Myogenic cell cycle duration in *Harpagifer* species with sub-Antarctic and Antarctic distributions: evidence for cold compensation

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Summary

In teleosts, the proliferation of myogenic progenitor cells is required for muscle growth and nuclear turnover. We measured the cell cycle and S-phase duration of myogenic cells in the fast myotomal muscle of two closely related *Harpagifer* species by cumulative S-phase labelling with 5-bromo-2'-deoxyuridine (BrdU). *Harpagifer antarcticus* is a stenothermal species from the Antarctic peninsula (experiencing temperatures of −2°C to +1°C) and *Harpagifer bispinis* is a eurythermal species from the Beagle Channel, Tierra del Fuego (living at +4°C in winter and up to 11°C in summer). Specific growth rates in the adult stages studied were not significantly different from zero. Myogenic progenitor cells were identified using an antibody against c-met. Seventy-five percent of the c-met⁺ve cells were in a proliferative state in both species. Cell cycle time was 150 h at 5°C and 81.3 h at 10°C in *H. bispinis* (*Q*₁₀ = 3.4). Cell cycle duration was 35% shorter in *H. antarcticus* at 0°C (111 h) than in *H. bispinis* at 5°C. The predicted cell cycle time for *H. bispinis* at 0°C (based on the *Q*₁₀ relationship) was 277 h, which was more than double that measured for the Antarctic species at this temperature. The results obtained are compatible with an evolutionary adjustment of cell cycle time for function at low temperature in the Antarctic species.

Key words: cell cycle, S-phase, *Harpagifer bispinis*, *Harpagifer antarcticus*, myogenic progenitor cell, cold compensation, Antarctic, temperature, nototheniid fish, satellite cell.

Introduction

The Antarctic Ocean has low stable temperatures (−2°C to +1.5°C) and experiences large seasonal variations in day length (Clarke, 1988). The suborder Notothenioidei (order Perciformes) dominates the fish fauna of the Antarctic marine ecosystem, its members exhibiting a diversity of morphological, ecological and life history types (Clarke and Johnston, 1996; Montgomery and Clements, 2000). Although most notothenioids live within the Antarctic region, 26 species are found outside Antarctica off the coasts of southern South America and New Zealand, where they experience considerably higher and more variable temperatures than their Antarctic counterparts (Eastman and Clarke, 1998). Antarctic and sub-Antarctic notothenioids have evolved from a common demersal perciform stock that lived before Antarctica became isolated and its temperature dropped in the Tertiary period (Eastman, 1993). Based on molecular phylogenies (Bargelloni et al., 1994; Ritchie et al., 1997; Stankovic et al., 2001), it is believed that the major radiation of the core Antarctic notothenioids took place after the establishment of the Antarctic Polar Front around 22–17 million years ago (Barker, 2001) whereas many sub-Antarctic notothenioids diverged only later, approximately 7–9 million years ago (Stankovic et al., 2001).

Modern Antarctic notothenioids are low temperature specialists possessing a number of unique physiological adaptations to cold water that allow them to survive at temperatures below 0°C. Some of these adaptations include the presence of antifreeze proteins and associated aglomerular kidneys (DeVries, 1984), a greater conformational flexibility of proteins and enzymes (Fields and Somero, 1998; Ciardiello et al., 2000) and a higher proportion of unsaturated phospholipid classes in cellular membranes (Cossins, 1994). Nevertheless, not all physiological traits show evolutionary adjustment in Antarctic fishes. Unloaded contraction velocity of the fast muscle fibres does not show cold compensation in a wide range of species (Johnston and Altringham, 1985; Johnson and Johnston, 1991) and maximum power output of the fast muscle is only 10–16% of that measured in Mediterranean and tropical Perciformes (Wakeling and Johnston, 1998). Similarly, comparative studies with isolated muscle mitochondria have found no evidence for upregulation in the maximum rate of respiration per mg mitochondrial
protein in Antarctic species (Johnston et al., 1998), and recent studies indicate that resting metabolic rate is not cold compensated in polar fish (Steffensen, 2002). Finally, annual growth rates of the Antarctic notothenioids are also generally believed to be slower than those of fish from temperate regions (DeVries and Eastman, 1981; Everson, 1984), although recent analyses of growth performance data have questioned this view (Kock, 1992; Kock and Everson, 1998). These new analyses suggest that only fish from the high-Antarctic zone, where water temperature is stable at −1.86°C, exhibit very slow growth rates, the growth performance of fish from lower-Antarctic latitudes/sub-Antarctic waters being similar to that of many temperate fish species (Kock and Everson, 1998; Morales-Nin et al., 2000; La Mesa and Vacchi, 2001). However, phylogenetic variation and the relatively few validations of age estimates complicate the interpretation of this type of analysis.

