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RESEARCH ARTICLE

Temperature-dependent modification of muscle precursor cell behaviour is an underlying reason for lasting effects on muscle cellularity and body growth of teleost fish

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SUMMARY

Temperature is an important factor influencing teleost muscle growth, including a lasting ('imprinted') influence of embryonic thermal experience throughout all further life. However, little is known about the cellular processes behind this phenomenon. The study reported here used digital morphometry and immunolabelling for Pax7, myogenin and H3P to quantitatively examine the effects of thermal history on muscle precursor cell (MPC) behaviour and muscle growth in pearlfish (*Rutilus meidingeri*) until the adult stage. Fish were reared at three different temperatures (8.5, 13 and 16°C) until hatching and subsequently kept under the same (ambient) thermal conditions. Cellularity data were combined with a quantitative analysis of Pax7+ MPCs including those that were mitotically active (Pax7+/H3P+) or had entered differentiation (Pax7+/myogenin+). The results demonstrate that at hatching, body lengths, fast and slow muscle cross-sectional areas and fast fibre numbers are lower in fish reared at 8.5 and 13°C than at 16°C. During the larval period, this situation changes in the 13°C-fish, so that these fish are finally the largest. The observed effects can be related to divergent cellular mechanisms at the MPC level that are initiated in the embryo during the imprinting period. Embryos of 16°C-fish have reduced MPC proliferation but increased differentiation, and thus give rise to larger hatchlings. However, their limited MPC reserves finally lead to smaller adults. By contrast, embryos of 13°C-fish and, to a lesser extent, 8.5°-fish, show enhanced MPC proliferation but reduced differentiation, thus leading to smaller hatchlings but allowing for a larger MPC pool that can be used for enhanced post-hatching growth, finally resulting in larger adults.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/214/11/1791/DC1

Key words: muscle growth, hyperplasia, hypertrophy, fibre type, temperature, Pax7, fish muscle, muscle precursor cell.

INTRODUCTION

Muscle development in the ectothermic teleost fish is strongly modulated by ambient temperature. Certainly the most exciting among a variety of complex and partly contradictory effects in this context is the phenomenon of 'thermal imprinting'. This means that the temperature experienced during embryonic life is likely to determine some sort of 'organismic memory', thus exerting a lasting (perhaps life-long) influence on the animals' muscle structure and whole body growth. Within an individual species, this may lead to a situation in which fish that are large at hatching end up as small adults, and *vice versa*, as a result of their early thermal history. Early comprehensive descriptions of such effects date back to studies from the 1990s in which fibre sizes and nuclei densities were measured in Atlantic salmon and herring transferred to natural habitat conditions after having been reared at different temperatures (Nathanilides et al., 1995; Johnston et al., 1998). Since then, the phenomenon has turned out to be more widespread and variable among teleosts. Thermally-dependent persistent programming of muscle growth has been shown to occur in Atlantic salmon, *Salmo salar* (Nathanilides et al., 1995; Johnston et al., 2000; Johnston et al., 2003; Albokhadaim et al., 2007; Macqueen et al., 2008), halibut, *Hippoglossus hippoglossus* (Galloway et al., 1999), haddock, *Melanogrammus aeglefinus* (Martell and Kieffer, 2007), sea bass, *Dicentrarchus labrax* (Alami-Durante et al., 2007) and zebrafish, *Danio rerio* (Johnston et al., 2009). As with the (non-memory) effects of continued direct thermal influence on teleost myogenesis (reviewed by Johnston and Hall, 2004; Johnston, 2006), thermal imprinting has been shown to interact with teleost muscle differentiation and growth in a complex manner, affecting cellular variables in various ways. These effects seem to depend upon factors such as species-specific response patterns, the duration of the imprint and the developmental interval tested. For example, fast fibre hyperplasia in the larval and/or juvenile periods may be promoted by embryonic rearing at either moderately cold (Nathanilides et al., 1995; Johnston et al., 2003; Albokhadaim et al., 2007; Macqueen et al., 2008) or intermediate (Johnston et al., 2009) or warm (Galloway et al., 1999; Martell and Kieffer, 2007) temperatures within the species' thermal range. Also, the capacity to recruit new fast fibres in response to the annual rise of water temperature in spring is higher in sea bass juveniles that were reared at a low temperature (Alami-Durante et al., 2007).

Although the studies on this issue to date have certainly provided most valuable 'mosaic pieces' of information, our understanding of the process of thermal imprinting as a whole is rather incomplete.

This is mainly because only limited allowance has been made for the true spectrum of cellular and molecular mechanisms upon which, and by which, thermal imprinting is likely to act. In regard to cellular mechanisms, studies are frequently confined to effects on fast fibres, which constitute the dominant mass-generating (i.e. economically important) tissue. The effects on slow fibres, although certainly being of high importance in relation to endurance swimming and flesh quality (Johnston, 1999), have been paid much less attention. Their existence is, however, very probable in the light of strong non-memory responses to continued thermal impact (e.g. Egginton and Sidell, 1989; Galloway et al., 1998; Stoiber et al., 2002) and of some imprinting studies (Galloway et al., 1999; Albokhadaim et al., 2007).

Also, the circumstances under which thermal imprinting modulates the supply of myogenic precursor cells (MPCs) to the growing muscle are incompletely understood. Combined evidence from two studies of Atlantic salmon suggests that MPC densities in juvenile fast muscle are highest when the embryos are imprinted within an 'optimum thermal range', whereas colder conditions and heating lead to reduced densities (Johnston et al., 2000; Johnston et al., 2003). This begs the question whether the phenomenon of thermal imprinting is simply caused by different rates of MPC accumulation until the end of the imprinting period or (also) depends on changes of the proliferation and differentiation rates of these cells later in development. Expression profiles of regulatory microRNAs in thermally imprinted zebrafish point towards sustained responses, including determination of when the adult fish finally stop investing MPCs into hyperplasia and continue muscle growth only by hypertrophy (Johnston et al., 2009).

A promising approach to clarify some of the uncertainties related to temperature-induced cellularity change in fish muscle comes from current advances in determining the origins of teleost MPCs. Recent work has demonstrated that early myogenic development in fish involves cell supply from a dermomyotome (DM), just as in the amniotes (Devoto et al., 2006; Stellabotte and Devoto, 2007). It has been shown that after primary myotome formation, it is DM cells that account for stratified hyperplasia (second phase myogenesis) at the dorsal and ventral extremes of the myotome and at the lateral boundaries of their fast muscle domains (Steinbacher et al., 2006; Steinbacher et al., 2007; Hollway et al., 2007; Stellabotte et al., 2007). Myogenic cells from the posterior DM lip that are transiently stockpiled in the lateral fast muscle continue to promote stratified growth even into the larval period, after the dissociation of the DM (Steinbacher et al., 2008; Marschallinger et al., 2009). Together, this seems to account for the well-known initial surge of teleost body growth until mosaic hyperplasia (third phase) is upregulated during the larval period (muscle growth during this phase is termed 'mosaic' because small new fibres become inserted between larger pre-existing fibres, thus giving cross sections a mosaic appearance). It is very plausible that this third phase of myogenesis also receives MPCs from the DM (Hollway et al., 2007), again as in the amniotes (cf. Gros et al., 2005; Relaix et al., 2005). From these results, it is also probable that during this phase the teleost myotome is additionally infiltrated by myogenic cells that do not immediately differentiate but remain as quiescent 'satellite cells' required for muscle growth and repair during later life (Hollway et al., 2007). All this identifies the DM as the pivotal growth engine of teleost myogenesis and suggests that the DM is the prime target for thermal influence on this process in the teleost embryo. Temperature modulation of DM cell dynamics would then be the fundamental determinant of the life-long 'imprinted' thermal effects on muscle phenotype and fish growth.

However, despite these advances, knowledge is still missing about whether the thermal memory effect involves modulation of as yet uninvestigated factors such as a possible genuine heterogeneity of the myogenic DM cells, or their detachment behaviour, migratory spread and proliferation–differentiation balance.

The present study investigates the long-term effects of embryonic temperature on muscle growth in the cyprinid pearlfish *Rutilus meidingeri* Heckel in the developmental period from hatching to adult stage. This is achieved using a combined approach involving, (1) digital analysis of muscle cellularity at high definition on semithin sections to unravel patterns of hyperplastic and hypertrophic growth, (2) an evaluation of myonuclear densities in fast fibres to monitor nuclear domain size and nuclei uptake during hypertrophy and (3) immunostaining-based characterisation of the proliferation–differentiation balance of MPCs.

