygen flux may be maintained in striped bass at cold temperature by virtue of the greater than 13-fold increase in volume of intracellular lipid droplets that occurs during cold acclimation (Egginton and Sidell, 1989; Hoofd and Egginton, 1997). Evidence is available to suggest that similar apparent mismatches in supply (capillary density) and demand (mitochondria) in Antarctic teleosts also may be overcome by increases in intracellular lipid density (Londraville and Sidell, 1990a). Finally, empirical measurements of oxygen movement through tissues of differing lipid content support this hypothesis, because the diffusion constant for oxygen increases in cold-acclimated striped bass in parallel with increased lipid density (Desaulniers et al., 1996).

Very low densities of intracellular lipid droplets were observed in fibres of *C. aceratus* and *C. rastrospinosus*. However, both of these species have extremely high densities of mitochondria, which increase the amount of lipid-rich intracellular membrane. The hydrocarbon core of biological membranes may function similarly to lipid droplets as an avenue for oxygen diffusion (Longmuir, 1980; Koyama et al., 1990). Three-dimensional analyses of mitochondria have shown that, in muscles containing high densities of mitochondria (>20%), some mitochondria form a continuous reticular structure, with mitochondria connected both longitudinally along the fibre axis and transversely, in regions adjacent to the I-band (Bakeeva et al., 1978; Kayar et al., 1988). In transverse sections of icefish pectoral adductor muscle, myofibrils appear as rosettes surrounded by mitochondria. This membranous network that spans the muscle fibre may be an important mechanism for delivering oxygen to the centre of fibres in the absence of Hb and Mb.

The unusually high densities of mitochondria in icefish oxidative muscle also reduce the diffusion distance that metabolites must travel between the mitochondria and myofibrils, and between mitochondrial clusters. Consequently, the mean diffusion distance between the cytoplasm and

mitochondria $\lambda_a$ was 1.23 μm in *C. aceratus* and 2.89 μm in *N. coriiceps*, the latter being similar to that found in the relatively sluggish goldfish ($\lambda_a$=2.9 μm; Tyler and Sidell, 1984). Similarly, the distance between mitochondrial clusters within icefish fibres is approximately one-half that of red-blooded species (data not shown).

### *Physiological significance of fibre size*

The size of muscle fibres generally increases as body temperature decreases in fishes, both within individuals in response to cold temperature acclimation and among species inhabiting different latitudes (Egginton and Johnston, 1984; Egginton and Sidell, 1989; Rodnick and Sidell, 1997; Johnston et al., 1998). Surprisingly, fibre size is also greater in species lacking haemoglobin (Fitch et al., 1984; Archer and Johnston, 1991; Johnston et al., 1998; this study) compared to red-blooded species. Oxygen delivery to mitochondria within the muscles of Antarctic fishes thus must overcome a two-pronged challenge. Not only is the distance that oxygen must travel to reach the centre of the fibre large, but also the cold body temperature of these animals results in a lower diffusion constant for the gas through a cytoplasm of high viscosity. The situation is further exacerbated by the lack of intracellular oxygen-binding myoglobin to facilitate oxygen diffusion in the skeletal muscles of these species (Moylan and Sidell, 2000). Constraints on intracellular delivery of oxygen seem particularly acute in the channichthyid icefish, which also lack oxygen-binding haemoglobin in their circulation. To avoid failure to meet aerobic demands of the tissue in these animals, one could reasonably expect to find specific mechanisms to enhance oxygen delivery to the tissue, and/or mechanisms that result in reduced oxygen demand. High mitochondrial densities within oxidative muscle of icefishes may serve such a function.

Despite large fibre radii in icefish, high densities of mitochondria are found at the centre of fibres in these species. In fact, mitochondrial densities at the centre of oxidative fibres of *C. aceratus* are equivalent to those nearest the capillary, unlike that observed in *G. gibberifrons*, where mitochondrial densities decline toward the centre of the fibre (Archer and Johnston, 1991). These results suggest oxygen diffusion is not limiting in icefish muscles, and thus, oxygen flux to the fibre centre may be maintained *via* the hydrocarbon cores of the reticular network of mitochondrial membranes in fish muscles (Egginton et al., 2000; O'Brien and Sidell, 2000).

