The Journal of Experimental Biology 210, 1170-1182
Published by The Company of Biologists 2007
doi:10.1242/jeb.002188

Persistent effects of incubation temperature on muscle development in larval haddock (*Melanogrammus aeglefinus* L.)

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Accepted 23 January 2007

Summary

Muscle development and growth were investigated in haddock larvae (*Melanogrammus aeglefinus* L.) incubated under controlled temperatures (4, 6, 8°C) and reared post-hatch through yolk-dependent and exogenous-feeding stages in a 6°C post-hatch environment. Changes in cell number and size in superficial and deep myotomes within the epaxial muscle were investigated for 28 days following hatch. Distinct and significant differences in muscle cellularity following separate developmental strategies were observed in superficial and deep myotomes. The number of superficial myofibres increased with time and, although not in a manner proportional to temperature during the first 21 days post hatch (d.p.h.), there was observed a trend during the final 7 days of greater mean cell size that was strongly associated with increased temperature. In addition, there was an apparent correspondence between increased temperature and increased size between 21 and 28 d.p.h. Among all temperature groups the superficial myotome not only demonstrated a consistent unimodal myofibre-size distribution but one that increased in range proportional to temperature. In the deep muscle, myotomes from higher incubation temperatures had a broader range of fibre sizes and greater numbers of myofibres. The onset of a proliferative event, characterized by a significant recruitment of new smaller myofibres and a bimodal distribution of cell sizes, was directly proportional to incubation temperature such that it occurred at 14 d.p.h. at 8°C but not until 28 d.p.h. at 4°C. The magnitude of that recruitment was also directly proportional to temperature. Following hatch, those embryos from the greatest temperature groups had the largest mean deep muscle size but, as a result of the proliferative event, had the smallest-sized cells 28 days later. The muscle developmental and growth strategy as indicated by sequential changes in cellularity and cell-size distributions between myotomes in response to temperature are also discussed in light of whole animal growth and development.

Key words: development, larva, muscle, temperature, myofibre.

Introduction

In fish, muscle development and growth is a complex process, related directly to variations in somatic growth rates (Johnston et al., 1996; Ayala et al., 2000; Stoiber et al., 2002) and to the body length at which recruitment of new muscle fibres stops (Weatherley and Gill, 1985). The main trunk muscles represent approximately 60% of a fish's mass (Johnston, 1981; Jobling, 1994; Johnston, 2001) and are the major site of protein synthesis and storage, exhibiting the highest growth rate efficiency of all tissues (Houlihan et al., 1995). Fish muscle is composed of two basic fibre types, a smaller proportion of slow aerobic 'red' and a larger portion of fast anaerobic 'white' (Mommsen, 2001; Sänger and Stoiber, 2001), whose development is separated spatially and temporally (Koumans and Akster, 1995; Patruno et al., 1998).

Muscle growth occurs through hyperplasia (an increase in fibre number) and hypertrophy (increase in fibre size) (Kundu and Mansuri, 1990; Johnston, 1999; Mommsen, 2001). Both processes are dependent upon the recruitment from progenitor stem cell populations (Koumans and Akster, 1995; Johnston, 2001; Stoiber et al., 2002), which are distinct for fast and slow muscle (Devoto et al., 1996). Compared to post-natal muscle development and growth in mammals and birds, fish continue to demonstrate hyperplasia of muscle tissue as an important avenue of muscle growth in post-embryonic larval and adult phases until long into mature adult life (Weatherley et al., 1988; Koumans and Akster, 1995; Johnston et al., 1998; Patruno et al., 1998; Mommsen, 2001). In addition, fish develop different muscle types within discrete myotomal zones and follow a multi-stage process that begins in the embryo. The embryonic stage of fish muscle differentiation and development involves

different progenitor cell populations, the activation of morphogenes such as Shh that regulate developmental patterning and events as well as inducing muscle regulatory factors (MRF) (Shilo, 2001), and the spatial and temporal sequential expression of MRFs (Rescan, 2005). In the late embryo, new myofibres are recruited in discrete dorsal and ventral germinal or proliferation zones (Koumans and Akster, 1995; Galloway et al., 1998; Stoiber et al., 2002), which soon thereafter become depleted of progenitor cells. This 'stratified' hyperplasia (Rowlerson and Veggetti, 2001; Rescan, 2005) represents the second phase of muscle development and growth. Subsequent muscle growth proceeds through hypertrophy and mosaic hyperplasia in which new fibres are recruited throughout the spatially separated myofibre zones in the myotome (Johnston et al., 1996; Stoiber et al., 2002; Rowlerson and Veggetti, 2001; Rescan, 2005).

Fish muscle is remarkably plastic in its response to changes in environmental conditions, primary among which is temperature (Johnston, 1993; Johnston, 2001; Johnston et al., 1997; Johnston et al., 1998; Martell et al., 2006). This plasticity often involves directional changes in the number and size of red and white myofibres (Johnston, 2001; Stoiber et al., 2002; Johnston and Hall, 2004), changes in the frequency (Johnston, 2001) and periodicity of each type of myofibre recruitment (Brodeur et al., 2003; Johnston, 2006), and changes in the myogenic progenitor cell populations (Johnston, 2006). At the cellular level, changes in temperature may also bring about variation in myofibril (Johnston, 2001; Johnston, 2006; Martell et al., 2006) and mitochondrial densities (Johnston, 1993; Galloway et al., 1998; Sänger and Stoiber, 2001; Johnston and Hall, 2004). Minor shifts in temperature have been shown to have significant effects on development in rapidly growing embryonic and larval phases (Blaxter, 1992; Kamler, 1992; Johnston, 2001; Johnston, 2006; Johnston and Hall, 2004) such that temperature variation during embryogenesis can differentially affect fast and slow muscle development and growth (Johnston et al., 1998; Stoiber et al., 2002; Johnston and Hall, 2004; Johnston, 2006). Studies have also revealed that these changes can persist through ontogeny, affecting subsequent production of somatic tissue (i.e. growth) (Johnston and Hall, 2004; Martell et al., 2005; Martell et al., 2006), swimming style, and performance (Johnston et al., 2001; Johnston and Temple, 2002; Johnston and Hall, 2004; Johnston, 2006). For example, increased incubation temperature affected the time taken to complete metamorphosis and the proportion of muscle fibre types present in the post-metamorphic juvenile turbot (*Scophthalmus maximus* L.) (Calvo and Johnston, 1992). Studies on larval haddock (*Melanogrammus aeglefinus* L.) (Martell et al., 2005; Martell et al., 2006) and herring (*Clupea harengus* L.) (Johnston, 1993) have also shown that different incubation temperatures affected shifts in developmental trajectories for different tissues, such as the eye, gut, notochord, muscle and nervous system, such that some were advanced and others retarded with respect to each other.

Very few studies have attempted to examine the phenotypic ramifications of resultant changes in muscle structure (cellularity) beyond early exogenous feeding (Johnston, 2006). Fewer still are studies that have controlled for post-hatch temperature conditions to examine the ramifications of temperature differences during embryonic development (Johnston, 2006). From our previous studies of haddock development over different temperatures, it is clear that incubation temperature affected general developmental rates, tissue-specific development, yolk absorption and growth (Martell et al., 2005; Martell et al., 2006), and that these effects persisted throughout larval and early juvenile phases.