MATERIALS AND METHODS

Rearing and sampling of fish

Investigations were carried out on European pearlfish *Rutilus meidingeri* Heckel (Cyprinidae) between the hatching and yearling stage. Parent animals were caught at the natural spawning sites in the Seeache River, a tributary to the Attersee, a lake at the northern rim of the Austrian Alps in April 2008. To perform imprinting, artificially inseminated eggs were kept at three different temperatures [8.5, 13 and 16°C (±0.3°C each)] until hatching. Temperatures chosen are all within the species' range of thermal tolerance during embryogenesis and may, depending upon weather conditions, all occur at the natural spawning sites (Siligato and Gumpinger, 2005). From hatching onwards, all fish were kept under the same (season-dependent) water temperatures as prevailing in the water supplies of the Scharfling Institute, where the experiments were carried out. Temperatures rose from 14.5°C in larval life (May–July) to 16°C towards the larva–juvenile transition (August, September), fell to 12°C during the following winter and rose again to 14.5°C in spring 2009. Fish were kept under natural photoperiod and fed *ad libitum* with live plankton until mid-August and then switched to commercial fish food of appropriate particle size. Rates of water flow and recirculation were kept equal for all thermal groups at any particular phase of the experiment. Random samples ($N \geq 5$ per group and technique) were taken of the following developmental stages: (1) hatching [0 days post-hatching (d.p.h.)], (2) onset of exogenous feeding at approximately 11 d.p.h., (3) mid-larval period at 40 d.p.h., (4) advanced larval stage at 82 d.p.h., (5) juvenile stage at 195 d.p.h. and (6) yearling stage at 360 d.p.h. All fish were overanaesthetised with MS-222 (3-aminobenzoic acid ethyl ester; Sigma, Vienna, Austria) and body lengths were measured ($N \geq 10$ at 0 and 11 d.p.h. and ≥ 30 at all further stages sampled) prior to any further treatment. Larger animals were cut into smaller pieces to allow sufficient fixative penetration.

Light microscopy and morphometry

Specimens for semithin sectioning for light microscopy and morphometry (five animals per thermal group and developmental stage) were immersion-fixed at 4°C using Karnovsky's paraformaldehyde–glutaraldehyde fixative (Karnovsky, 1963) diluted to half-concentration with PBS, postfixed in 1% osmium tetroxide (3 h, 4°C), dehydrated in a graded series of ethanol and embedded in glycid ether 100 epoxy resin (Serva, Heidelberg, Germany). Semithin transverse sections (1.5 μm) at the level of the anus were cut on a Reichert (Vienna, Austria) Ultracut S microtome and mounted on glass slides. For clearer visualisation of cell borders, the osmium contrast was amplified by treatment

with 1,4-paraphenylendiamine (Merck-Schuchardt, Hohenbrunn, Germany) (Böck, 1984). Results were photographed through a Reichert Polyvar microscope.

For morphometric analysis of cellularity, contours of the muscle cells within one epaxial and one hypaxial quadrant of the trunk per individual were digitally traced from the semithin sections and measured using an adapted version of ImageJ software. To precisely analyse the local variations in hyperplastic growth within the myotome (cf. Steinbacher et al., 2006), quadrants were divided into distinct subregions according to a body-size-dependent schedule (Fig. 1): apical, lateral (stratified growth) and central (mosaic growth) in fast muscle, apical and central (stratified growth) in slow muscle. From the 82 d.p.h. stage onwards, fibre size measurement in the central fast muscle region was confined to a representative transect (Fig. 1). Fast fibre numbers were always determined for the entire central subregion. Slow muscle subdivision was begun when a multi-layered slow muscle wedge at the level of the horizontal septum had formed (from 82 d.p.h. onwards). To exclude measuring errors of the ImageJ software, a few implausibly small values ($\ll 2\,\mu m^2$) were deleted from the data set.

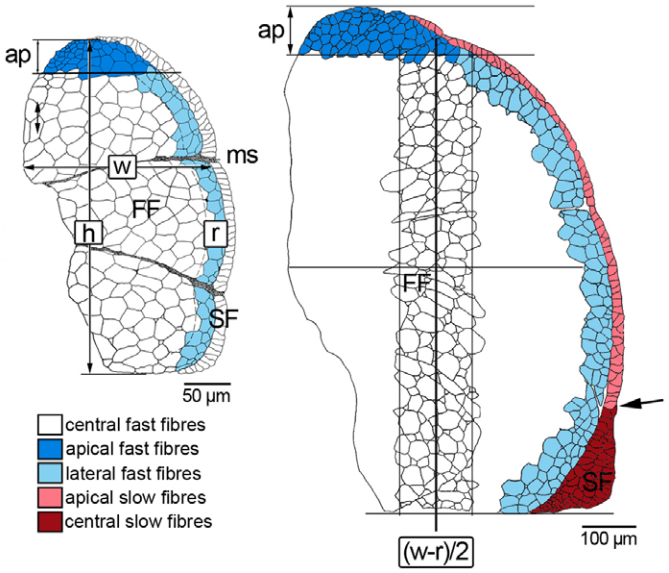

Fig. 1. Zonal subdivision of fast and slow muscle for fibre size measurement as exemplified for epaxial quadrants of pearlfish at onset of exogenous feeding (left) and at 82 d.p.h. (right): *w*, width (maximum mediolateral extension of fast muscle); *h*, height (maximum dorsoventral extension); *r*, boundary of lateral fast muscle growth zone delineated at *r*=1/10*w*; *ap*, apical growth zone, with the boundary set at *ap*=1/10*h*. From 82 d.p.h. onward, examination of the central fast fibre zone was confined to a representative transect centred at (*w*–*r*)/2, width defined as (*w*–*r*)/4, height as *h*–*ap*. Also beginning at 82 d.p.h., the slow muscle domain was subdivided into monolayered apical zones and a multilayered central zone at the horizontal septum (indicated by arrow). FF, fast fibres; ms, myoseptum; SF, slow fibres.

Myonuclei counting

The protocol for evaluation of myonuclear densities was modified from that of Brack et al. (Brack et al., 2005). Briefly, five to ten myotomes were dissected from the anal area of fish previously fixed in 4% paraformaldehyde at 4°C for 8 h. After removal of the slow fibre layer and the adhering skin, with fine forceps, the myotomes were incubated in 40% NaOH for 5–10 min, rinsed several times in PBS + 0.1% Tween 20, and pipetted up and down vigorously to separate individual fibres. Fibre nuclei were stained with 10 µg ml^{-1} Hoechst 33258 (Sigma). The nuclei of 40–50 fibres per individual fish, from five individuals per developmental stage and thermal group were counted, and fibre lengths and diameters measured using Photoshop CS3. Results are expressed as the ratio of myonuclei number to fibre volume ($1r^2\pi$), hereafter termed nuclei/volume ratio.

Immunolabelling

Immunolabelling of transverse cryostat sections was used to analyse the temperature-dependent variation in paired box transcription factor 7-positive (Pax7+) MPCs within the DM and the myotome. For immunolabelling procedures, freshly killed animals were coated with cryostat embedding medium (Tissue-Tek OCT compound; Miles, Elkhart, IN, USA), cryofixed by plunging into 2-methylbutane cooled to near its freezing point (158°C) with liquid nitrogen, and sectioned as described by Stoiber et al. (Stoiber et al., 2002). All sectioning was confined to positions close to the anal vent.

To discriminate between different subsets of MPCs (proliferating or differentiating cells), the Pax7 antibody was combined with either a marker of proliferation (H3P) or a marker of myogenic differentiation (myogenin). The following primary antibodies were used: monoclonal mouse anti-chicken Pax7 IgG1 (1:20; DSHB, Iowa City, IA, USA), polyclonal rabbit anti-phospho-histone H3 (H3P, 1:100; Upstate, Lake Placid, NY, USA) and polyclonal rabbit anti-rat myogenin (Myog, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Alexa 488-conjugated goat anti-rabbit (1:800) and Alexa 546-conjugated goat anti-mouse IgG1 (1:800; Invitrogen, Lofer, Austria) were applied as secondary antibodies. Nuclei were counterstained with Hoechst 33258. Detailed protocols are provided in Steinbacher et al. (Steinbacher et al., 2006).

