Muscle growth in Polar fish: a study of *Harpagifer* species with sub-Antarctic and Antarctic distributions

IAN A JOHNSTON,¹ VERA L A VIEIRA,¹ DANIEL A FERNANDEZ,¹ MARGUERITE ABERCROMBY¹ JULIE C BRODEUR,¹ LLOYD PECK³ AND JORGE CALVO²

¹ Gatty Marine Laboratory, School of Biology, University of St Andrews, St Andrews, Fife KY16 8LB, Scotland, ² CADIC-CONICET, (9410) Ushuaia, Tierra del Fuego, Argentina and ³ British Antarctic Survey, High Cross, Madingley Road, Cambridge, CB3 0ET.

SUMMARY: Muscle growth was investigated in *Harpagifer* species with sub-Antarctic (*Harpagifer bispinis*, environmental temperature (ET) 4–11°C) and Antarctic (*Harpagifer antarcticus*, ET -1.5 to +1°C) distributions. The trunk musculature of 1 d-old larval *H. antarcticus*, 0.84–0.93 cm standard length (SL), contained 1025 ± 76 fast muscle fibres per cross-section (Mean $\pm$ SE, $n=6$). Zones of muscle fibre formation were restricted to the dorsal and ventral cones of the myotomes. The maximum diameter of fast muscle fibres increased linearly with standard length and was 270 μ m at 10.1 cm SL. The density of myogenic progenitor cells, determined by staining sections for c-met and myogenic regulatory factors (MyoD, myf-5, myf-6, myogenin), decreased with increasing body length. The number and size distribution of fast muscle fibres with respect to standard length was indistinguishable in *H. bispinis* and *H. antarcticus*, although the number of c-met⁺ cells and the maximum body size was higher in the Antarctic species. Comparative studies indicate that a large maximum fibre diameter is a general characteristic of the Notothenioidei. This may reflect the radiation from a small bodied ancestor with a relatively low fibre number, although adaptive explanations related to metabolic rate reduction cannot be excluded.

KEY WORDS: Antarctic fish, muscle growth, cold adaptation, myogenic regulatory factors

INTRODUCTION

The Antarctic fish fauna is dominated by the perciform sub-order Notothenioidei which comprise more than 100 species showing a wide range of trophic specialisations.¹⁾ They are thought to have evolved from a bottom living ancestor prior to the formation of the sub-Antarctic current around 20 million years ago.²⁾ Extant Antarctic Notothenioids are highly stenothermal (-1.86°C to +2°C) and show a range of adaptations to low temperature, including glycopeptide antifreeze molecules and associated aglomerular kidneys.¹⁾ There is a tendency for reduced haematocrit, which reaches an extreme in the family Channichthyidae which lack haemoglobin and myoglobin in the skeletal muscles.³⁾ Enzymes such as myosin ATPase are specialised for low temperature and have flexible tertiary structures.^{4,5)} Antarctic Notothenioids have relatively low resting metabolic rates and modest scopes for aerobic activity.⁶⁾ Studies with isolated mitochondria have shown that state 3 respiration rates are not up-regulated relative to temperate and sub-Antarctic species assayed at the same low temperatures.⁷⁾ The volume density of mitochondria in the aerobic muscle fibre types can

reach 35–50% even in relatively sluggish species, reflecting the relatively low rate of oxygen uptake per unit volume of mitochondria.⁷⁾ Muscle growth is also unusual in these fish with most species having a relatively low number of muscle fibres.^{8,9)} Fibre recruitment ceases early in the life cycle in *Notothenia coriiceps* and consequently the majority of growth is by hypertrophy.⁸⁾ The maximum diameter of slow and fast muscle fibres in Antarctic notothenioid have been reported to exceed 120 and 550 μ m respectively.^{8,9)}

Notothenioids living outside Antarctica experience much higher temperatures.¹⁾ Some Sub-Antarctic Notothenioids recruit muscle fibres for comparable periods to temperate species, indicating a diversity of muscle growth patterns.¹⁰⁾ Comparative studies of species with Antarctic and sub-Antarctic distributions provide one method of investigating which specialised features of their physiology are associated directly with adaptation to sub-zero temperatures. In the present study, muscle growth has been investigated in two ecologically similar species from the genus *Harpagifer* (plunderfishes). The species studied were *H. antarcticus* from the Antarctic

peninsular living at around -1°C all year round and *H. bispinis* from the Beagle Channel, Tierra del Fuego which experiences temperatures of $+4^{\circ}\text{C}$ in the winter and 11°C in the summer months.