Even in light of the above arguments, the effects of both cold-temperature and oxygen-binding protein expression on fibre size seem anomalous. However, one positive outcome from increasing fibre size may be to conserve energy. Approximately 20–40% of an organism's energy budget is used to maintain ionic gradients (Jobling, 1994). Minimising ion leakage is one mechanism animals use to reduce metabolic costs and endure environmental stress. Cell membranes are less permeable to ions in cold-tolerant animals (hibernators) compared to those that are cold-sensitive (Hochachka, 1986), and turtles reduce membrane permeability to help them survive long periods of hypoxia (Hochachka et al., 1996). Large fibre size in icefish decreases the surface-to-volume ratio of the cell, and the relative surface area available for the diffusion of ions. Increasing fibre size thus may decrease the energetic demands of icefish, and may be possible only because the mitochondrial reticulum helps to maintain oxygen diffusion within these large fibres.

Two characteristics of their vascular systems may provide additional insurance that oxygen supply is maintained to the centre of large muscle fibres in icefish. First, icefish have a well-developed hypobranchial circulatory system, which delivers freshly oxygenated blood directly from the gills to the pectoral muscles (Egginton and Rankin, 1998). Flow capacity of the hypobranchial system in icefish is approximately 30% of total cardiac output. In contrast, in red-blooded nototheniods, the hypobranchial system is formed from fewer and smaller efferent branchial arteries compared to icefishes, and is a less important component of the pectoral vasculature system (Egginton and Rankin, 1998). Second, the capillary bed of icefish oxidative muscle is highly tortuous, forming extensive loops along the muscle fibres, which maximises the surface area for gas exchange (Egginton and Rankin, 1998).

### *Oxidative capacity*

Our measurements of enzyme activities indicate that the lower aerobic metabolic capacity of pectoral adductor muscle in icefish correlates with lower capillarity of the musculature. Maximal activities per gram tissue of citrate synthase and cytochrome oxidase, two enzymes indicative of aerobic metabolic capacity, are lower in muscles of icefish compared to red-blooded species. CPT-I catalyzes an important reaction in the metabolism of fatty acids, and is a good indicator of potential for fatty-acid oxidation. Maximal activity of CPT-I is greater in *N. coriiceps* and *G. gibberifrons* compared to the two icefish. Despite an apparently lower capacity for aerobic metabolism, icefish do not compensate with a greater reliance on anaerobic metabolic pathways to fuel swimming. In general, Antarctic fish species have a lower capacity for anaerobic metabolism than temperate zone species, possibly because of the greater thermal sensitivity of these enzymes compared to aerobically poised enzymes (Crockett and Sidell, 1990).

In conclusion, low capillary densities relative to mitochondrial densities in pectoral adductor of icefish reflect differences in ultrastructure and in metabolic requirements compared to those of red-blooded Antarctic fishes. Differences in mitochondrial architecture between icefish and red-blooded species indicate that oxidative capacity per mitochondrion may be lower in icefish. Thus, equivalent volumes of mitochondria in channichthyids and red-blooded nototheniids may not require equivalent lengths of capillary to maintain oxidative phosphorylation (Fig. 4). Although large mitochondrial density does not increase the oxidative metabolic capacity of icefish muscle, lipid-rich membranes may maintain oxygen flux because oxygen is more soluble in their hydrocarbon core than in cytoplasm. The reticular structure of the mitochondrial population may further provide an important avenue for oxygen diffusion in icefish that have two obstacles to overcome

in maintaining oxygen flux: lack of oxygen-binding proteins and large-sized fibres. Despite ultrastructural characteristics that may enhance oxygen diffusion, aerobic metabolic capacities per gram muscle are lower in icefish than in red-blooded species, thus restricting them to low energy niches and making them potentially more sensitive to changes in their thermal environment.

The technical assistance of Sandra Cordiner, and support from US NSF (grants OPP 94-21657 and OPP 99-09055) and UK NERC (grant GR3/10522) is gratefully acknowledged. We thank the Masters and crew of *R/V Polar Duke* and personnel of Antarctic Support Associates, both aboard ship and at Palmer Station, and members of the British Antarctic Survey for their invaluable support in collecting and maintaining Antarctic specimens.

## References

**Archer, S. D. and Johnston, I. A.** (1989). Kinematics of labriform and subcarangiform swimming in the Antarctic fish *Notothenia neglecta*. *J. Exp. Biol.* **143**, 195-210.

**Archer, S. D. and Johnston, I. A.** (1991). Density of cristae and distribution of mitochondria in the slow muscle fibers of Antarctic fish. *Physiol. Zool.* **64**, 242-258.