Mean habitat temperature has an important influence on growth rate in ectotherms, and the apparent slow growth of Antarctic fishes has often been intuitively linked to the low temperatures of the Southern Ocean. However, evidence for seasonality in Antarctic fish growth (North, 1988; Ashford and White, 1993, Coggan, 1997a), despite the quasi constancy of Antarctic water temperature, has led to the suggestion that resource (i.e. food) limitation rather than temperature may restrict growth rate in the field (Clarke, 1988; Clarke and North, 1991). This hypothesis is supported by laboratory experiments which show that fish maintained under conditions characteristic of the austral winter tend to have less appetite and consume less food than fish maintained under summer conditions (Targett, 1987; Coggan, 1997b).

In fish, growth is closely linked to the dynamics of muscle growth because the fast muscle constitutes a large proportion of the body mass (Mommsen, 2001). The notothenioids have an unusual pattern of muscle growth in that muscle fibre recruitment generally ceases early in the life cycle and subsequent growth in muscle mass is entirely by fibre hypertrophy (Battram and Johnston, 1991; Fernandez et al., 2000; Johnston et al., 2002). The proliferation of a population of myogenic progenitor cells provides a source of additional nuclei to support this process (Koumans and Akster, 1995; Fauconneau and Paboeuf, 2001). These cells are equivalent to the satellite cells described in mammals (Mauro, 1961), and both quiescent and activated progenitor cells can be identified by their expression of c-met, the receptor for hepatocyte growth factor (Cornelison and Wold, 1997; Johnston et al., 2000), which is believed to be involved in their activation (Tatsumi et al., 1998). The proliferation of myogenic precursors is furthermore required in adult stages for nuclear turnover and for muscle repair following injury (Rowlerson et al., 1997).

A crucial element of the proliferative potential of a cell population is the time needed to proceed through a complete cell cycle. Therefore, a previously unexamined way by which low temperature could restrict Antarctic fish growth rate and nuclear turnover is by limiting cell cycle progression rate in myogenic cells. The present study reports the first measurements of myogenic cell cycle duration in fish. Cell cycle duration was determined in two closely related species of Notothenioidei from the genus Harpagifer (family Notothenidae). H. antarcticus is a stenothermal species from the Antarctic peninsula that lives at temperatures between −2°C and +1°C throughout the year, while H. bispinis is a eurythermal species from the Beagle Channel, Tierra del Fuego that experiences temperatures of +4°C in winter and up to 11°C in summer. These two species have similar morphology, ecology and life history strategies, although the Antarctic species reaches a somewhat greater body size. The absence of an upward adjustment of cell cycle time at low temperatures in the Antarctic species would represent a fundamental constraint of low temperature on growth and the rate of turnover of muscle nuclei.

Materials and methods

Fish

Harpagifer antarcticus (Nybelin 1947) were collected by SCUBA divers near Rothera Point, Adelaide Island, Antarctica (67°34′S, 68°08′W). Fish were held in flow-through seawater aquaria at the British Antarctic Survey research station at Rothera before being transported by ship to the Gatty Marine Laboratory in St Andrews, UK, where they were maintained at 0°C (12h:12h L:D) for several months before the experiment began. Average body mass and fork length of the fish used were 21.1±4.3 g and 9.4±0.6 mm (N=12, mean ± S.D.), respectively. Harpagifer bispinis (Forster 1801) were collected on the shelf at low tide in the Beagle channel, Tierra del Fuego, Argentina. Fish were acclimatized to either 5°C or 10°C (12h:12h L:D) for a minimum of 15 days prior to the experiment. Average body mass and fork length of the fish used were 3.15±0.77 g and 5.3±0.4 mm (N=33, mean ± S.D.), respectively. H. bispinis were fed chopped hake (Merluccius hubbsi) muscle, and H. antarcticus were fed chopped squid flesh ad libitum twice weekly throughout the acclimatization period. No food was offered after the experiment had begun. Specific growth rates of both fish species were not significantly different from zero when the experiment began (H. antarcticus at 0°C: −0.20% day–1, N=8; H. bispinis at 5°C: −0.39% day–1, N=30; H. bispinis at 10°C: −0.24% day–1, N=30). This is as would be expected as the fish were at least three-quarters maximum size and show very little annual growth at this size (Daniels, 1983; Kock, 1992).