Numbers of MPCs were evaluated in the area immediately lateral to the slow fibres (site occupied by the DM, the external cell layer in the embryos) and in the lateral fast muscle immediately underneath the slow fibres (lateral stratified growth zone). At hatching, analysis of these two areas was undertaken in the entire epaxial and hypaxial quadrants. In all other stages, evaluation was confined to individual somites, i.e. areas delimited by two successive myosepta (cf. Steinbacher et al., 2008). One set of specimens was used to determine numbers of Pax7+/H3P– and Pax7+/H3P+ cells (10–20 somites or myotomes per individual, resulting in a total of approximately 60 somites or myotomes from five to seven individuals per developmental stage and thermal regime). A second set of specimens was used to evaluate numbers of Pax7+/Myog– and Pax7+/Myog+ cells within the lateral fast muscle (cell numbers given per 100 µm along the lateral fast muscle surface on myotome cross sections). Note that the central areas of the myotomes (mosaic growth zone) were not included into Pax7+ cell evaluation because, with a very few exceptions, such cells were not detected at these sites (see also Steinbacher et al., 2006; Marschallinger et al., 2009).

Statistical analysis

Standard software packages (SPSS 16; SPSS Inc., Chicago, Illinois, USA) were used for statistical analyses. In all cases, a Kolmogorov–Smirnov test was applied to test for normal distribution of the data. Levene's test was used to ascertain that the homogeneity of variances assumption was satisfied. Differences between temperature groups were analysed using a one-way ANOVA combined with Levene's test for homogeneity of variances. Fisher's least significant difference (LSD) procedure was used to evaluate differences between groups where equivalence of variances was

assumed, a Games–Howell test was used if variances were heterogeneous. Differences were considered statistically significant at $P \leq 0.05$.

RESULTS

Fish growth

Onset of substantial hatching (approximately 10% of embryos hatched) was achieved at 7, 11 and 23 days post-fertilisation (d.p.f.) in the 16, 13 and 8.5°C rearing groups, respectively. Mean body size (standard length; SL) at hatching was significantly larger in the 16°C group than in the 13 and 8.5°C groups ($P<0.01$ each; Fig. 2). Hatchlings at 13°C, in turn, were significantly larger than hatchlings at 8.5°C ($P<0.01$; Fig. 2). During the following larval period, the 13°C-reared fish (13°C-fish) and the 8.5°C-fish both exhibited substantial catch-up growth, which resulted in, at 40 d.p.h. onwards, the 13°C-fish always being the largest (Fig. 2). The 8.5°C-fish, being the smallest at hatching, were approximately the same size as the 16°C-fish at 40 and 195 d.p.h. and were larger than the 16°C-fish at 82 and 360 d.p.h. (Fig. 2).

Muscle cellularity

At hatching, the total cross-sectional area (c.s.a.) of the fast muscle within one half of the trunk was significantly larger in fish incubated at 16°C than in fish incubated at either 8.5 or 13°C ($P<0.01$ each; Fig. 3A), which have similar fast muscle c.s.a. At 40 d.p.h., the 16°C-fish continued to have the largest fast muscle c.s.a., but the differences to the other thermal groups were no longer statistically significant. The situation changed at 82 d.p.h., when 16°C-fish fast muscle c.s.a. was smaller than that of both other groups and values for the 8.5°C-fish were transiently largest. Further development was characterised by a long-lasting surge of fast muscle growth in the 13°C-fish, which attained the largest fast muscle size at the late juvenile stage (195 d.p.h., $P<0.001$) and keep it until the yearling stage (360 d.p.h., $P<0.001$). By contrast, the 8.5°C-fish at these two stages had the smallest fast muscle c.s.a. In other words, the increase in fast muscle mass in the three thermal groups in the time between hatching and 360 d.p.h. was 171-fold for 13°C-fish, 135-fold for 8.5°C-fish, but only 84-fold for 16°C-fish.

Similar to fast muscle, slow muscle c.s.a. at hatching was larger in the 16°C-fish than in the 13 and 8.5°C-fish ($P=0.01$ for 13°C-fish but not significant for 8.5°C-fish; Fig. 3B). At 40 and 82 d.p.h., there were no significant differences between the slow muscle c.s.a. of the three groups. At 195 and 360 d.p.h., the largest slow muscle c.s.a. was found in the 13°C-fish. This means that the increase in slow muscle mass between hatching and 360 d.p.h. was 41-fold in 13°C-fish, but only 28-fold and 25-fold in 8.5 and 16°C-fish, respectively. The relatively small increase in the 8.5°C-fish corresponds with the observation that these fish have a very high proportion of slow muscle at hatching, a typical effect of cold acclimation in cyprinids (Stoiber et al., 2002). This is also reflected in the low fast/slow muscle ratio of the 8.5°C-fish at hatching (Fig. 3C). Regression lines of fast/slow ratio data of the whole period of investigation lie close together with only a slight difference in slope. There were no statistically significant differences between both fast and slow muscle c.s.a. from the epaxial and hypaxial quadrants in all fish analysed (not shown).

The other determinant of muscle cellularity is muscle fibre number, which evolved along a roughly similar course. At hatching and at 40 d.p.h., there was a trend towards more fast fibres in the 16°C-fish (Fig. 4A), although this was not statistically significant. A clear increase in fast fibre numbers was then seen in all temperature groups at 82 d.p.h. when mosaic hyperplasia had begun. This development was similarly intense in 8.5 and 13°C-fish, but clearly weaker in 16°C-fish. From 195 d.p.h. onwards until the end of the investigation, fast fibre numbers of 13°C-fish were always higher than those of the two other thermal groups (Fig. 4A). Increase in fast fibre number over the entire period investigated (from hatching to 360 d.p.h.), taken as a measure of hyperplastic growth intensity, was 10.0-fold for 13°C-fish (corresponding to an increase of 8.0 fibres per day), but only 7.8-fold for 8.5°C-fish (6.5 fibres per day) and 7.3-fold for 16°C-fish (6.6 fibres per day). In contrast to fast fibres, there were almost no differences in slow fibre numbers between thermal groups in the time from hatching to 82 d.p.h. (Fig. 4B). The course of quantitative changes of these fibres was also similar for the three groups, with constant values until 40 d.p.h. followed by a sharp increase. Slow fibre numbers in both juveniles at 195 d.p.h. and yearlings at 360 d.p.h. were highest in the 13°C group. Plotting the ratio of fast to slow fibre numbers against the total number of fibres demonstrated the similar courses of the numbers of the two fibre types in the thermal groups (Fig. 4C). A minor divergence is only seen with the 8.5°C-fish, which had a slightly higher proportion of slow fibres in large fish.

Plotting the numbers of fast fibres within size (c.s.a.) classes showed that 16°C-fish clearly had more larger fibres at hatching (peak density at 100 μm^2) than the fish of both other thermal groups (peak densities at 60–70 μm^2; Fig. 5A). During ongoing development, these distributions became quite similar for all groups, except that there were more small fibres of $\leq 50\ \mu m^2$ in 8.5 and 13°C-fish than in 16°C-fish in most of the stages (Fig. 5B–E; fibres of $\leq 50\ \mu m^2$ were assumed to be recently formed – upper limit of size category was arbitrary). At 360 d.p.h., proportional data continued to indicate similar trends for all three groups (Fig. 5E) and plotting of absolute fibre numbers per size class showed that 13°C-fish had the most fibres over almost the entire size range; only the 8.5°C-fish had more smaller fibres ($\leq 50\ \mu m^2$; Fig. 5E′). By contrast, 16°C-fish had the fewest fibres, indicating lowest rates of both hypertrophy and hyperplasia.