These changes may have a large potential impact upon larval function and capability (i.e. predator avoidance, prey capture, swimming performance, etc.) and, through these, survival. We considered it necessary next to examine how changes in muscle development, so crucial to larval function and growth, are manifested and how they are related to changes observed in earlier studies. We hypothesize that differences in incubation temperature will result in considerable and persistent alterations in larval haddock muscle phenotype and that the effects will differ between superficial and deep myotomes. A novel reductionist/integrative experimental approach that consisted of a series of interrelated and nested studies (see also Martell et al., 2005; Martell et al., 2006) was undertaken to investigate how temperature variation during embryogenesis can affect phenotypic plasticity in haddock. The commercially desirable gadid haddock inhabits broad regions of the North Atlantic in waters that range from 1–13°C (Scott and Scott, 1988). Whether the result of climate change (Jobling, 1997; Kennedy and Walsh, 1997; Rombough, 1997), natural environmental variation (Johnston, 2006), or changes in aquaculture protocols (Aiken, 2003), haddock are likely exposed to subtle changes in temperature during their embryonic development.

The present study will examine and integrate analyses of cellularity and fine-scaled cell-size distributions in both superficial and deep myotomes at multiple incubation temperatures over several crucial early life history phases and muscle developmental stages. This approach will yield a more complete picture of the reaction norm (Johnston, 2006), permitting a more thorough interpretation of muscle developmental strategy through ontogeny and its impacts on larval muscle phenotype.

Materials and methods

A single batch of fertilized eggs was collected following protocols as described (Martell et al., 2005) from haddock *Melanogrammus aeglefinus* L. broodstock held in temperature-controlled tanks at the St Andrews Biological Station (Canada). Zygotes were determined to be ~95% four-cell and ~5% eight-cell stage. The rearing system consisted of twelve 5 l flow-through incubators divided into four replicates for each of three temperatures, 4°C, 6°C and 8°C (±0.5°C) (see Martell et al., 2005). The batch of ~120 000 fertilized zygotes was divided evenly among the twelve rearing units. Once 50% hatch was

observed, the contents of each incubator were each carefully transferred to one of twelve 60 l flow-through polypropylene tanks with seawater set to 6°C. Following tank transfer, samples were collected according to procedures as detailed elsewhere (Martell et al., 2005). Beginning at 1 d.p.h., random samples of 10–15 yolk-dependent and exogenously feeding larvae were collected from each of the four replicate tanks per temperature. Daily sampling continued until 14 d.p.h. and once every week thereafter. Sampling was terminated when cumulative sampling attrition and mortality depleted all larvae from grow-out tanks (i.e. at 28 d.p.h. for 4°C and 6°C treatments but 21 d.p.h. at 8°C).

Sampled free-embryos and larvae were anaesthetized with 0.1 mg l^{-1} tricaine methanesulfonate (Sigma-Aldrich Canada Ltd, Oakville, Ontario, Canada) then fixed in seawater-buffered 4% formaldehyde. Five fixed specimens were selected at random consistently from one of the four replicate samples in each of the three larval grow-out temperature groups at 2, 7, 14, 21 and 28 d.p.h. (no 28 d.p.h. samples available from 8°C). Each specimen was individually embedded in glycomethacrylate resin – either JB4+ (Polysciences Inc, Warrington, PA, USA) or Technovit 7100 (Energy Beam Sciences, Agawam, Massachusetts, USA). Mounting procedures are detailed elsewhere (Martell et al., 2006). Cross-sections (2 μm) were cut at the level of the posterior gut using a Reichert-Jung Autocut 2040 microtome (Leica Canada, Ontario, Canada). Adjacent sections were stained with 1% Toluidine Blue O (Presnell and Schreibman, 1997) and modified Paragon stain (see Martin et al., 1966; Spurlock et al., 1966).

Images of each section were taken in triplicate using a Nikon 990 camera mounted on a Zeiss Photomicroscope III (Zeiss Canada, ON, Canada). All triplicate image groups were subsequently stacked and averaged to eliminate random colour-channel noise and enhanced (for cell membrane contrast) in Adobe PhotoShop (v. CS) using software plug-ins from Fovea Pro and Optipix (Reindeer Graphics, Asheville, NC, USA). Outlines of all muscle cells on one side of the dorsal epaxial myotome were digitally traced over the high-resolution averaged images in PhotoShop using a graphics tablet (Wacom, Vancouver, WA, USA). Separate drawing layers were created for both the superficial single cells and the deep cells. Each cellular outline layer was subsequently processed to create detached outlines of each cell, calibrated for magnification, and all cells counted and measured for equivalent diameter and area using Fovea Pro.

Statistics

The number and equivalent diameters of superficial and deep muscle cells, as measured by image analysis procedures, were log transformed and analyzed over time (categorical factor) by saturated two-way Model I ANOVA (P=0.05) and, due to significant interactions among factors, subsequently by one-way Model I ANOVA using GLM procedures (JMP software, SAS Institute, Cary, NC, USA). Tukey *post-hoc* multiple comparisons (P=0.05) were employed following ANOVA analyses. Equivalent diameters of superficial and deep muscle cells were binned into 2.5 μm categories for each specimen at each temperature and sampling period. The resulting frequency distributions were analyzed using a series of one-way ANOVAs (P=0.05) due to significant interactions among the main effects temperature, time and frequency class, with *post-hoc* Tukey HSD tests (P=0.05) (i.e. among frequency classes at temperature and day, among temperatures at day and frequency class, among days at temperature and frequency class). Total muscle cross-sectional area for each sampled larva was calculated as the sum of the products of cell number and mean cell area for each myofibre type. These data were analyzed by temperature and time using a saturated two-way ANOVA (P=0.05) and, due to a significant interaction between factors, subsequently by two one-way ANOVAs (i.e. by temperature and by time). Regressions of mean equivalent deep cell diameters as a function of total deep cell number were fitted to a logarithmic function of the form $\ln(D)=b+m(N)$ (where D represented the mean equivalent deep muscle cell diameter and N, the total number of deep muscle cells), using least-squares methods (JMP software). Regression slopes among temperature treatments were compared statistically using standard methods (Zar, 1999).

Results

The post-hatch larval haddock epaxial myotome consisted of two visually distinct groups of myofibres (Fig. 1). As fibre types were not characterized by immunocyto- or histochemical techniques but by location and structure, these two types were termed ‘superficial’ and inner or ‘deep’ fibres [terminology after Koumans and Akster (Koumans and Akster, 1995)]. Superficial fibres were observed initially in a peripheral myotomal monolayer that extended from dorsal to lateral apexes, lateral to the developing neural tube (Fig. 1). These cells had a rectangular shape, high mitochondrial volume density (mitochondria determined by cellular architecture and staining), and a peanut-shaped arrangement of myofibrils set against the interior-facing surface of the myofibre. By day 21 at 6°C (day 14 at 8°C and not observed by 28 days at 4°C), this monolayer had begun to retreat from the dorsal and ventral apexes, which coincided with an intrusion of deep cell proliferation (Fig. 2). The remainder of the myotome was occupied by deep myofibres. These cells were irregular or polygonal in shape and possessed a radial arrangement of myofibril bundles, and a low volume density of subsarcolemmal (most) and intermyofibrillar (few) mitochondria (Figs 1, 2). During subsequent development, many small myofibres (<2.5 μm in diameter) were observed either at the dorsal apexes of the epaxial myotome or between the superficial and deep muscle layers (Fig. 2). Each small myofibre possessed a heterochromatic nucleus and, in some cases, a small bundle of myofibrils. During larval development, the time to the appearance of these small cells was found to vary negatively with temperature, such that they were first observed in the apical proliferation zone at ~0.5 d.p.h. in the