**Bakeeva, L. E., Chentsov, Y. S. and Skulachev, V. P.** (1978). Mitochondrial framework (reticulum mitochondriale) in rat diaphragm muscle. *Biochim. Biophys. Acta* **501**, 349-369.

**Battino, R., Evans, F. D. and Danforth, W. F.** (1968). The solubilities of seven gases in olive oil with reference to theories of transport through the cell membrane. *J. Am. Oil Chem. Soc*. **45**, 830-833.

**Beenakkers, A. T., Dewaide, J. E., Henderson, P. T. and Lutgerhorst, A.** (1967). Fatty acid oxidation and some participating enzymes in animal organs. *Comp. Biochem. Physiol*. **22**, 675-682.

**Blake, R. W.** (1979). The mechanics of labriform locomotion. *J. Exp. Biol.* **82**, 255-271.

**Crabtree, B. and Newsholme, E. A.** (1972a). The activities of phosphorylase, hexokinase, phosphofructokinase, lactate dehydrogenase and glycerol 3-phosphate dehydrogenases in muscles from vertebrates and invertebrates. *Biochem. J.* **126**, 49-58.

**Crabtree, B. and Newsholme, E. A.** (1972b). The activities of lipases and carnitine palmitoyltransferase in muscles from vertebrates and invertebrates. *Biochem. J.* **130**, 697-705.

**Crockett, E. L. and Sidell, B. D.** (1990). Some pathways of energy metabolism are cold adapted in Antarctic fishes. *Physiol. Zool*. **63**, 472-488.

**Cruz-Orive, L. M. and Weibel, E. R.** (1981). Sampling designs for stereology. *J. Microsc.* **122**, 235-257.

**Desaulniers, N., Moerland, T. S. and Sidell, B. D.** (1996). High lipid content enhances therate of oxygen diffusion through fish skeletal muscle. *Am. J. Physiol*. **271**, R42-R47.

**Dunn, J. F., Archer, S. D. and Johnston, I. A.** (1989). Muscle fibre types and metabolism in post-larval and adult stages of notothenioid fish. *Polar Biol*. **9**, 213-223.

**Eastman, J. T.** (1993). *Antarctic Fish Biology: Evolution in a Unique Environment*. San Diego: Academic Press.

**Egginton, S.** (1990). Morphometric analysis of tissue capillary supply. In *Vertebrate Gas Exchange from Environment to Cell*. Advances in Comparative and Environmental Physiology, vol. **6** (ed. R. G. Boutilier), pp. 73-141. Berlin: Springer Verlag.

**Egginton, S.** (1994). Stress response in two Antarctic teleosts (*Chaenocephalus aceratus* Lönnberg and *Notothenia coriiceps* Richardson) following capture and surgery. *J. Comp. Physiol. B* **164**, 482-491.

**Egginton, S. and Cordiner, S.** (1994). Effect of fixation protocols on muscle preservation and *in situ* diffusion distances. *J. Fish Biol.* **47**, 59-69.

**Egginton, S. and Johnston, I. A.** (1984). Effects of acclimatization temperature on routine metabolism muscle mitochondrial volume density and capillary supply in the elver (*Anguilla anguilla*). *J. Therm. Biol*. **9**, 165-170.

**Egginton, S. and Rankin, J. C.** (1998).Vascular adaptations for a low pressure / high flow blood supply to locomotory muscles of icefish. In *Antarctic Fishes: A Biological Overview* (ed. G. DiPrisco, A. Clarke and E. Pisano), pp. 185-195. Berlin: Springer Verlag.

**Egginton, S. and Sidell, B. D.** (1989). Thermal acclimation induces adaptive changes in subcellular structure of fish skeletal muscle. *Am. J. Physiol*. **256**, R1-R9.

**Egginton, S., Cordiner, S. and Skilbeck, C.** (2000). Thermal compensation of peripheral oxygen transport in skeletal muscle of seasonally acclimatized trout. *Am. J. Physiol*. **279**, 375-388.

**Egginton, S., Ross, H. F. and Sidell, B. D.** (1988). Morphometric analysis of intracellular diffusion distances. *Acta Stereologica* **6**, 449-454.

**Ellsworth, M. L. and Pittman, R. N.** (1984). Heterogeneity of oxygen diffusion through hamster striated muscles. *Am. J. Physiol*. **246**, H161-H167.

**Fitch, N. A., Johnston, I. A. and Wood, R. E.** (1984). Skeletal muscle capillary supply in a fish that lacks respiratory pigments. *Resp. Physiol*. **57**, 201-211.