Cumulative S-phase labelling

Total cell cycle (t_c) and S-phase (t_s) duration of the satellite cells were estimated using cumulative S-phase labelling with 5-bromo-2’-deoxyuridine (BrdU; Sigma Chemicals, Poole, UK). BrdU is a thymidine analogue that is incorporated into DNA during the S-phase of the cell cycle. Cells labelled with BrdU can be visualized by immunohistochemistry. The theoretical and mathematical considerations of the cumulative S-phase labelling approach have been described in detail by
Nowakowski et al. (1989). Briefly, the technique involves sequentially labelling a proliferating cell population with pulses of BrdU. As BrdU is only incorporated during the S-phase, the proportion of cells labelled in the total population (labelling index, Li) after the first pulse of BrdU is equal to $t_s/t_c$. With each new pulse of BrdU, the fraction of labelled cells increases linearly as new cells enter S-phase while previously marked cells are still visible (see Fig. 1). This increase in Li continues until the cells that were at the end of the S-phase at the time of the first injection re-enter S-phase. The time needed to reach this point is equivalent to $t_c-t_s$ and corresponds to the moment when Li reaches a plateau as all the proliferating cells have been labelled (Fig. 1). The value at which the Li reaches a plateau represents the proportion of the total population that is proliferating and is called the ‘growth fraction’ (GF). If the growth fraction is different from 1, the proportion of cells marked after the first pulse of BrdU ($L_i(0)$) is more correctly expressed by:

$$L_i(0) = GF \times t_s/t_c.$$  \hspace{1cm} (1)

Furthermore, since $GF/t_c$ is equal to the rate at which the Li increases during repeated S-phase labelling, equation 1 can be rearranged so that $t_s$ is obtained from the slope ($m$) and the y-intercept ($L_i(0)$) of the labelling index curve (see Fig. 1) obtained by repeated S-phase labelling:

$$t_s = L_i(0)/m.$$  \hspace{1cm} (2)

Once $t_s$ is known, it is also possible to estimate $t_c$ from the parameters of the curve shown in Fig. 1 by rearranging equation 1 to obtain:

$$t_c = GF \times t_s/L_i(0).$$  \hspace{1cm} (3)

Experimental design

All experiments were carried out under Home Office License following national requirements for animal care. Repeated S-phase labelling of the satellite cells was achieved for *H. antarcticus* at 0°C and *H. bispinis* at 5°C and 10°C by repeatedly injecting the animals intraperitoneally with BrdU. BrdU was diluted to 10 ng ml$^{-1}$ in phosphate-buffered saline (PBS), and a volume of this solution was injected so that the animal received 250 μg g$^{-1}$ of body mass. *H. antarcticus* were injected every 12 h until 132 h after the first injection. *H. bispinis* were injected every 8 h until 72 h and 48 h after the first injection for fish at 5°C and 10°C, respectively. However, as more time was needed for the labelling index to reach a plateau, a second group of *H. bispinis* was injected every 12 h until 144 h and 120 h after the first injection for fish at 5°C and 10°C, respectively. To monitor the evolution of the labelling index and establish a curve like the one shown in Fig. 1, one animal from each group was killed by a sharp blow to the head 1 h after each injection. Fish were, however, not sampled after the first injection because the number of labelled cells was usually too low to be accurately measured at that time. A 5-mm thick transverse slice of the trunk was taken at 0.7 fork length. The slices were frozen on cork strips in isopentane cooled near its freezing point in a mixture of acetone and dry ice and were stored at −20°C until sectioning. Frozen trunk slices were cut on a cryostat at 7-μm thickness and mounted on glass slides coated with poly-L-lysine. The sections were air-dried and stored at −20°C until processing for immunohistochemistry.