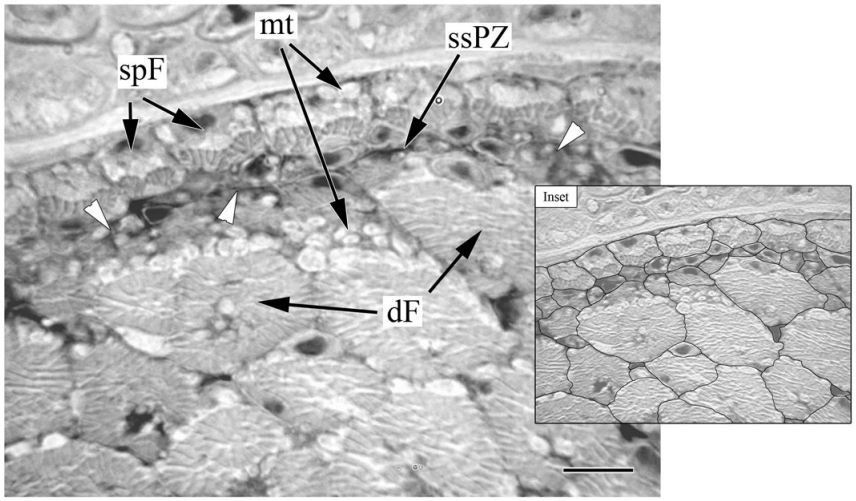

Fig. 1. Cross-section of the outer epaxial musculature of a 2 d.p.h. larval haddock incubated at 6°C, stained with Paragon, demonstrating distinctive cellular and structural features. Bar, 20 μm. spF, superficial myofibres; dF, deep myofibres; ssPZ, superficial-deep septum proliferation zone; mt, mitochondria; white arrowheads, small new myofibres. For descriptive purposes, the inset (50% size of the main figure) illustrates the outlines of individual myofibres.

8°C treatment embryos but not until ~2.7 d.p.h. in the 4°C embryos. Similarly, the proliferation zone between the superficial and deep muscle cells was first distinguished at ~1.4 d.p.h. in 8°C embryos but not until about ~2.7 d.p.h. at 4°C. The appearance of these two ontogenetic events was inversely proportional to incubation temperature and occurred just prior to exogenous feeding at 8°C, but following exogenous feeding at 4°C, and corresponded to a general delay in the timing of cellular proliferation with decreased temperature. Unlike the deep muscle proliferation zone, small superficial muscle fibres, when observed late in the experiment, were found exclusively in the proliferation zone located at the horizontal myoseptum, interior of the lateral line (Fig. 3).

Superficial myofibres

There was no significant difference in myofibre mean equivalent diameters among temperature groups (Fig. 4A) except at 21 d.p.h. ($F_{2,16}$=7.33, P=0.006), wherein the 8° group had a significantly greater mean myofibre diameter than that in the 6°C group (Tukey, P=0.05). The mean diameter of the 4°C treatment was not significantly different from either the 6° or 8°C treatments. There was a significant change in mean equivalent diameter over time in the 6°C group ($F_{4,23}$=4.57, P=0.007); however, overall this was neither consistent nor directional as there was an initial significant decrease in diameter followed by an increase at 21 d.p.h. (Tukey HSD, P=0.05). The 8°C group also showed a significant ($F_{3,15}$=6.78, P=0.004) and directional increase in diameter over time with the final 21 d.p.h. sample having the greatest mean diameter over time (Tukey HSD, P=0.05).

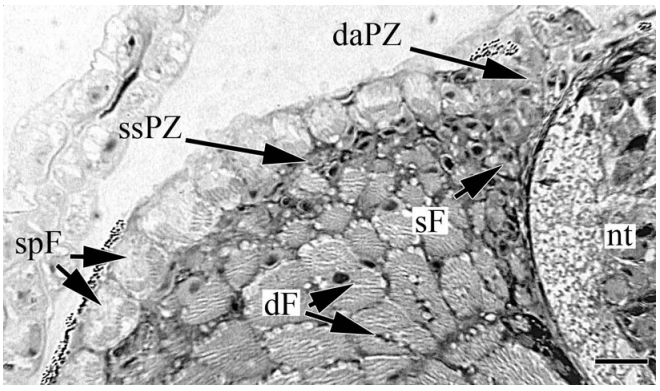

Fig. 2. Cross-section of the dorsal epaxial musculature at the level of the posterior gut from a 21 d.p.h. larval haddock incubated at 6°C, stained with Paragon, indicating distinctive cellular features. Bar, 30 μm. spF, superficial myofibres; dF, deep myofibres; sF, small myofibres; mt, mitochondria; ssPZ, superficial-deep septum proliferation zone; daPZ, dorsal apical proliferation zone; nt, neural tube.

Numbers of superficial cells (Fig. 4B) increased with temperature ($F_{2,53}$=4.62, P=0.014) and over time ($F_{3,53}$=23.58, P<0.0001) for the first 21 d.p.h. (i.e. over four sampling periods). The 6°C treatment had significantly greater numbers of superficial cells than the 4°C treatment beyond 7 d.p.h. At day 28, the 6°C treatment was also significantly greater than the 4°C treatment ($F_{1,10}$=6.15, P=0.033). However, the temperature effect was not directly proportional as the numbers of myofibres in the 8°C group was not significantly different from either the 4°C or 6°C (Tukey, P=0.05).

The multiple analyses of myofibre diameter frequency distributions among temperatures, days and frequency classes (Fig. 5) were integrated and revealed general trends among experimental groups. There was the tendency for the superficial myotome to be composed of a greater number of myofibres in the larger size-classes and of a broader range of fibre sizes that were proportional to both temperature and time. This was especially apparent in the 6°C and 8°C groups. Within each frequency distribution, by temperature and day, the peak of the distribution was significantly different from the 'shoulders' of the distribution, which were also significantly different from the outer distribution classes (Tukey HSD, P=0.05). The equivalent diameters at the distribution maxima at all temperatures and days were found to occur at ~15 μm (mode between frequency class 12.5–15 μm and 15–17.5 μm),

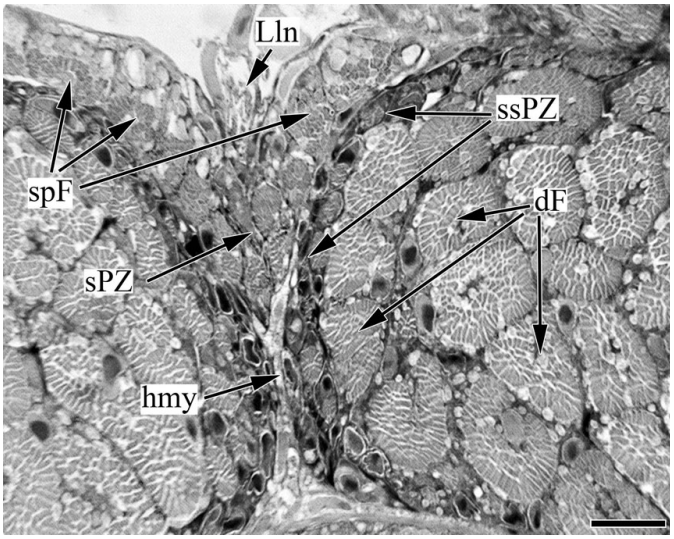

Fig. 3. Cross-section of the dorsal epaxial musculature at the level of the posterior gut of a 28 d.p.h. larval haddock incubated at 6°C, stained with Paragon, illustrating distinctive myotomal features about the horizontal myotome. Bar, 30 μm. spF, superficial myofibres; dF, deep myofibres; sPZ, superficial proliferation zone; ssPZ, superficial-deep septum proliferation zone; hmy, horizontal myoseptum; Lln, lateral line nerve.