**Hansen, C. A. and Sidell, B. D.** (1983). Atlantic hagfish cardiac muscle: metabolic basis of tolerance to anoxia. *Am. J. Physiol*. **244**, R356-R362.

**Hochachka, P. W.** (1986). Defense strategies against hypoxia and hypothermia. *Science* **231**, 234-241.

**Hochachka, P. W., Buck, L. T., Doll, C. J. and Land, S. C.** (1996). Unifying theory of hypoxia tolerance: molecular/ metabolic defense and rescue mechanisms for surviving oxygen lack. *Proc. Natl. Acad. Sci. USA* **93**, 9493-9498.

**Holeton, G.** (1970). Oxygen uptake and circulation by a haemoglobinless Antarctic fish (*Chaenocephalus aceratus* Lönnberg) compared with three red-blooded Antarctic fish. *Comp. Biochem. Physiol*. **34**, 457-471.

**Hoofd, L. and Egginton, S.** (1997). The possible role of intracellular lipid in oxygen delivery to fish skeletal muscle. *Respir. Physiol.* **107**, 191-202.

**Hoppeler, H. and Billeter, R.** (1991). Conditions for oxygen and substrate transport in muscles in exercising mammals. *J. Exp. Biol.* **160**, 263-283.

**Hoppeler, H. and Lindstedt, S. L.** (1985). Malleability of skeletal muscle in overcoming limitations: structural elements. *J. Exp. Biol.* **115**, 355-364.

**Hoppeler, H., Mathieu, O., Weibel, E. R. Krauer, R., Lindstedt, S. L. and Taylor, C. R.** (1981a). Design of the mammalian respiratory system. VIII. Capillaries in skeletal muscles. *Resp. Physiol.* **44**, 129-150.

**Hoppeler, H., Mathieu, O., Krauer, R., Claasen, H., Armstrong, R. B. and Weibel, E. R.** (1981b). Design of the mammalian respiratory system. IV. Distribution of mitochondria and capillaries in various muscles. *Resp. Physiol*. **44**, 87-111.

**Jobling, M.** (1994). *Fish Bioenergetics*. London: Chapman and Hall.

**Johnston, I. A.** (1989). Antarctic fish muscles – structure and function and physiology. *Ant. Sci*. **1**, 97-108.

**Johnston, I. A. and Harrison, P.** (1985). Contractile and metabolic characteristics of muscle fibres from Antarctic fish. *J. Exp. Biol.* **116**, 223-236.

**Johnston, I. A., Calvo, J., Guderley, H., Fernandez, D. and Palmer, L.** (1998). Latitudinal variation in the abundance and oxidative capacities of muscle mitochondria in perciform fishes. *J. Exp. Biol.* **201**, 1-12.

**Kayar, S. R., Hoppeler, H., Mermod, L. and Weibel, E. R.** (1988). Mitochondrial size and shape in equine skeletal muscle: a three-dimensional reconstruction study. *Anat. Rec.* **222**, 333-339.

**Koyama, T., Zhu, M. Y., Araiso, T., Kinjo, M., Kitagawa, H. and Sugimura, M.** (1990). Dynamic microstructure of plasma and mitochondrial membranes from bullfrog myocardium- a nanosecond time-resolved fluorometric study. *Jap. J. Physiol*. **40**, 65-78.

**Lindsey, C. C.** (1978). Form, function, and locomotory habits in fish. In *Fish Physiology*, vol. VII (ed. W. S. Hoar and D. J. Randall), pp. 1-88. New York: Academic Press.

**Londraville, R. L. and Sidell, B. D.** (1990a). Ultrastructure of aerobic muscle in Antarcticfishes may contribute to maintenance of diffusive fluxes. *J. Exp. Biol.* **150**, 205-220.

**Londraville, R. L. and Sidell, B. D.** (1990b). Maximal diffusion-distance within skeletal muscle can be estimated from mitochondrial distributions. *Resp. Physiol*. **81**, 291-302.

**Longmuir, I. S.** (1980). Channels of oxygen transport from blood to mitochondria. *Adv. Physiol. Sci*. **25**, 19-22.

**Mathieu-Costello, O., Suarez, R. K. and Hochachka, P. W.** (1992). Capillary-to-fiber geometry and mitochondria density in hummingbird flight muscle. *Resp. Physiol*. **89**, 113-132.