MATERIALS AND METHODS

Fish

Harpagifer bispinis were collected from the shoreline in the Beagle Channel, Tierra del Fuego, Argentina during February 2000 and 2001. A total of 28 fish were studied ranging from 2.45 cm standard length (SL) (0.36 g) to 7.1 cm SL (7.2 g). *Harpagifer antarcticus* were collected by scuba divers at the British Antarctic Survey base at Rothera on the Antarctic peninsula and transported to the Gatty Marine Laboratory, St Andrews, Scotland where they were maintained at -1 to 0°C (12h light: 12h dark). Fish were fed Antarctic krill and squid *ad libitum*. Some fish spawned in the aquarium and larvae hatched 100–120 d after fertilisation. A total of 20 *H. antarcticus* were studied ranging from 0.84 cm SL (8 mg) to 10.1 cm SL (24.8 g).

Structural studies and immunohistochemistry

A 5 mm steak through the trunk muscle was prepared at 0.7 SL. For larvae and juveniles the whole cross-section was frozen in isopentane cooled in liquid nitrogen (-159°C). For larger specimens up to 3 blocks were prepared per cross-section prior to freezing. Frozen sections, 7 μm thick, were cut at -20°C using a cryostat. The different muscle fibre types were identified as previously described.⁹⁾ For quantitative measurements sections were stained with haematoxylin-eosin or Sirius Red stain. The total cross-sectional area of fast muscle and the cross-sectional area and average diameter of 600–800 individual fibres were determined by digital planimetry using an image analysis system (Kontron Elektronik, Basel, Switzerland). The total number of fast muscle fibres and myonuclei were also quantified from the same sections. Immunohistochemistry was carried out on sections fixed for 10 min in 4% (m/v) paraformaldehyde in phosphate-buffered saline.¹²⁾ c-met was used as a marker of myogenic progenitor cells.¹¹⁾ The density of mononuclear cells staining for a mixture of myogenic regulatory factors (MyoD, myogenin, Myf-5, Myf-6) was also determined as previously described.¹³⁾ Anti-rabbit polyclonal IgG primary antibodies, which were all obtained from Santa Cruz Biotechnology Inc. USA, were used. Peroxidase

activity was developed using 3-amino-9-ethylcarbazole (Sigma Chemicals, Poole, UK). Control sections were prepared in which the primary and/or secondary antibodies were omitted.

Statistics

In order to facilitate the statistical analysis of fibre size distribution smooth nonparametric estimates of the probability density function of fibre diameter were calculated using a kernel function. Bootstrap techniques were used to distinguish underlying structure in the distributions from random variation as described in Johnston *et al.*¹²⁾ Values of the smoothing parameter (h) used ranged from 0.083 to 0.125. Muscle cellularity parameters were compared using ANCOVA with fish standard length as covariate.

RESULTS

The increase in fast muscle cross-sectional area (CSA) at 0.7 SL is shown in Fig. 1.

Fig. 1. The relationship between the total cross-sectional area of fast myotomal muscle fibres and standard length in *H. antarcticus* (open circles) and *H. bispinis* (closed circles). The fitted line is a 2nd order polynomial.

There was no significant difference between the species and the data could be fitted by a single 2nd order polynomial [$\text{CSA} = 0.0072 (\pm 0.0176) - 0.103 (\pm 0.0074) \text{SL} + 0.0090 (\pm 0.0007) \text{SL}^2 (\pm \text{SD})$] (Adj.Rsq. = 0.97, residual degrees freedom = 44, $P < 0.001$). In the larvae of *H. antarcticus* there was a superficial layer of slow muscle, 2 to 3 fibres thick, along the entire lateral surface of the trunk (Fig. 2A).

Fig. 2. Myotomal muscle in the Antarctic plunderfish, *Harpagifer antarcticus*. A) larva, B) juvenile 3.7 cm SL and C) adult 10.1 cm SL. The arrows in A show the locations of the zones of new muscle fibre formation in the ventral myotome. Arrows in B show gradation in fibre size. Abbreviations: f: fast muscle fibre; hs: major horizontal septum; s: slow muscle fibre; t: tonic muscle fibre; sk: skin and sc: spinal chord

The average number of fast muscle fibres per trunk cross-section in *H. antarcticus* larvae, 0.84–0.93 cm standard length (SL), was 1025 ± 76 (Mean $\pm$ SE, $n=6$). Distinct germinal zones of new fibre

production were restricted to the dorsal and ventral cones of the myotomes in larvae (arrows in Fig. 2A).

The number of fast muscle fibres showed no significant increase with SL for juvenile and adult stages (SL 3.7 – 10.1 cm, *H. antarcticus*, $3,800 \pm 140$, $n=13$, SL 2.5 – 7.1 cm *H. bispinis*, $3,600 \pm 140$, $n=26$) (Mean $\pm$ SE) (Fig. 3A).