although the distributional range increased over time. The 4°C distributions exhibited no significant difference over time in any frequency class and there was no observed significant increase in distribution range. In the 6°C treatment at 14 d.p.h., the frequencies expressed in the 10–12.5 μm and 12.5–15 μm classes were significantly greater compared to earlier samples (Tukey HSD, P=0.05). Following this, at 21 and 28 d.p.h., frequencies in the larger size classes (i.e. >20 μm) displayed a significant increase over time. The distributions in the 8°C fish were also observed to have significant maxima (P<0.05) at 12.5 μm but this was found at 2 and 14 d.p.h., while it shifted to 15 μm at 7 and 21 d.p.h. For both 6°C and 8°C treatments there was a significant and gradual increase in distributional breadth or range and in the number of larger myofibres over time (Tukey, P=0.05) (arrows on Fig. 5). This was especially apparent in the 6°C treatment at 28 d.p.h.

Deep myofibres

Immediately following hatch, mean muscle cell diameter was greatest in the 8°C treatment at 2 d.p.h. ($F_{2,10}$=4.6, P=0.038) and 7 d.p.h. ($F_{2,12}$=5.5, P=0.02) and decreased with reduced temperature (Tukey, P=0.05) (Fig. 6A). However, this pattern was reversed in later ontogeny with the largest mean cell diameters found the 4°C treatment at both 21 d.p.h. ($F_{2,16}$=12.22, P=0.0006) and 28 d.p.h. ($F_{1,9}$=33.67, P=0.0003) (no 8°C sample at 28 d.p.h.). This pattern reversal (i.e. negative effect of temperature on mean myofibre diameter) confirmed the significant interaction between temperature and time ($F_{7,56}$=9.86, P<0.0001) first noted in the initial two-way ANOVA. Analyses also showed that at 14 d.p.h., the midpoint in the post-hatch experimental timeline, there was no significant difference (P=0.66) in deep muscle mean diameters among temperature groups. The data (Fig. 6A) also displayed a trend of greater decrease in mean cell size with temperature such that the highest temperature group showed the greatest rate and depth of decline, further clarifying the significant interaction noted above. All temperature groups showed a significant effect of time on mean cell size: 4°C ($F_{4,19}$=6.73, P=0.0011), 6°C ($F_{4,22}$=51.31, P<0.0001), 8°C ($F_{3,15}$=32.08, P<0.0001).

Changes in myofibre number over time for the first 21 days of sampling (common for all temperature treatments), differed for each temperature treatment (two-way ANOVA, temperature×time interaction; $F_{11,47}$=2.73, P=0.023). Examination of the interaction plots revealed that there was an earlier increase in myofibre numbers as a function of increased incubation temperature. Overall, subsequent one-way ANOVAs for each temperature showed an increase in myofibre number over time, but *post-hoc* analyses revealed specific differences. In the 4°C treatment, the mean total number of myofibres in the 28 d.p.h. sample was significantly greater from only the 14 d.p.h. sample; all other samples were similar

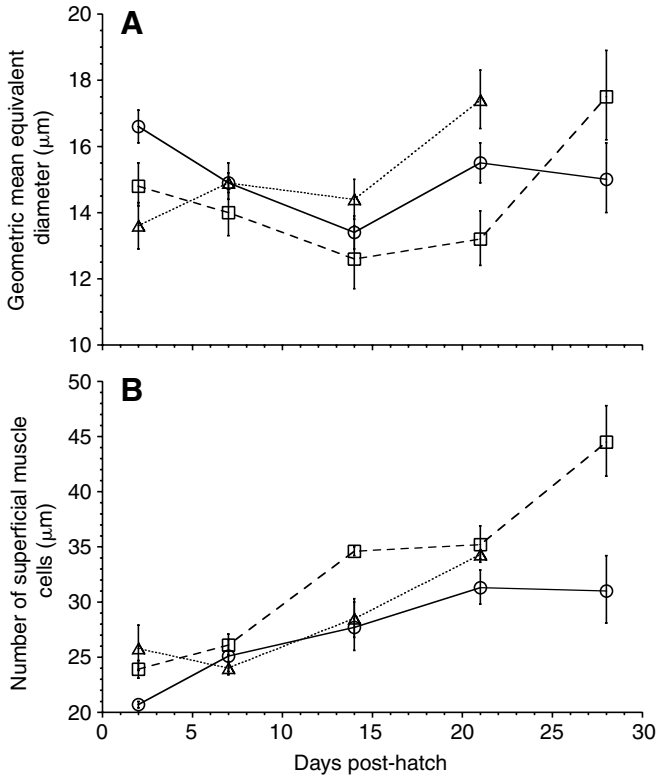

Fig. 4. Effect of incubation temperature on the post-hatch (A) geometric mean equivalent diameter and (B) mean number of superficial myofibres (note log y-axis) in one quadrant of the dorsal epaxial myotome in haddock reared in a constant 6°C post-hatch environment. Values are means ± 1 s.e.m. (N=71). Incubation temperature treatments: 4°C, circles; 6°C, squares; 8°C, triangles.