Numbers of small fibres ($\leq 50\ \mu m^2$) were also examined as a measure of how thermal imprinting affects the stratified and mosaic hyperplastic growth modes. This was undertaken separately within the three fast muscle zones defined in Fig. 1, because stratified growth occurs in the apical and lateral zones, and mosaic growth in the central zone. Results demonstrate that at hatching, there were

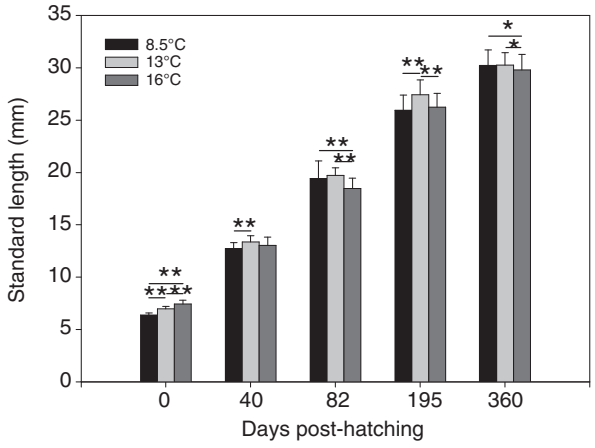

Fig. 2. Mean body lengths of thermally imprinted fish groups throughout the experiment; Error bars indicate s.d.; asterisks indicate significant differences (*$P \leq 0.05$ and **$P \leq 0.01$) using Fisher's LSD test.

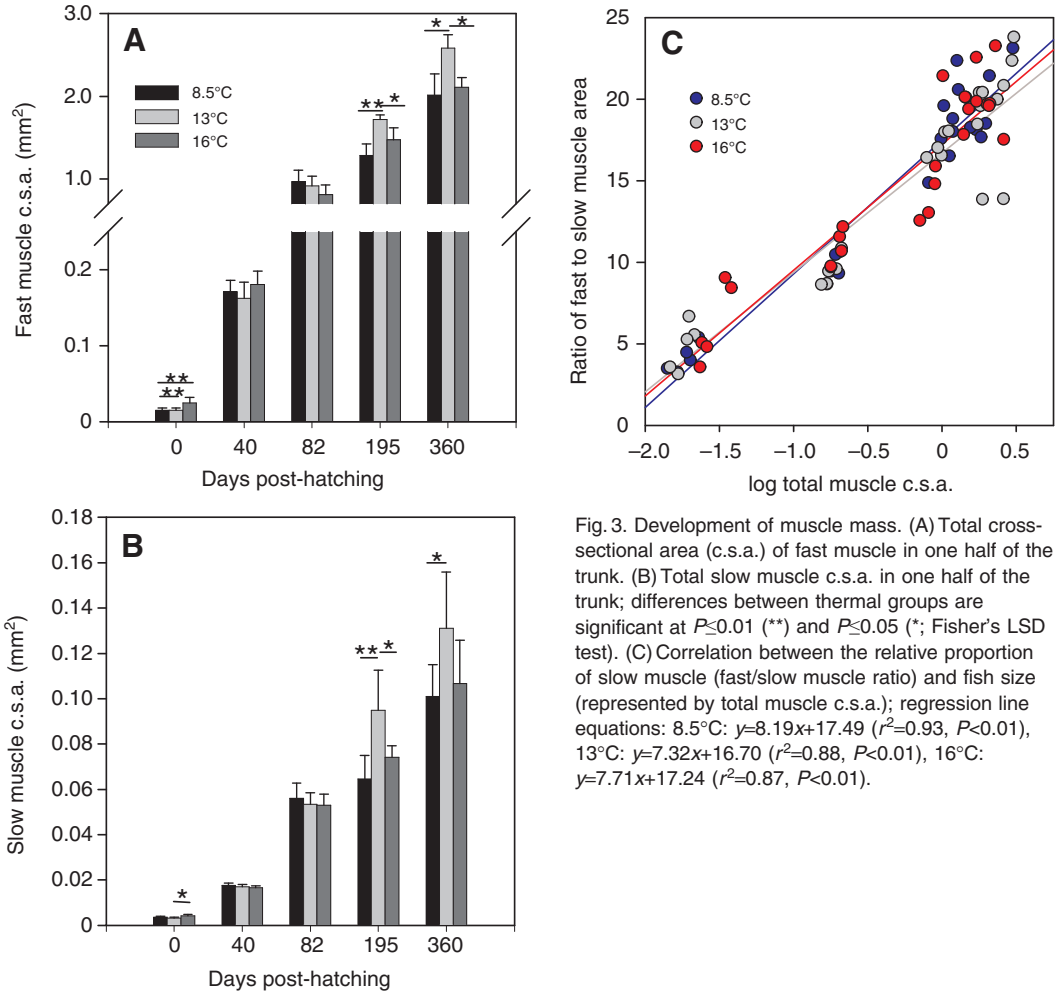

Fig. 3. Development of muscle mass. (A) Total cross-sectional area (c.s.a.) of fast muscle in one half of the trunk. (B) Total slow muscle c.s.a. in one half of the trunk; differences between thermal groups are significant at $P \leq 0.01$ (**) and $P \leq 0.05$ (*; Fisher's LSD test). (C) Correlation between the relative proportion of slow muscle (fast/slow muscle ratio) and fish size (represented by total muscle c.s.a.); regression line equations: 8.5°C: $y=8.19x+17.49$ ($r^2=0.93$, $P<0.01$), 13°C: $y=7.32x+16.70$ ($r^2=0.88$, $P<0.01$), 16°C: $y=7.71x+17.24$ ($r^2=0.87$, $P<0.01$).

slightly more small fibres in the apical growth zone in 16°C-fish, and in the lateral growth zone in the 8.5°C-fish (supplementary material Fig. S1A,B). There were more small fibres in the central zone of both 8.5 and 13°C-fish than of 16°C-fish ($P<0.01$ each, supplementary material Fig. S1C). In terms of relative amounts, 16°C-fish had lower proportions of small fibres in all three zones (apical: 87.9%; lateral: 91.6%; central: 39.7%) than 8.5 and 13°C-fish (apical: 97.3 and 97.2%; lateral: 98.7 and 99.1%; central: 76.7 and 70.7%, respectively). During subsequent development, fast fibre formation in the apical zone appeared to be unaffected by thermal history, except at the 82 d.p.h. stage, when 8.5°C-fish launched a sudden and conspicuous surge in the number of small fibres, which had no parallel in the 13 and 16°C-fish ($P=0.02$ and $P<0.01$, respectively, supplementary material Fig. S1A–C). This differs from the situation in the lateral zone. In this zone, there was a clear trend towards fewer small fibres in the 16°C-fish than in the 8.5 and 13°C-fish. Similar to the apical zone, 8.5°C-fish showed an increase in fast fibre recruitment at 82 d.p.h., which was followed by a prominent decline at 195 d.p.h. In most stages, the lowest numbers of the small fibres in the central zone were observed in the 16°C-fish, whereas highest numbers were, with one exception, always found in the 8.5°C-fish (supplementary material Fig. S1C). Note that the values in supplementary material Fig. S1C from the 82 d.p.h. stage onwards are fibre numbers from a dorsoventral transect (see Fig. 1). Note also that transect-derived numbers at the 82 d.p.h. stage are higher than those from full-myotome measurement at 40 d.p.h., thus indicating a period of intense mosaic hyperplasia in the period in between.

To specifically examine slow muscle hyperplasia, numbers of small fibres were separately analysed in the whole slow fibre layer until 40 d.p.h. and separately for two distinct zones (central, apical, see Fig. 1) in all following stages. From this it emerged that the fish of the 8.5°C group tended to have more small slow fibres than the fish of the other two thermal groups throughout the experiment. Zonal evaluation in the more advanced stages showed the same trend, quite homogeneously in the central zone and most distinctly in the apical zone at 195 d.p.h. ($P<0.01$ for 8.5 *vs* 16°C-fish; supplementary material Fig. S1D,E).

Mean c.s.a. of the 100 largest fast muscle fibres within one half of the trunk served as a measure of hypertrophy. Using this measure, it was found that the fast muscle fibres of 16°C-fish were clearly more hypertrophic than those of the 13 and 8.5°C-fish at hatching ($P=0.01$ each; Fig. 6A). However, this advance was subsequently compensated for by enhanced fast fibre growth in both of the other groups, so that at 195 and 360 d.p.h. 13°C-fish had the largest fibres. Size increase of these 100 largest fibres between hatching and 360 d.p.h., taken as a measure of hypertrophic growth intensity, was 27.7-fold for 8.5°C-fish (corresponding to an increase in mean fibre size of 5.0 $\mu m^2 day^{-1}$), 29.3-fold for 13°C-fish (5.5 $\mu m^2 day^{-1}$), but only 17.5-fold for 16°C-fish (4.8 $\mu m^2 day^{-1}$).