**Immunohistochemistry**

Muscle sections were doubly stained against BrdU and c-met to localize labelled myogenic cells (Fig. 2). The primary antibodies used were a rabbit polyclonal antibody against c-met (m-met, SP260; Santa Cruz Biotechnology, Santa Cruz, USA) and a mouse monoclonal antibody against BrdU (Clone BU-33, Sigma Chemicals). Sections were first fixed in acetone for 10 min and then placed in a solution containing 5% (v/v) normal goat’s serum, 1% (v/v) Triton X-100 and 1% (m/v) bovine serum albumin (BSA) in PBS for 15 min to rehydrate them and block non-specific binding sites. Sections were subsequently washed for 3×2 min in PBS and incubated overnight at 4°C with both primary antibodies diluted 1:20 in a solution containing 1% Triton X-100 and 1% BSA in PBS. After three washes in PBS, sections were incubated for 30 min at room temperature with biotinylated goat anti-mouse secondary antibody (Sigma Chemicals) diluted 1:20 in a solution containing 5% (v/v) normal goat’s serum, 1% (v/v) Triton X-100 and 1% (m/v) bovine serum albumin (BSA) in PBS for 15 min to rehydrate them and block non-specific binding sites. Sections were subsequently washed for 3×2 min in PBS and incubated for 30 min at room temperature with alkaline phosphatase-conjugated extraAvidin (Sigma Chemicals, Poole, UK) diluted 1:20 in a solution containing 1% Triton X-100 and 1% BSA in PBS. After washing the sections for 3×2 min in Tris buffer, alkaline phosphatase activity was developed using a solution containing Fast Blue BB, levamisole, naphtol-ASMX-phosphate and N,N-dimethylformamide in Tris buffer, which gives a blue end product (Van der Loos, 1999). Peroxidase activity was afterward developed using 3-amino-9-ethylcarbazole, which

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Fig. 1. Theoretical curve obtained during repeated S-phase labelling. See text for details.
gives a red end product (Van der Loos, 1999). Counts of the number of red (c-met positive), blue (BrdU positive) or purple (c-met and BrdU positive) cells were made from 60 fields of 0.024–0.031 mm² per fish.

**Statistical analysis**

The increase of the labelling index over time was modelled at each temperature by linear regression analysis. The slopes of the regression lines fitted at each temperature were compared by analysis of covariance using the general linear model (GLM) of the SPSS statistical software.

**Results**

In both *Harpagifer* species, the number of c-met positive cells staining for BrdU increased linearly over time with repeated S-phase labelling. The individual plots are shown in Fig. 3, and the fitted regression parameters are given in Table 1. An analysis of covariance demonstrated that the slopes from the three regression lines were significantly \((P<0.05)\) different from one another. The labelling index reached a plateau at a value of 0.75 at all three temperatures (Fig. 3), indicating that 75% of the satellite cell population was in a proliferative state.

Total cell cycle and S-phase duration were determined by solving equations 2 and 3 (see Materials and methods). Both cell cycle and S-phase duration were longer at lower temperatures in *H. bispinis* (Table 2), with a \(Q_{10}\) for cell cycle progression of 3.4. Using this \(Q_{10}\), a cell cycle time of 277 h was predicted for *H. bispinis* at 0°C, which is much more than the cell cycle time of 111 h measured in *H. antarcticus* at this temperature (Table 2).

**Discussion**

The constant low temperature of the Southern Ocean is often believed to limit growth rate in Antarctic fish, although clear evidence supporting this view is still lacking (Clarke and North, 1991). One possible way for low temperature to limit growth and nuclear turnover would be by reducing myogenic cell proliferation rate, since this process plays a predominant role in muscle growth and maintenance (Fauconneau and Paboeuf, 2001) and the muscle constitutes the major fraction of body mass in fish (Mommsen, 2001). This possibility was examined in the present study by comparing myogenic cell cycle duration in two closely related *Harpagifer* species: the Antarctic *H. antarcticus*, which lives at temperatures between –2°C and +1°C and the sub-Antarctic *H. bispinis*, which lives at temperatures between +4°C and 11°C. The absence of an upward adjustment of cell cycle time at low temperatures in the Antarctic species would represent a fundamental constraint of low temperature on growth and the rate of turnover of muscle nuclei.