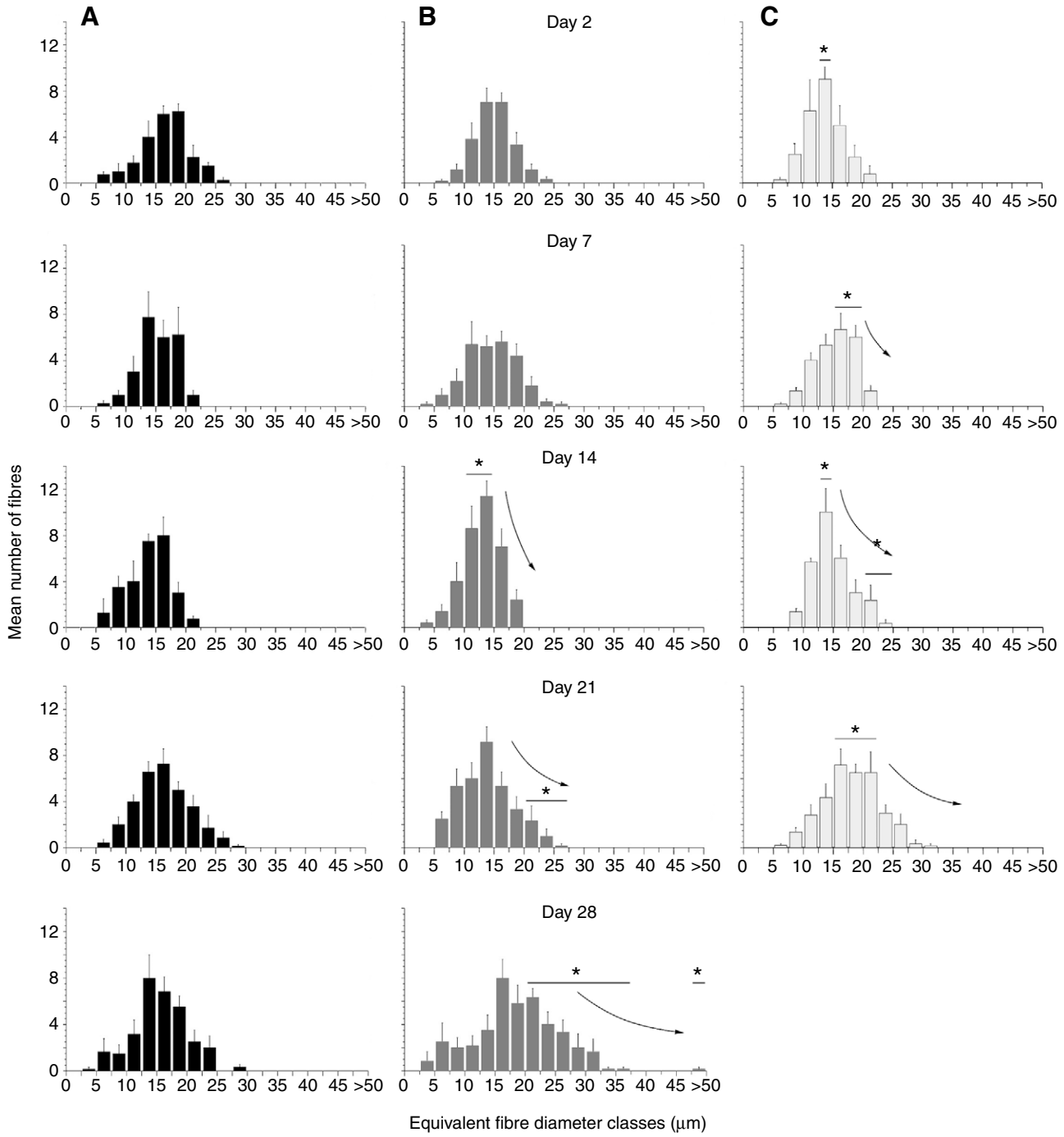

Fig. 5. Frequency distributions of superficial equivalent diameters of all myofibres present in one quadrant of the haddock dorsal epaxial myotome binned into 2.5 μm diameter classes from 2 until 28 d.p.h. among the three incubation temperature treatments (A, 4°C; B, 6°C; C, 8°C). Values are means ± 1 s.e.m. (N=71) *Bars marked by a horizontal line are significantly different between consecutive days (P<0.05). Arrows (→) indicate temporal trends and are described in text.

statistically (Tukey, P=0.05). However, for both 6°C and 8°C treatments the mean total number of myofibres in the final sample (28 and 21 d.p.h., respectively) were significantly greater than all those of all other sampling periods, which were all similar statistically (Tukey, P=0.05). Analyses also revealed that muscle cell number did not differ significantly among temperature groups at either 2 d.p.h. ($F_{2,10}$=1.38, P=0.30), 7 d.p.h. ($F_{2,10}$=2.54, P=0.12) or 14 d.p.h. ($F_{2,9}$=2.12, P=0.18). However, by 21 d.p.h. there significantly greater cell numbers with increased temperature ($F_{2,16}$=3.51, P=0.05) such that 8°C had significantly greater numbers (c. 25%) than either the 4°C or 6°C treatments (Tukey, P=0.05). Similarly, by 28 d.p.h. the

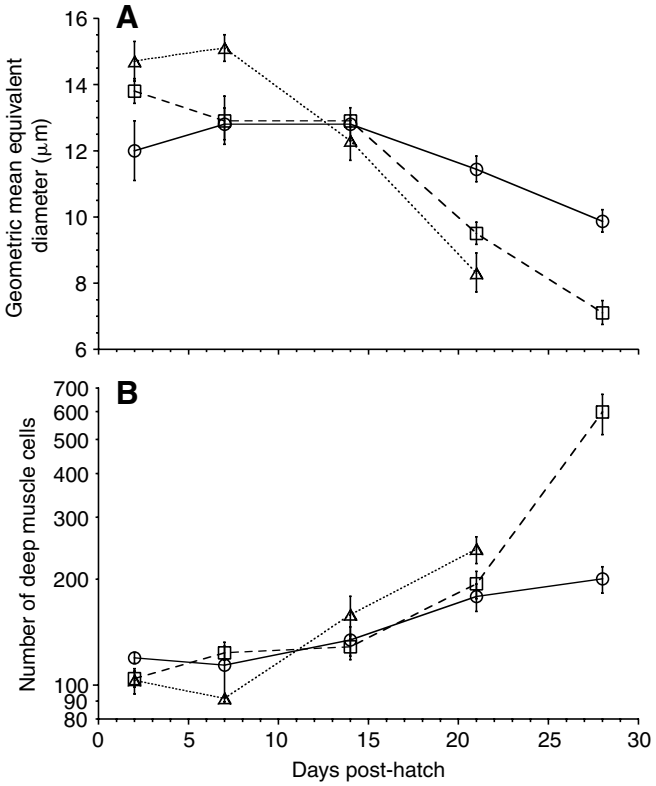

Fig. 6. Effect of incubation temperature on the post-hatch (A) geometric mean diameter and (B) mean number of deep myofibres (note log *y*-axis) in one quadrant of the dorsal epaxial myotome in haddock reared in a constant 6°C post-hatch environment. Values are means ± 1 s.e.m. (N=71) Incubation temperature treatments: 4°C, circles; 6°C squares; 8°C, triangles.

two remaining temperature treatments were significantly different (P=0.0005, $F_{1,9}$=28.43) with larvae from the 6°C incubation possessing almost threefold greater numbers of deep muscle cells compared to those from 4°C (Tukey, P=0.05).

Analysis of frequency distributions of deep muscle cell equivalent diameters revealed that distributions contained one or two maxima that were significantly greater (Tukey, P=0.05) than adjacent size classes (Fig. 7). Higher temperature resulted in an increase in the number of myofibres in the size classes, an earlier appearance of both larger and smaller muscle cells, and an increased distributional range. Specifically, analysis of frequencies by temperature among frequency classes and days showed a trend of increased mean numbers of myofibres in size class over time. Significant differences were found among frequency maxima between days at each temperature beginning at 21 d.p.h. at 4°C, 14 d.p.h. at 6°C, and 7 d.p.h. at 8°C. Of importance was the significant increase (P<0.05) in the numbers of the smallest cells (0–7.5 μm in diameter) between 14 and 21 d.p.h. at 8°C, ~21 d.p.h. at 6°C and ~28 d.p.h. at 4°C. Analysis by day among size classes and temperature revealed that the size distribution in the 8°C group at 14 and 21 d.p.h. differed significantly from those of the 6°C and 4°C groups (which were similar statistically). At 28 d.p.h., however, the distribution at 6°C differed significantly from that of 4°C. The significant increases in frequencies in the 0–5 μm size classes observed at 21 and 28 d.p.h. at 6°C were found to be similar to that observed in 8°C at 14 and 21 d.p.h., respectively.

Logarithmic regressions (Fig. 8) describing the relationship between deep muscle cell size (equivalent diameter) and number were significant for each temperature treatment (4°C, P=0.026; 6°C, P<0.001; 8°C, P<0.001). The regression of the 4°C data accounted for some of the observed variation (r^2_{adj}=0.17), while those of 6°C and 8°C accounted for much of the variation (r^2_{adj}=0.62 and 0.78, respectively). The rate of decline in cell diameter was found to be directly proportional to incubation temperature and cell number. Multiple comparisons between cell number–diameter regressions showed that the slopes of the 4°C and 6°C regressions were not significantly different ($q_{2,47}$=0.136; P>0.5) but were significantly less than that of 8°C (4°C, $q_{2,39}$=5.65; P<0.001; 6°C, $q_{2,42}$=6.59; P<0.001).