Analyses of myonuclear density reveal that at hatching, the 16°C-fish had a lower nuclei/volume ratio than 13 and 8.5°C-fish

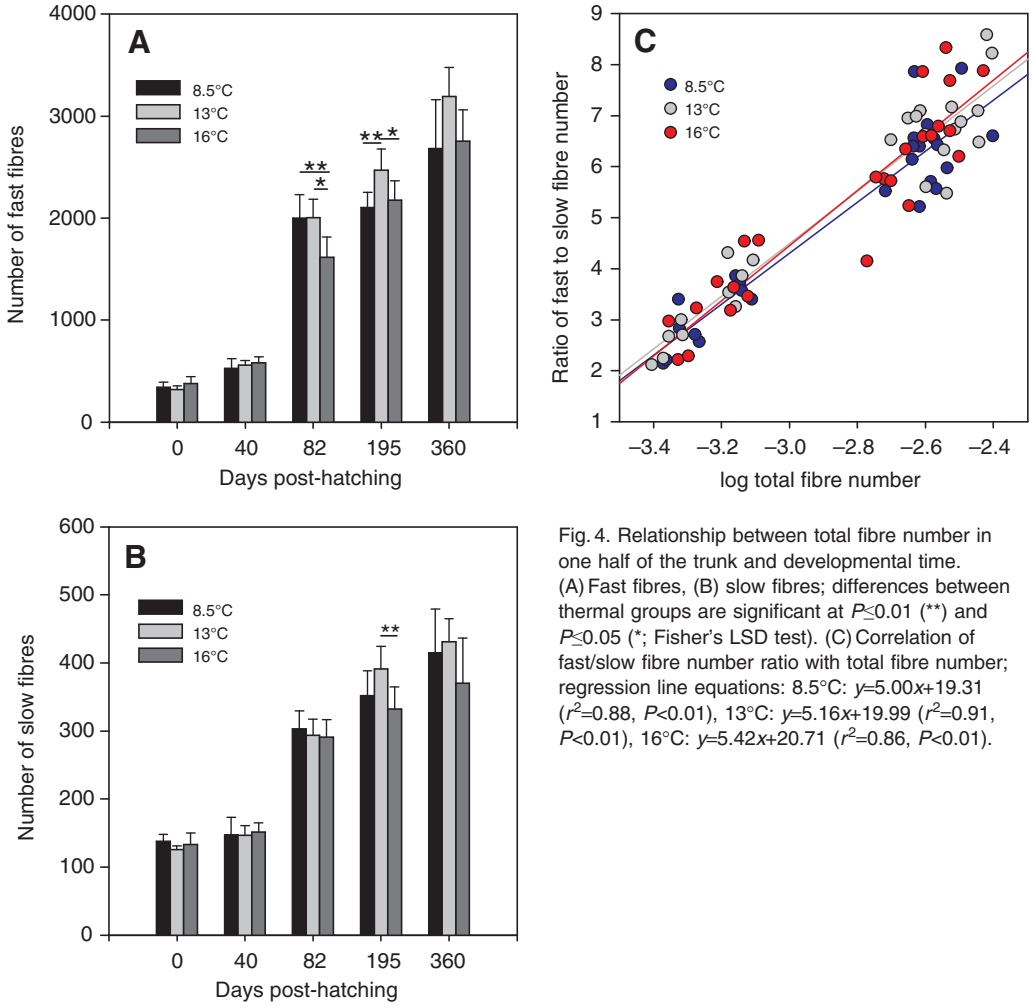

Fig. 4. Relationship between total fibre number in one half of the trunk and developmental time. (A) Fast fibres, (B) slow fibres; differences between thermal groups are significant at $P \leq 0.01$ (**) and $P \leq 0.05$ (*; Fisher's LSD test). (C) Correlation of fast/slow fibre number ratio with total fibre number; regression line equations: 8.5°C: $y=5.00x+19.31$ ($r^2=0.88$, $P<0.01$), 13°C: $y=5.16x+19.99$ ($r^2=0.91$, $P<0.01$), 16°C: $y=5.42x+20.71$ ($r^2=0.86$, $P<0.01$).

(Fig. 7A). Thus, at the end of the imprinting period, 16°C-fish had fewer nuclei per given volume (or larger fibres per given number of nuclei), i.e. larger nuclear domains than the fish of the two other thermal groups. Similar to the 'largest fibre' development, this situation became reversed during the post-imprinting period, with the lowest nuclei/volume ratios occurring in the 8.5°C-fish of all further stages investigated until 360 d.p.h. (Fig. 7B).

The 50 largest fibres within the slow muscle of one half of the trunk were measured to determine slow muscle hypertrophy. Similar to fast fibres, hypertrophy at hatching was most advanced in the 16°C-fish ($P<0.01$ *vs* both other groups; Fig. 6B). Subsequently, a period in larval development without significant differences (40 and 82 d.p.h.) gave rise to a phase in which large slow fibre hypertrophy was reduced in the 8.5°C-fish, which lasted until the end of the investigation ($P<0.01$ *vs* 13 and 16°C-fish at 195 d.p.h.; ANOVA test only at $P=0.07$ at 360 d.p.h.).

Muscle precursor cells

Molecular tracing of MPCs was undertaken to assess how patterns of precursor cell behaviour correlate with those of the temperature-induced modulation of cellularity. MPCs were detected by immunolabelling for Pax7, a reliable and currently the most widely used marker of such cells in vertebrates (e.g. Buckingham and Relaix, 2007). Co-staining for H3P served to assess whether thermal history also affects the mitotic activity of these cells. All data are given in relative numbers (labelled cells per 100 µm of myotome cross-section length). Results demonstrate that by the time of hatching, Pax7+ MPCs almost exclusively occurred within the DM epithelium constituting the lateral covering of the slow muscle layer of the myotomes (hence, cell counts at this stage were confined to the DM of one trunk quadrant; see also Fig. 8A). Highest numbers of Pax7+ MPCs were found in the DM of the 13°C-fish ($P<0.01$ *vs* 16°C-fish; Fig. 9A), 16°C-fish contained the lowest numbers ($P<0.01$ *vs* 8.5 and 13°C-fish). Double-immunolabelling for Pax7 and H3P showed that the DM of the 13°C-fish also had the largest fraction of proliferating (Pax7+/H3P+) MPCs (6.5%, compared with 4.4 and 4.5% in the 16 and 8.5°C-fish, respectively). Note that differentiating (Pax7+/Myog+) MPCs within the DM were only rarely observed and not quantified. Immunostaining results further revealed that throughout the larval and juvenile periods, Pax7+ cells were present at the position of the previous DM layer (i.e. along the lateral surface of the myotome; Fig. 8B). Similar to the hatching stage, the 13°C-fish had the most Pax7+ cells throughout the investigated period (Fig. 9A). In contrast to the hatching stage, relative numbers of H3P+ (mitotically active) Pax7+ cells at the original DM site no longer significantly varied between the three thermal groups, indicating that proliferation kinetics of such residual DM cells are directly temperature-dependent rather than pre-determined by thermal imprinting.

From 40 d.p.h. onwards, immunostaining demonstrated that Pax7+ MPCs also occurred within the lateral fast muscle growth zone beneath the slow muscle layer (Fig. 8B), a main target of MPC