Fig. 3. The relationship between standard length (cm) and (A) fibre number and (B) the maximum diameter of fast myotomal muscle fibres in *H. antarcticus* (open circles) and *H. bispinis* (closed circles)

Only the smallest *H. bispinis* studied (2.5 cm SL) had fibres in the smallest size category ($<10 \mu\text{m}$) indicating relatively recent recruitment. It seems likely that the zones of new muscle fibre became gradually exhausted. In fish less than 4.0 mm localised areas showing a gradation of fibre diameters could still be observed (arrows in Fig. 2B).

*Harpagifer antarcticus**Harpagifer bispinis*

Fig. 4. Probability density functions of the diameter of fast muscle fibres for (A, B) and adult (C, D) stages of *Harpagifer antarcticus* and *H. bispinis*. The dotted lines represent the smooth distributions fitted to 600 measurements of fibre diameter from individual fish. The arrow in B indicates the distribution of fibre size from the smallest fish *H. bispinis* (2.5 cm SL).

There was no evidence for fibre recruitment outwith the germinal zones. The smoothed distributions of fibre diameter for *H. antarcticus* (Fig 4A, C) and *H. bispinis* (Fig. 4B, D) are illustrated. The distribution of fibre diameter was unimodal, shifting to a higher median diameter with increasing body length (Fig 4 A-D). The maximum fibre diameter, calculated from the 99% percentiles of the smoothed distributions, increased with SL and was identical for the two species (Fig. 3B). For *H. antarcticus* the relationship between maximum fibre diameter (MFD) and SL

were as follows: $MFD = -9.06 (\pm 2.19) + 28.09 (\pm 0.44) SL$; Adj. $R^2 = 0.996$; $P < 0.0001$, residual degrees freedom = 17 and for *H. bispinis* $MFD = 13.52 (\pm 7.28) + 24.58 (\pm 1.49) SL$; Adj. $R^2 = 0.910$; $P < 0.001$, (Mean $\pm$ SE). Both maximum SL and maximum fibre diameter are greater in *H. antarcticus* (10.1 cm and 270 μm) than in *H. bispinis* (7.1 cm, 210 μm) (Fig. 3B). The total density of nuclei decreased with increasing SL (Fig. 5A). The density of mononuclear cells immunopositive for c-met, a myogenic marker, was 3-7% of the total myonuclei

and was significantly higher for *H. antarcticus* than *H. bispinis* ($P < 0.05$) (Fig. 5B). However, the proportion of the c-met⁺ cells staining for myogenic regulatory factors was similar between the species, representing 1–4% of the total myonuclei (Fig. 5C).

Fig. 5. The relationship between fish standard length and (A) total myonuclear density, (B) the density of cells immunopositive for c-met and (C) the density of cells immunopositive for myogenic regulatory factors in *Harpagifer antarcticus* (open circles) and *H. bispinis* (closed circles).

DISCUSSION

Two phases of postembryonic myogenesis have been described in teleosts. The first phase has been termed “stratified hyperplastic growth” and involves new fibre production from distinct germinal layers or proliferative zones.¹⁴ This phase of growth begins in late embryonic stages and may extend through the larval to juvenile stages.¹⁴ Starting in the larval or juvenile stage new fibres are formed on the surface of existing fibres giving a characteristic mosaic pattern.^{12,15} This second phase of post-embryonic myogenesis has been termed “mosaic hyperplasia.”¹⁴ The number of muscle fibres recruited per unit muscle mass varies considerable between different genera of teleosts.¹⁵ The myotomal muscle of Antarctic Notothenioids is characterised by very large diameter fibres compared to other teleost families and is associated with a relatively low fibre number.⁸ The main finding of the present study was that the mosaic hyperplastic phase of myogenesis was missing in the two *Harpagifer* species studied. Comparative studies indicate that a large maximum fibre diameter is a general feature of the sub-order.^{8,9,10,11}

In some species that reach a larger body size than *Harpagifer*, the stratified hyperplastic growth phase continues for longer and the mosaic hyperplastic phase is present.¹⁰ (unpublished results). However, since the patterns of muscle growth are similar in the stenothermal Antarctic plunderfish and the more eurythermal sub-Antarctic plunderfish this would argue against a low fibre number being some specific adaptation to low temperature. Nevertheless, it remains possible that the relatively low fibre number and large average diameter of muscle fibres in these species contributes to their reportedly low resting metabolic rates.⁶ and therefore adaptive explanations for the observed growth patterns cannot be excluded. Longevity and slow rates of annual growth are characteristic of many Antarctic fish.¹⁶ The nuclei required for postembryonic growth are derived from the proliferation of myogenic progenitor cells and presumably growth potential is reflected in the number, clonal life and proliferative capacity of these cells. In this respect, it is interesting that the density of c-met positive cells was significantly greater in *H. antarcticus* than *H. bispinis* (Fig. 5B) perhaps reflecting the greater ultimate body size of the former species.