There was no significant difference in total muscle cross-sectional area among temperature treatments (i.e. 4°C, 6°C, 8°C) during the common first 21 days of sampling (Tukey, P=0.05). Also, for each temperature, there was no significant difference in total area over the first 21 days of sampling (P>0.05). Analysis of 4°C and 6°C treatments over the full 28 days of sampling (no samples of 8°C at 28 d.p.h.) revealed that there was a significant overall increase in muscle area in the 6°C treatment ($F_{4,27}$=21.3572, P<0.0001), but again no significant change in the 4°C group (P>0.05).

Discussion

Differences in incubation temperature were shown to have significant and persistent effects on epaxial muscle development and growth in post-hatch yolk-dependent and exogenous-feeding haddock larvae. Importantly, the persistent alterations in muscle cellularity for superficial and deep myofibres followed different developmental programs. As the post-hatch grow-out environments for all embryonic temperature treatments were controlled at a constant 6°C, differences observed herein could be attributed solely to the effects of temperature during embryonic development with no confounding effects. Devoto et al. (Devoto et al., 1996) proved that there were separate populations of myogenic progenitor cells (MPC) for superficial and deep myofibres, and Johnston (Johnston, 2006) argued that increased fibre number during early myogenesis would result from the promotion of MPC proliferation over terminal differentiation, a possible result of changes in molecular signals in response to environmental alteration. A reduction in MPCs in association with reduced myofibre recruitment has been observed in Atlantic salmon (Johnston et al., 1999). Although changes in cellularity associated with differences in incubation temperature have been reported in other studies of marine, freshwater and anadromous fish (for reviews, see Koumans and Akster, 1995; Johnston, 2001; Johnston, 2006), the duration and extent of the persistence reported herein is new. Persistent effects of

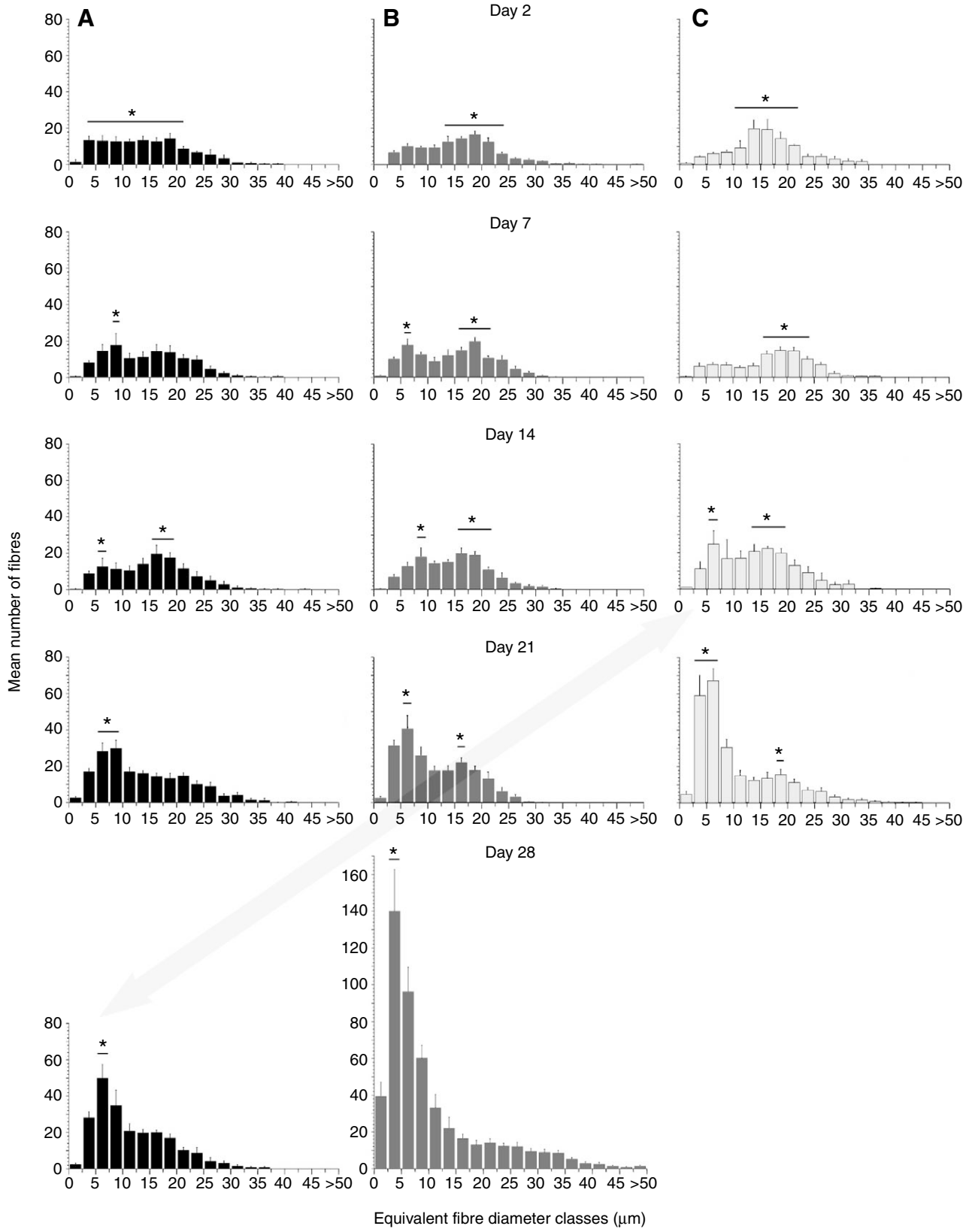

Fig. 7. Frequency distributions of superficial equivalent diameters of all myofibres present in one quadrant of the haddock dorsal epaxial myotome binned into 2.5 μm diameter classes from 2 until 28 d.p.h. among the three incubation temperature treatments (A, 4°C; B, 6°C; C, 8°C). Values are means ± 1 s.e.m. (*N*=71). *Bars marked by a horizontal line are significantly different between consecutive days ($P<0.05$). The diagonal grey arrow indicates the temporal shift in the occurrence of the hyperplastic event and is described in text.