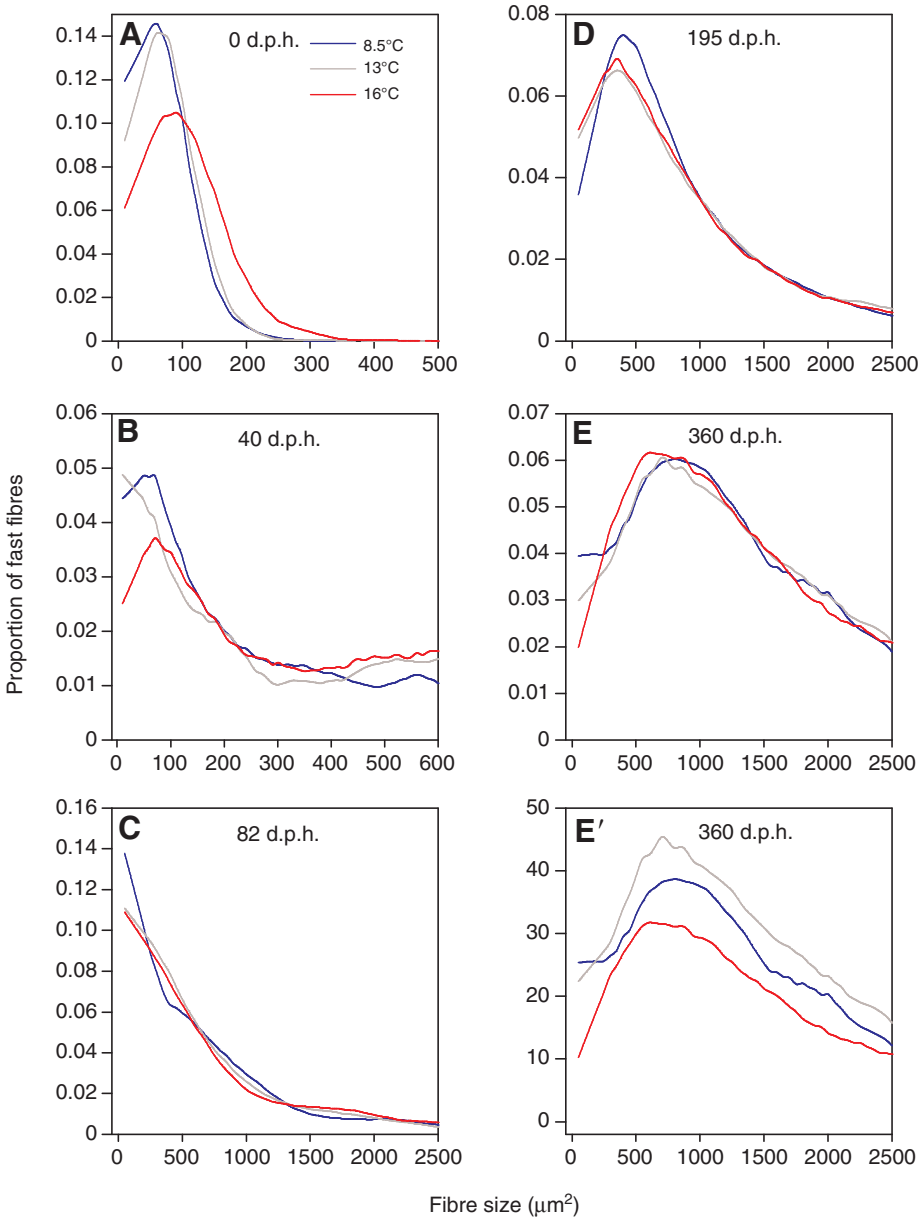

Fig. 5. Smoothed distributions of fast fibre c.s.a. of the developmental stages investigated: (A) 0 d.p.h.; (B) 40 d.p.h.; (C) 82 d.p.h.; (D) 195 d.p.h.; (E,E′) 360 d.p.h. Fibre numbers in c.s.a. classes are given as a proportion of total number of fibres in A–E, but in absolute numbers in E′. Curves were fitted to the data points by a Sigma-Plot-9-based local smoothing technique using polynomial regression and weights computed from the Gaussian density function.

translocation from the DM (Steinbacher et al., 2008; Marschallinger et al., 2009). The results are in agreement with the above findings for non-translocated (DM-residing) MPCs, in that the 16°C-fish always had the lowest numbers of Pax7+ cells (not shown). Co-staining for H3P was used to analyse whether the early thermal experience of the fish influences MPC proliferation at this site. The H3P+ subpopulation of these cells did not vary between groups at 40 d.p.h., but did so at 82 and 195 d.p.h. when Pax7+/H3P+ cells were clearly less numerous in 16°C-fish than in the other groups (22.4, 15.1 and 9.7% at 82 d.p.h., and 14.4, 17.7 and 9.3% at 195 d.p.h. for 8.5, 13 and 16°C-fish, respectively, Fig. 9B). Note that, because of the absence of Pax7+ cells in the central areas of the myotomes, no conclusions can be drawn concerning MPC behaviour other than the observed imprinting responses of the mosaic growth mode (see cellularity results).

Double immunolabelling for Pax7 and Myog served to trace a possible influence of thermal imprinting on the differentiation of lateral fast muscle MPCs. The highest relative amounts of Pax7+/Myog+ cells were found in the 16°C-fish at 40 and 82 d.p.h. (49.7, 54.9 and 58.5% at 40 d.p.h., and 36.2, 39.3 and 42.3% at 82 d.p.h. for 8.5, 13 and 16°C-fish, respectively; Fig. 9B), whereas no relevant inter-group differences were detected later, at 195 d.p.h. (74.1, 71.7 and 71.6%).

Provided that differentiating (Myog+) MPCs are not at the same time dividing (H3P+; highly likely but not tested in the present study), the ratio of such cells (percentage Pax7+/H3P+ divided by percentage Pax7+/Myog+) provides further valuable information about the modulatory effects exerted by thermal imprinting on the proliferation–differentiation balance of the MPCs. At 40, 82 and 195 d.p.h., these ratios were 0.14, 0.19 and 0.13 for 16°C-fish, but 0.23, 0.38 and 0.24 for 13°C-fish, and 0.15, 0.63 and 0.19 for the 8.5°C-fish. This suggests that in the developmental period from 40 to 195 d.p.h., the differentiation–proliferation equilibrium of Pax7+ cells in 16°C-fish lies stably further towards differentiation than in

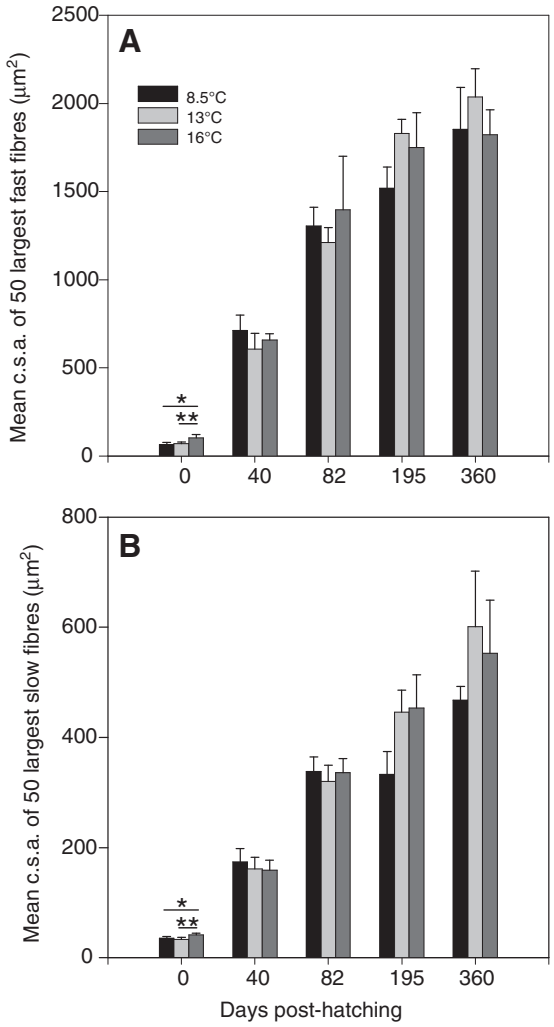

Fig. 6. Relationship between mean size of largest fibres (c.s.a. + s.d.) and developmental time. (A) The 100 largest fast fibres and (B) the 50 largest slow fibres; intergroup differences significant at $P \leq 0.01$ (**) and $P \leq 0.05$ (*; Fisher's LSD test).