**Montgomery, J. C. and Macdonald, J. A.** (1984). Performance of motor systems in Antarctic fishes. *J. Comp. Physiol*. **154**, 241-248.

**Moylan, T. J. and Sidell, B. D.** (2000). Concentrations of myoglobin and myoglobin mRNA in heart ventricles from Antarctic fishes. *J. Exp. Biol.* **203**, 1277-1286.

**O'Brien, K. M. and Sidell, B. D.** (2000). The interplay among cardiac ultrastructure, metabolism and the expression of oxygen-binding proteins in Antarctic fishes. *J. Exp. Biol*. **203**, 1287-1297.

**Opie, L. H. and Newsholme, E. A.** (1967). The activities of fructose 1,6-diphosphate, phosphofructokinase and phosphoenol pyruvate carboxykinase in white muscle and red muscle. *Biochem. J*. **103**, 391-399.

**Read, G., Crabtree, B. and Smith, G. H.** (1977). The activities of 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase in hearts and mammary glands from ruminants and non-ruminants. *Biochem. J*. **164**, 349-355.

**Rodnick, K. J. and Sidell, B. D.** (1994). Cold acclimation increases carnitine palmitoyltransferase I activity in oxidative muscle of striped bass. *Am. J. Physiol*. **166**, R405-R412.

**Rodnick, K. J. and Sidell, B. D.** (1997). Structural and biochemical analyses of cardiac ventricular enlargement in cold-acclimated striped bass. *Am. J. Physiol*. **42**, R252-R258.

**Ruud, J. T.** (1954). Vertebrates without erythrocytes and blood pigment. *Nature* **173**, 848-850.

**Sänger, A. M.** (1999). Morphometric analysis of axial muscle in cyprinid fish provides support that intracellular lipid plays a role in slow fibre oxygen supply. *J. Fish Biol.* **54**, 1029-1037.

**Schwerzmann, K., Hoppeler, H., Kayar, S. R. and Weibel, E. R.** (1989). Oxidative capacity of muscle and mitochondria: correlation of physiological, biochemical, and morphometric characteristics. *Proc. Natl. Acad. Sci. USA* **86**, 1583-1587.

**Sidell, B. D.** (1998). Intracellular oxygen diffusion: the roles of myoglobin and lipid at cold body temperature. *J. Exp. Biol.* **201**, 1118-1127.

**Smith, P. K., Krohn, R. I., Hermanson, G. T., Malia, A. K., Gartner, M. D., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olsen, B. J. and Klenk, D. C.** (1985). Measurement of protein using bicinchoninic acid. *Anal. Biochem*. **150**, 76-85.

**Srere, P. A., Brazil, A. and Gonen, L.** (1963). The citrate condensing enzyme of pigeon breast muscle and moth flight muscle. *Acta Chem. Scand*. **17**, S219-S234.

**Tyler, S. and Sidell, B. D.** (1984). Changes in mitochondrial distribution and diffusion distances in muscle of goldfish upon acclimation to warm and cold temperatures. *J. Exp. Zool.* **232**, 1-9.

**Walesby, N. J. and Johnston, I. A.** (1980). Fibre types in the locomotory muscles of an Antarctic teleost, *Notothenia rossii*. *Cell Tiss. Res*. **208**, 143-164.

**Webb, P. W.** (1990). How does benthic living affect body volume, tissue composition, and density of fishes? *Can. J. Zool*. **68**, 1250-1255.

**Weibel, E. R.** (1979). *Stereological Methods*, vol 1. New York: Academic Press.

**Weibel, E. R.** (1984). *The Pathway for Oxygen*. Cambridge: Harvard University Press.

**Wharton, D. C. and Tzagoloff, A.** (1967). Cytochrome oxidase from beef heart mitochondria. *Meth. Enzym*. **10**, 245-260.

**Wittenberg, B. A. and Wittenberg, J. B.** (1989). Transport of oxygen in muscle. *Ann. Rev. Physiol*. **51**, 857-878.

**Zammit, V. A. and Newsholme, E. A.** (1976). The maximum activities of hexokinase, phosphorylase, phosphofructokinase, glycerol phosphate dehydrogenases, lactate dehydrogenase, octopine dehydrogenase, phosphoenolpyruvate, carboxykinase, nucleoside diphosphate kinase, glutamate-oxaloacetate transaminase and arginine kinase in relation to carbohydrate utilization in muscles from marine invertebrates. *Biochem. J*. **160**, 447-462.