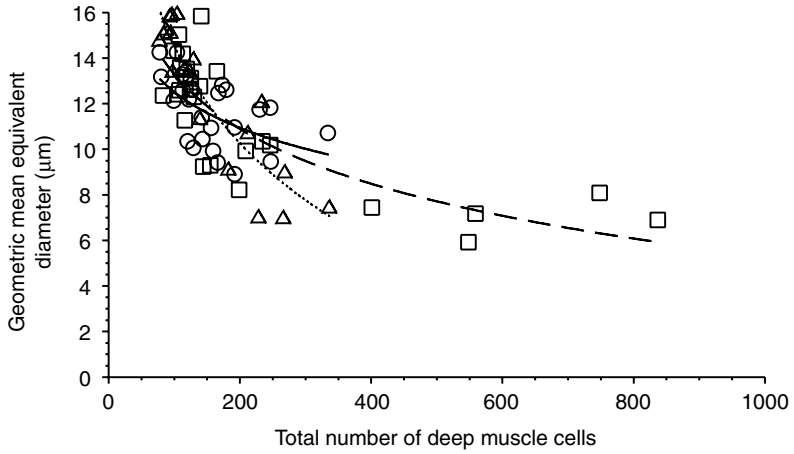

Fig. 8. Logarithmic regressions describing the relationship between total number of deep myofibres and their mean equivalent diameter in one quadrant of the post-hatch larval dorsal epaxial myotome among the three incubation treatments (4°C, 6°C, 8°C). Circles, 4°C ($\ln y=2.60–0.001x$, $r^2_{adj}=0.17$); squares, 6°C ($\ln y=2.63–0.001x$, $r^2_{adj}=0.62$); triangles, 8°C ($\ln y=3.00–0.003x$, $r^2_{adj}=0.79$). Logarithmic regressions were fitted to raw data by least-squares methods. All regressions were significant ($P<0.05$).

embryonic temperature on post-hatch muscle cellularity beyond initiation of exogenous feeding have been demonstrated in only a few studies such as in turbot (*Scophthalmus maximus* L.) for 26 d.p.h. (Gibson and Johnston, 1995), herring (*Clupea harengus* L.) for almost 150 d.p.h. (Johnston et al., 1998) and Danube bleak (*Chalcalburnus chalcoides mento* Agassiz) for up to 80 d.p.h. (Stoiber et al., 2002). However, unlike the present study, the initial former study reared turbot through the post-hatch experimental period at the same temperature as incubation (i.e. 12°C and 16°C) and both latter studies employed gradually rising temperatures in the post-hatch environment to simulate natural conditions. In addition, studies that have encompassed a narrower ontogenetic range have proposed, but not been able to demonstrate, the occurrence of this phenomenon: Atlantic salmon *Salmo salar* L. (Nathanailides et al., 1995; Johnston et al., 2000a; Johnston et al., 2000b), sea bass *Dicentrarchus labrax* L. (Ayala et al., 2000), and Atlantic cod *Gadus morhua* L. (Galloway et al., 1998).

Superficial myotome

Superficial muscle exhibited similar cellularity patterns among all temperature groups, with an increase in cellular recruitment and, to some extent, size across temperature and time (Fig. 4A,B). A continuous low-level recruitment of new small cells and growth of existing myofibres was observed in all treatments over time, as illustrated by the consistent unimodal distribution among temperature groups with larger cell size class frequencies increasing over time (Fig. 5). This resulted in an increased distributional range in weeks 3 and 4 that also progressed with temperature (arrowed trends in Fig. 5). Danube bleak also displayed a unimodal pattern of cell size distributions unaffected by embryonic temperature treatments (Stoiber et al., 2002), with a similar delay in the superficial proliferation event until long after this was noted in deep muscle. Throughout the present experiment, superficial epaxial muscle was maintained in a monolayer extending from the dorsal apex to the horizontal myoseptum (Figs 1–3). There was no indication of the subsequent retreat of the monolayer to form a wedge-shaped superficial muscle zone at the level of the horizontal myoseptum, a characteristic of superficial muscle development and prelude to gill functionality (El-Fiky and Wieser, 1988). However, outside the time frame of the present study, the wedge-shaped superficial proliferation zone was present in haddock muscle at 50 d.p.h. (sample not large enough to be included within this study). The superficial monolayer is thought to act as the medium for gas exchange during early ontogeny until the gills have developed sufficiently to take over this function. This development has been reported to be a function of larval size (El-Fiky and Wieser, 1988; Koumans and Akster, 1995; Rombough, 2002) although Wells and Pinder (Wells and Pinder, 1996) concluded that gill development in Atlantic salmon was more closely related to developmental phase than to size (mass). El-Fiky and Wieser (El-Fiky and Wieser, 1988) also reported that the species that had the longest delay before exhibiting free-swimming behaviour had the longest period wherein gill development was delayed and the superficial layer served as the respiratory organ (El-Fiky et al., 1987; Koumans and Akster, 1995). This would imply that gill functionality was a requirement for free-swimming behaviour. Given that the entire myotome is still functioning aerobically in early larval phases (El-Fiky and Wieser, 1988; Wieser, 1995), the occurrence of the superficial proliferation event may signal the development of a juvenile/adult muscle structure, the advent of an anaerobic metabolic function in the deep muscle zone (Wieser, 1995), and an active free-swimming lifestyle. The slow continual addition of cells and increase in mean cell size observed as a function of increased temperature herein, may be simply proportional to and a function of the increased growth rate also reported to be proportional to increased incubation temperature (Martell et al., 2005). Thus, the observed increase in superficial muscle growth with temperature would maintain the monolayer coverage with its crucial respiratory function over the gradually increasing myotome volume.

Deep myotome

Deep myofibre zone development (Figs 1, 2) was affected significantly by embryonic temperature (Fig. 6), such that the extent of the proliferation of small new myofibres was

positively influenced by increased incubation temperatures. In addition, the onset of the major proliferative event (Fig. 7) was advanced with higher temperatures. Both of these responses were also directly proportional to incubation temperature and were maintained despite the lack of temperature differences in the post-hatch environment. Although modifications in larval deep muscle cellularity as a function of incubation temperature change have been reported widely in the literature they are consistent neither in direction nor in pattern (reviewed by Johnston, 2006). This inconsistency may be a consequence of a failure to properly establish the reaction 'norm' as discussed (Johnston, 2006), but may also be a function of differences among the genomes within such a diverse group of freshwater, anadromous and marine species. Detailed analysis of the fine-scale cell-size distribution patterns herein revealed that the temperature-related proliferation event strongly affected both cellularity and cell size distributions (Figs 6, 7). This event was confined primarily to the dorsal proliferation zones and was characteristic of the 'stratified hyperplasia' phase of muscle growth (Rowlerson and Veggetti, 2001; Rescan, 2005). The associated reduction in mean cell size and increase in number as well as the bimodal distribution of cell sizes (Fig. 7) was reflective of that proliferation. Bimodal distributions associated with proliferation have also been reported for sea bass (Ayala et al., 2000), Atlantic salmon (Johnston et al., 1999; Bjørnevik et al., 2003), and for Danube bleak (Stoiber et al., 2002). However, detailed analyses of the changes in the distributions herein, indicated that the proliferation event was advanced by approximately 7 days for each 2°C increment in incubation temperature (illustrated by the arrow in Fig. 7). Muscle in herring (Vieira and Johnston, 1992), plaice (*Pleuronectes platessa* L.) (Brooks and Johnston, 1993), sea bass (Ayala et al., 2000) and cod (Hall and Johnston, 2003), and in culture (Fauconneau and Paboeuf, 2001), have exhibited increased proliferation in response to increased incubation temperature. In addition, an embryonic stem cell line (HEW) cultured from haddock reared for this study exhibited significantly greater growth at increased temperatures (approx. threefold after 14 days; 4°C *vs* 12°C) (Bryson et al., 2006). However, the opposite effect following hatch was observed for cod (Galloway et al., 1998), Atlantic halibut *Hippoglossus hippoglossus* L. (Galloway et al., 1999), whitefish *Coregonus lavaretus* L. (Hanel et al., 1996) and Atlantic salmon (Usher et al., 1994).

How do we reconcile these different observations? Is there a fundamental strategy underlying both sets of responses? Increased incubation temperatures for Atlantic salmon resulted in increased hyperplasia in later ontogeny associated with increased growth (Higgins and Thorpe, 1990; Usher et al., 1994); also swim-up fry from Atlantic salmon embryos incubated at greater temperatures grew faster during the post-exogenous feeding phase (Peterson and Martin-Robichaud, 1989). Atlantic salmon expressed a differential incubation temperature-related effect on muscle growth strategies such that at hatch Atlantic salmon from lower incubation temperatures had more and larger muscle fibres, but in alevins from greater incubation temperatures, hyperplasia was the more dominant muscle growth process during the endogenous-feeding phase (Johnston and McLay, 1997).