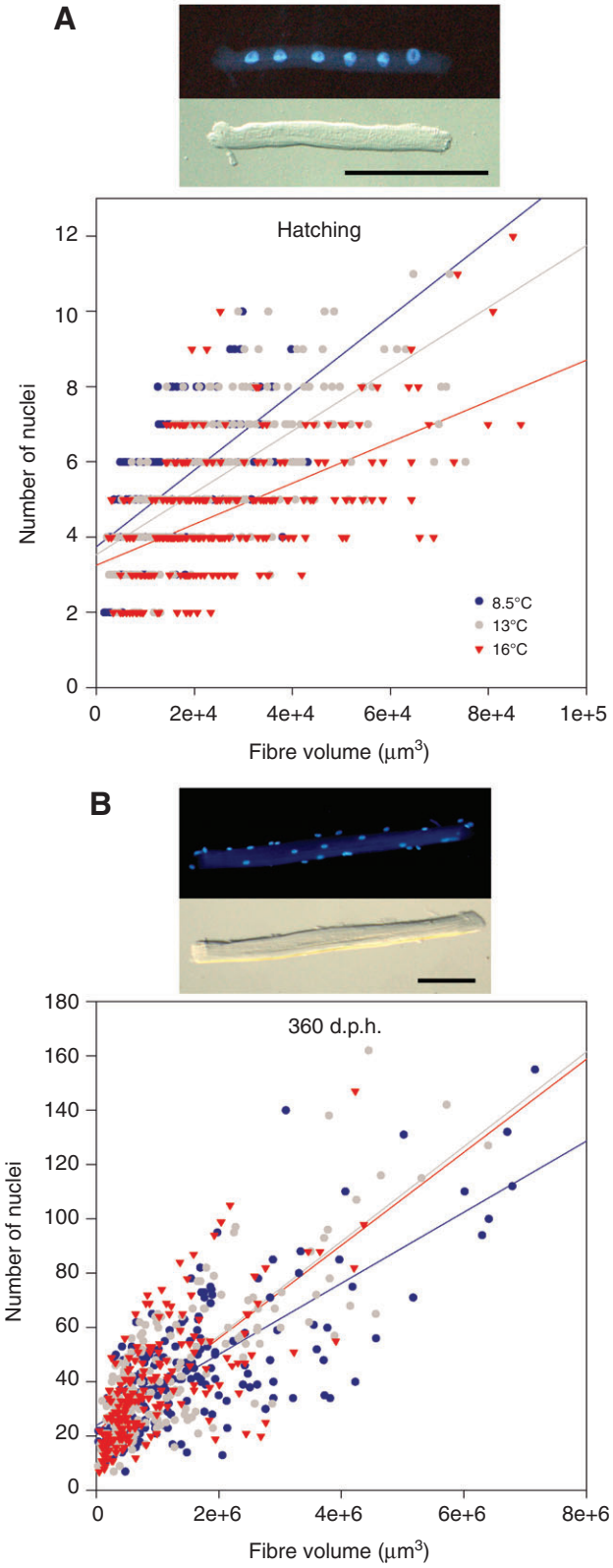

Fig. 7. Myonuclear densities of isolated fast fibres. Relationship of nuclei/fibre volume (A) at hatching and (B) at 360 d.p.h. Images show a typical isolated single fast muscle fibre of a 13°C-fish stained with Hoechst 33258. Scale bars, 100 μm. Regression line equations: (A) 8.5°C, $y=1.02x+3.74$ ($r^2=0.36$, $P<0.01$); 13°C, $y=8.21x+3.54$ ($r^2=0.48$, $P<0.01$); 16°C, $y=5.46x+3.25$ ($r^2=0.29$, $P<0.01$); (B) 8.5°C, $y=1.31x+24.00$ ($r^2=0.55$, $P<0.01$); 13°C, $y=1.74x+21.94$ ($r^2=0.66$, $P<0.01$); 16°C, $y=1.71x+21.72$ ($r^2=0.48$, $P<0.01$).

13°C-fish, whereas it shifts back and forth, with a prominent shift towards proliferation at 82 days, in 8.5°C-fish (Fig. 9B).

DISCUSSION

This study demonstrates clearly that the European pearlfish is susceptible to thermal imprinting, which exerts a variety of effects on body growth and muscle cellularity that persist into adult life; such imprinting also occurs, although in slightly different ways, in other species.

The most readily identifiable effect is that differences in fish size between thermal groups, arising during the imprinting period become, can be compensated for in post-imprinting life when all the groups are in the same thermal conditions (Fig. 2). In the pearlfish, this leads to the situation in which animals reared at 16°C, being significantly larger with more muscle mass (fast and slow muscle c.s.a.) at hatching, were subsequently smaller than the fish of both the other groups; the 13°C-fish being the most efficient in catch-up growth, followed by the 8.5°C-fish (Figs 2, 3).

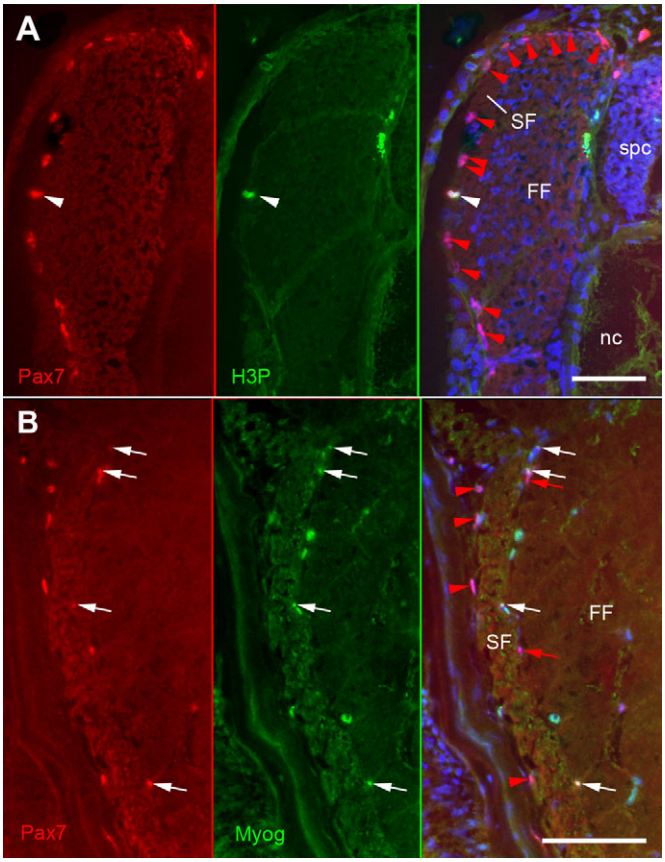

Fig. 8. Images of lateral myotome from sections immunostained for Pax7 (red) illustrating muscle precursor cell assessment. (A) 8.5°C-fish at hatching, section co-stained for H3P (green) to test for mitotically active Pax7+ cells (white arrowheads). Note that all Pax7+ cells (arrowheads) are exclusively located at the site of the previous dermomyotome (DM). (B) 13°C-fish at 195 d.p.h., section co-stained for Myog (green) to test for Pax7+ cells entering myogenic differentiation (white arrows). Note that Pax7+ cells occur in both the area of the previous DM (arrowheads) and the lateral fast muscle (arrows). Nuclei were counterstained with Hoechst 33258. FF, fast fibres; nc, notochord; SF, slow fibres; spc, spinal cord. Scale bars, 50 μm.

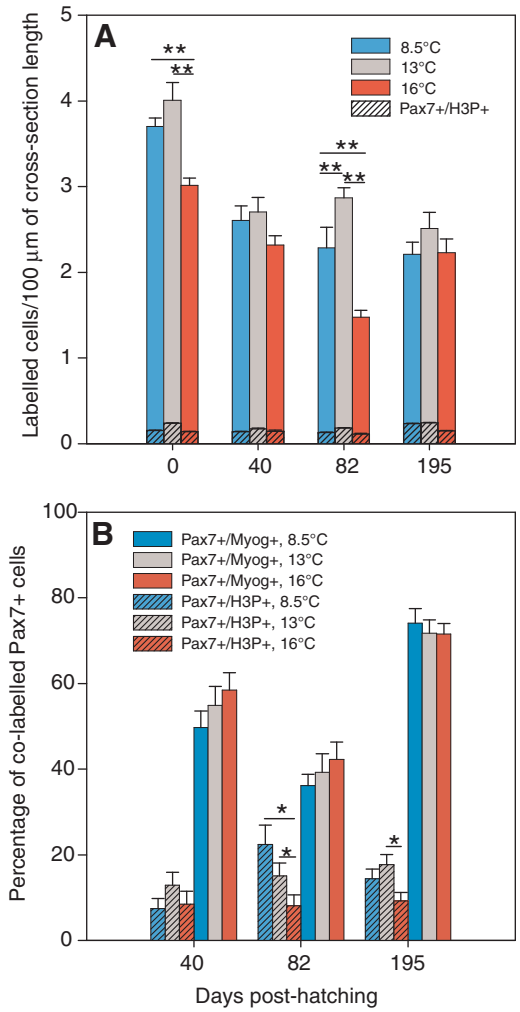

Fig. 9. Quantification of MPCs in double-immunostained 10 μm myotomal cross sections for the developmental period from hatching to 195 d.p.h. (A) Numbers of Pax7+ cells and of Pax7+/H3P+ cells at the site of the previous DM, per 100 μm distance in the section plane. (B) Percentage of Pax7+ cells in the lateral fast muscle growth zone that have entered proliferation (Pax7+/H3P+) or differentiation (Pax7+/Myog+). Intergroup differences were significant at $P \leq 0.01$ (**) and $P \leq 0.05$ (*); values are means + s.e.m.