The present study showed that cell cycle duration increases with decreasing temperatures in *H. bispinis* (Table 2). However, in contrast with this finding, cell cycle time was 1.35 times shorter in the Antarctic *H. antarcticus* at 0°C than in the sub-Antarctic species at 5°C (Table 2), suggesting a cold compensation of cell cycle progression rate in the Antarctic species. Two features of the data obtained suggest that this adjustment of satellite cell cycle duration in *H. antarcticus* represents an evolutionary adaptation. Firstly, the adjustment is in the direction to elevate cell cycle time in the polar species when compared with the sub-Antarctic species. Secondly, the

<table>
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<tr>
<th>Species</th>
<th>Temperature (°C)</th>
<th>Slope</th>
<th>Intercept</th>
<th>(r^2)</th>
<th>(P)</th>
<th>(N)</th>
</tr>
</thead>
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<td>0</td>
<td>0.0068</td>
<td>0.25</td>
<td>0.978</td>
<td>&lt;0.001</td>
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<tr>
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<td>0.20</td>
<td>0.972</td>
<td>&lt;0.001</td>
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<tr>
<td><em>Harpagifer bispinis</em></td>
<td>10</td>
<td>0.0091</td>
<td>0.19</td>
<td>0.966</td>
<td>&lt;0.001</td>
<td>11</td>
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</table>
cell cycle time of the two species is almost identical when compared at the median annual habitat temperature at which each species lives (H. antarcticus 111 h at 0°C and H. bispinis 110 h at its median life temperature of 7.5°C, estimated by interpolation using the observed Q10 of 3.4). Taken together, the data obtained in this study therefore suggest that evolution has been able to adjust the basic molecular machinery of cell proliferation to offset the rate-limiting effect of low temperatures.

Comparative data on myogenic cell cycle time in vivo are confined to a single study on 30-day-old rats (Schultz, 1996). This study found that 80% of fast skeletal muscle satellite cells proliferated with a cell cycle duration of 32 h and an S-phase of 14 h (Schultz, 1996). This percentage of proliferating satellite cells is similar to the 75% measured in the two Harpagiferidae species. However, whereas Schultz (1996) suggested that the remaining 20% of satellite cells were also dividing, although at a slower rate, the presence of linear labelling and a distinct plateau in Fig. 3 suggests a more homogeneous cell cycle time in the present study (Nowakowski et al., 1989). The relatively high proportions of dividing myogenic cells measured in both rats and fish may be associated with nuclear turnover as well as growth, since studies in the rat have shown that 2% of the nuclei are replaced each week in adult stages (Schmalbruch and Lewis, 2000). The cell cycle times measured in the present study probably reflected this nuclear turnover since the Harpagifer used were not actively growing.

Numerous extracellular signals that influence growth rate (e.g. growth factors, mitogen antagonists, differentiating inducing factors) are involved in regulating the cell cycle (Beijersbergen and Bernards, 1996; Jones and Kazlauskas, 2001). In the present study, the Harpagifer species had growth rates that were not significantly different from zero. The observed differences in cell cycle time are therefore most likely due to differences in temperature. Although the specific molecular and physiological adaptations responsible for the cold compensation of cell cycle progression rate remain to be identified, it is likely that already described cold adaptations of Antarctic fish such as the structural alteration of tubulins (Dietrich, 1997) and the greater conformational flexibility of proteins and enzymes (Fields and Somero, 1998; Ciardiello et al., 2000) play a part in this phenomenon. Interestingly, whereas total cell cycle time was considerably shorter in H. antarcticus at 0°C than in H. bispinis at 5°C, the duration of the S-phase was similar between the two species (Table 2), indicating that cold compensation was less important for DNA replication rates than for other parts of the cell cycle.

Although it is still debated whether or not growth is slower in Antarctic fishes, the present study shows that, if such a restriction of growth rate exists, it is unlikely to be due to a limitation of muscle growth by low temperature since myogenic cell cycle progression rate appears to show cold compensation. However, it remains to be examined whether cell cycle progression rate also shows cold compensation in myogenic progenitors of high-Antarctic fishes, as there are indications that these fish may be the only notothenioids with a truly reduced growth rate (Kock and Everson, 1998; Morales-Nin et al., 2000; La Mesa and Vacchi, 2001).

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### References


### Table 2. Total cell cycle duration and S-phase duration estimated by repeated S-phase labeling with BrdU in Harpagifer antarcticus and Harpagifer bispinis

<table>
<thead>
<tr>
<th>Species</th>
<th>Temperature (°C)</th>
<th>Cell cycle time (h)</th>
<th>S-phase time (h)</th>
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<tbody>
<tr>
<td>H. antarcticus</td>
<td>0</td>
<td>111</td>
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<tr>
<td>H. bispinis</td>
<td>5</td>
<td>150</td>
<td>40</td>
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<td>10</td>
<td>81.3</td>
<td>20.9</td>
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