Both plunderfish species build nests which are guarded by the males. In some temperate teleost species, egg incubation temperature influences the number of c-met⁺ cells together with fibre recruitment and hypertrophy in subsequent stages of

the life cycles.^{17,18)} The environmental temperature experienced during embryonic development is significantly lower for *H. antarcticus* (-1°C) than for *H. bispinis* (5-11°C). The very similar relationships between SL and the number and size of muscle fibres for these species indicate either limited phenotypic plasticity in muscle growth patterns and/or relatively recent divergence from a common ancestor.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Natural Environment Research Council of the United Kingdom and from the British Council – Antorchas Foundation (Argentina – Project 13817/4).

REFERENCES

1. Eastman JT. *Antarctic Biology: Evolution in an unique environment*. New York: Academic Press, 1993, 322pp.
2. Clarke A, Johnston IA. Evolution and adaptive radiation of Antarctic fishes. *Trends Ecol. Evol* 1996; **11**: 212-213.
3. Sidell BD, Vayda, ME, Small DJ, Moylan TJ, Londraville RL, Yuan ML, Rodnick KJ, Eppley ZA, Costello L. Variable expression of myoglobin among the haemoglobinless Antarctic fishes. *Proc. Natl. Acad. Sci. USA*. 1997; **97**: 3420-3424.
4. Johnston IA, Walesby, NJ, Davison W, Goldspink G. Temperature adaptation in myosin of Antarctic fish. *Nature* 1975; **254**: 74-75.
5. Gauvry L, Enion S, Ettelaie C, Goldspink G. Characterisation of red and white muscle myosin heavy chain gene coding sequences from antarctic and tropical fish. *Comp. Biochem. Physiol. B* 2000; **127**: 575-588.
6. Johnston IA, Clarke A, Ward, P. Temperature and metabolic rate in sedentary fish from the Antarctic, North Sea and Indo-West Pacific Ocean. *Mar Biol*. 1991; **109**: 91-95.
7. Johnston IA, Calvo J, Guderley H, Fernández DA, Palmer L. Latitudinal variation in the abundance and oxidative capacities of muscle mitochondria in Perciform fishes. *J. Exp. Biol*. 1998; **101**: 1-12.
8. Battram JC, Johnston IA. Muscle growth in the Antarctic teleost *Notothenia neglecta* (Nybelin). *Antarctic Sci*. 1991; **3**: 29-33.
9. Dunn J, Archer SD, Johnston, IA. Muscle fibre types and metabolism in post larval and adult stages of Notothenioid fish. *Polar Biol*, 1989; **9**: 213-223.
10. Fernández DA. Histochemistry, distribution and growth of axial muscle in sub-Antarctic notothenioids. Preliminary analysis of two factors related to swimming performance: buoyancy and temperature. Thesis, University of Buenos Aires, 250pp.
11. Fernández DA, Calvo J, Franklin CE, Johnston IA. Muscle fibre types and size distribution in sub-antarctic notothenioid fishes. *J. Fish Biol*, 2000; **56**: 1295-1311.
12. Johnston IA, Strugnell G, McCracken ML, Johnstone R. Muscle growth and development in normal-sex-ratio and all-female diploid and triploid Atlantic salmon. *J. Exp. Biol*. 1999; **202**: 1991-2016.
13. Johnston, IA, McLay HA, Abercromby M, Robins D. Phenotypic plasticity of early myogenesis and satellite cell numbers in Atlantic salmon spawning in upland and lowland tributaries of a river system. *J. Exp. Biol*. 2000; **203**: 2539-2552.
14. Rowlerson A, Veggetti, A. Cellular mechanisms of post-embryonic muscle growth in aquaculture species. In: Johnston IA (ed). *Fish Physiology*. Vol.18, *Muscle development and growth*. Academic Press, New York. 2001; 103-140.
15. Johnston IA. Genetic and environmental determinants of muscle growth patterns. In: Johnston IA (ed). *Fish Physiology*. Vol.18, *Muscle development and growth*. Academic Press, New York. 2001; 141-186.
16. Clarke A, North AW. Is the growth of polar fish limited by temperature? In: di Prisco G, Maresca B, Tota B (eds). *Biology of Antarctic fish*. Springer-Verlag, Berlin. 1990; 54-69.
17. Johnston IA, McLay HA, Abercromby M, Robins D. Early thermal experience has different effects on growth and muscle fibre recruitment in spring- and autumn-running Atlantic salmon populations. *J. Exp. Biol*. 2000; **203**: 2553-2564.
18. Johnston IA, Cole NJ, Abercromby M, Vieira VLA. Embryonic temperature modulates muscle growth characteristics in larval and juvenile herring. *J. Exp. Biol*. 1998; **201**: 623-646.