Lifestyle might be an important consideration in understanding overall muscle growth strategy among fish species (Usher et al., 1994). Specifically, this could refer to the length of the yolk-dependent stage, the lack of a requirement for coordinated swimming activity, and the subsequent requirement for swimming necessitated by the need for prey capture and predator avoidance. These conditions are similar to lifestyle characteristics argued to be of significance for superficial muscle growth (*op. cit.*). Muscle growth differences among species and within species among phenotypes may be a function of the duration of the yolk-dependent stage. The three-stage yolk absorption process in embryonic and yolk-dependent haddock was variously condensed and accelerated in a manner directly proportional to incubation temperature (Martell et al., 2005). It may be that this advance triggered the earlier onset of the hyperplastic event associated with increased incubation temperatures observed herein (Fig. 7). In species that have a long yolk-dependent stage, such as the 30–90 days for Atlantic salmon (Peterson et al., 1977) or the 50 days for halibut (Galloway et al., 1999), significant recruitment of deep muscle fibres was observed either towards the end of or following the complete absorption of the yolk (Johnston and McLay, 1997; Galloway et al., 1999). Within this yolk-dependent larval stage, hypertrophy was the dominant process. In those species with a very short yolk-dependent stage, such as 7–14 days at 8°C to 4°C, respectively, for haddock (Martell et al., 2005), or similarly in cod (Hall and Johnston, 2003) and Danube bleak (Stoiber et al., 2002), hyperplasia is the dominant growth process very soon after hatch and the end of yolk-dependency. The initiation of exogenous feeding has been argued to be a myogenic event, characterized by increased hyperplasia (Stoiber et al., 2002). Species such as turbot (Gibson and Johnston, 1995) and curimatã-paću *Prochilodus marggravii* Walbaum (Brooks et al., 1995) hatch in a primitive developmental stage but possess a small yolk. For these species, muscle hyperplasia is also delayed until after rapid development and yolk utilization. We propose that comparisons among species regarding muscle growth would be better made with respect to the depletion of the yolk reserves and the onset of exogenous feeding.

Hyperplasia and growth strategy

The overall developmental strategy followed in response to differences in temperature was also manifested in the significant differences among logarithmic cellularity relationships (i.e. number *vs* size), which illustrated a developmental strategy that favoured increased proliferation (i.e. greater slope/hyperplasia) over hypertrophy at greater incubation temperatures (Fig. 8). However the question remains, what governed the preference of hyperplasia as the favoured strategy for muscle development under situations of increased overall growth and as a result of increased incubation temperatures? The fastest-growing fish expressed the greatest

hyperplasia (Weatherley and Rogers, 1978; Weatherley et al., 1979; Weatherley et al., 1980). Nathanailides et al. (Nathanailides et al., 1995) proposed that larvae with fewer but larger fibres would have less potential for subsequent hypertrophic growth compared to those with more numerous and smaller cells. The increase in cellular recruitment associated with increased temperature herein encapsulated a strategy of increasing functional units in early ontogeny, upon which to build by hypertrophy in subsequent life history. Hanel et al. (Hanel et al., 1996) reported that, compared to hypertrophy, hyperplasia was an energetically more expensive cellular growth process. In addition, overall production efficiency of protein and new tissue increased with temperature, despite the increase in both routine metabolic rates and cost of growth (Hanel et al., 1996; Pedersen, 1997). Higgins and Thorpe (Higgins and Thorpe, 1990), citing von Bertalanffy (von Bertalanffy, 1960), argued that the decreased surface area:volume ratio associated with increased cellular growth limited cellular physiological processes. They also noted, citing Goss (Goss, 1966), that growth required to maintain organ physiological capacity within the growing organism depended primarily upon the reproduction of its functional units, such as cells or tissues. Weatherley and Rogers (Weatherley and Rogers, 1978) proposed that the application of von Bertalanffy's hypothesis would result in the fastest growth being achieved by means of hyperplasia, which was shown to be correct (Higgins and Thorpe, 1990; Weatherley et al., 1979; Weatherley et al., 1980). It was also argued that decreased energy intake, a function of smaller body size and reduced temperature, would compromise nuclear division (e.g. hyperplasia) but not necessarily hypertrophy (Usher et al., 1994). They also proposed that protein synthesis associated with hypertrophy might be the more efficient process by which to increase growth.

Immediately following hatch, individuals from greater temperature treatments had the greatest mean superficial and deep fibre diameters but, with the earlier onset of proliferation, had the smallest mean deep and largest superficial fibre sizes by 28 d.p.h. In a concurrent study of haddock development, it was found that myofibrillar densities in embryonic and larval phases also decreased with increasing temperature (Martell et al., 2006). Arendt proposed that the interaction between growth and development determined the tissue-specific (i.e. muscle) developmental strategy (Arendt, 2000). Further, he argued that either an earlier onset or later offset of differentiation would produce smaller, and more numerous and but less mature, fibres in faster-growing individuals. However, this rapid early muscle growth may limit subsequent muscle growth in later ontogeny. In an analysis of total muscle cross-sectional area in the post-hatch larvae herein, it was shown that although there was no significant difference among temperature treatments during the first 21 d.p.h., muscle did show significant growth in the last 7 days proportional to greater incubation temperature. Arendt also argued that the reduced myofibre maturity (i.e. reduced or delayed myofibrilargenesis) could result in a potential loss of muscle power compared to that of similarly sized but slower-growing individuals (Arendt, 2000). Studies have shown that compared to slower-growing conspecifics, faster-growing individuals, which for transgenic salmon (Farrell et al., 1997) expressed increased myofibre proliferation, exhibited reduced swimming performance (Kolok and Oris, 1995; Farrell et al., 1997; Arendt, 2000; Hill et al., 2000). However, herring reared at increased incubation temperatures, although possessing reduced myofibril densities (Vieira and Johnston, 1992), showed greater fast-start swimming performance and a 'more developmentally advanced sub-carangiform style' (Johnston et al., 2001), demonstrated by reduced yaw during normal swimming undulations. It remains to be discovered in future studies how incubation temperature-affected differences in muscle cellularity and maturity may affect muscle power, and metabolic and swimming performance.

The change in muscle development associated with increased rearing temperatures, as noted herein and previously (Martell et al., 2006), synchronized with the advanced development of the eye, gut, blood cells and notochord, and the increased growth rates and earlier depletion of the yolk (Martell et al., 2005), seem to point to an earlier onset in the capability to feed exogenously and avoid/escape predation. Perhaps as well, in higher energy environments the acceleration of and advance in hyperplasia is energetically favoured and results in a greater growth potential. These two points are worthy of much further investigation in future studies.

The authors acknowledge the financial and logistical support provided to D.J.M. and EAT by Fisheries and Oceans Canada (St Andrews Biological Station, St Andrews, NB, Canada). J.D.K. thanks the Natural Sciences and Engineering Research Council (Discovery Grant) for funding. We also are grateful to E. Heikkila for her contributions to this project and to two anonymous reviewers for their helpful comments on the manuscript. All experimental protocols followed Canada Council on Animal Care guidelines for the care and use of experimental animals.

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