This macroscopic effect is reflected in the cellularity. The 16°C-fish have the largest fast and slow fibres and highest overall numbers of fast fibres at hatching, but the 8.5°C-fish and especially the 13°C-fish catch up with them, or surpass them, subsequently (Figs 4–7). However, combined analysis of fibre size distributions and myonuclear densities (Figs 5, 7) reveals that the advanced fast fibre hypertrophy in 16°C-fish at hatching comes with larger nuclear domains, indicated by reduced myonuclear density. Assuming that the nuclear domains of these fish are to some extent 'elastic' (cf. Brack et al., 2005), this can be interpreted in two ways: (1) imprinting at 16°C favours instant utilisation of this elasticity without requirement of enhanced nuclear uptake from MPCs, whereas imprinting at lower temperatures has no such effect; (2) warm imprinting reduces the availability of MPCs for fusion to growing fibres, thus enforcing early nuclear domain expansion. Although the present data do not distinguish between these possibilities, they do show that muscle precursor cells of 16°C-fish until hatching have a higher differentiation rate than those of their colder reared counterparts. This enables these fish to run hypertrophy and hyperplasia most efficiently as long as the direct impact of warm rearing is present. However, this advantage cannot be permanently sustained under thermal conditions after hatching, when the 13 and 8.5°C-fish grow faster, although by different cellular mechanisms. The 13°C-fish upregulate both fast fibre hypertrophy (29.3-fold increase in size of the 100 largest fibres) and hyperplasia (10.0-fold increase in fibre number). The 8.5°C-fish, by contrast, favour fast fibre hypertrophy (27.7-fold increase), while hyperplasia is moderate (6.5-fold increase in fibre number). These different mechanisms conform with those of other fish from temperate habitats, e.g. Atlantic salmon (Nathanailides et al., 1995), herring (Johnston et al., 1998) and haddock (Martell and Kiefer, 2007), thus suggesting that the imprinting responses of muscle cellularity of such fish follow a common trend: cold-imprinting favours hypertrophy over hyperplasia, warm-imprinting favours hyperplasia over hypertrophy, imprinting at intermediate temperatures fosters both. A different developmental course is also apparent in relation to fast fibre nuclei/volume ratios. These ratios are similar for 13 and 16°C-fish,

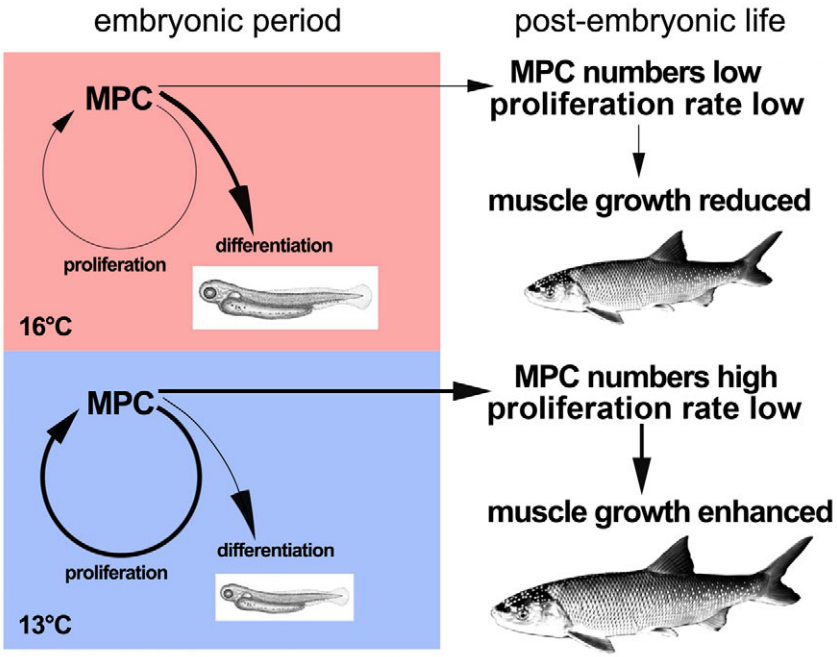

Fig. 10. Diagram summarising and explaining the main results of the present study at the MPC level. Warm-imprinted (16°C) pearlfish embryos tend to reduce MPC proliferation in favour of early differentiation. This generates large hatchlings but leaves only limited MPC reserves with reduced proliferative activity for further growth, finally resulting in smaller adults. Embryos imprinted at the middle temperature (13°C) and, to a lesser extent, also at the cold temperature (8.5°C) tend to enhance MPC proliferation at the cost of early differentiation. This leads to smaller hatchlings but ongoing proliferation allows for sustained MPC reserves to compensate for the initially delayed growth, finally resulting in larger adults.

but because the 13°C-fish have much larger fibres there was more nuclear uptake from the MPC pool to keep their nuclear domains at the same size as the 16°C-fish. By contrast, the clearly lower ratios of the 8.5°C-fish at this developmental stage indicate that their fast fibre hypertrophy preferentially utilises the nuclei already stockpiled within the fibres and thus occurs at the cost of domain expansion (as in 16°C-fish at hatching). Interestingly, these diverging response patterns of nuclear domains bear some resemblance to those found in thermally imprinted (warm *vs* ambient-reared) Atlantic salmon (Johnston et al., 2003), specifically if larger domains were a long-term consequence of sub-optimal thermal imprint (i.e. cold in pearlfish, but warm in salmon).

Regarding the question of whether the slow muscle plays a role on its own in the imprinting responses of the teleost muscle, the present data show clearly that this is not the case. Slow muscle trends are broadly similar to fast muscle trends in terms of both overall mass and cellularity, thus leaving the relative proportion of the slow muscle compartment (fast/slow ratio) largely unaffected (Fig. 3).

As regards the question of whether cellularity responses to thermal imprinting involve relevant shifts of muscle cell recruitment between myotomal growth zones (lateral, apical and central), the present data show that this is clearly not the case (supplementary material Fig. S1). This provides an indication that all the different types of MPCs that have been found in the teleost myotome (e.g. Devoto et al., 1996; Barresi et al., 2001; Groves et al., 2005; Marschallinger et al., 2009) are equally influenced by thermal history.

Results from immunostaining for Pax7, H3P and Myog, together with morphometric analyses suggest that the driving forces behind the cellularity differences include two mechanisms at the MPC level: one is the influence on the proliferation behaviour of MPCs during the imprinting period, with the consequence that 13°C-fish at hatching have the highest number of residual Pax7+ cells and proliferating Pax7+/H3P+ cells within the DM, whereas the 16°C-fish have the lowest numbers (Fig. 8A). The second is the influence on the differentiation behaviour of the MPCs. This seems to come into operation during the imprinting period, because 16°C-fish have more muscle fibres at hatching than the 13°C-fish (Fig. 4, see above). Perhaps more importantly, the present results provide clear evidence that thermal imprinting exerts a lasting effect on MPC proliferation in the lateral fast muscle domains of the post-hatching larvae. At this site, at which MPCs have been shown to be accumulated in the post-embryonic period (Steinbacher et al., 2008; Marschallinger et al., 2009), the proliferation–differentiation balance of the Pax7+ MPCs is shifted towards proliferation in the 13°C-fish (and partly also in 8.5°C-fish) but not in the 16°C-fish (Fig. 9B).

Together these findings suggest that proliferation of the DM-derived Pax7+ MPCs of the warm-reared 16°C-fish is reduced in favour of early differentiation, resulting in limited MPC reserves being available for further growth, and finally in smaller muscle mass and total body size (Fig. 10). The trend is reversed in the 13°C-fish and, to a lesser extent, also in the 8.5°C-fish. These animals exhibit more intense MPC proliferation, generating sustained reserves that provide them with a higher muscle growth potential than in the 16°C-fish (cf. amounts of Pax7+/Myog+ at hatching and 195 d.p.h. in Fig. 9B) and to finally attain larger muscle mass and total size. All this is in agreement with evidence from two studies of Atlantic salmon suggesting that densities of c-met+ MPCs in juvenile fast muscle are highest when the embryos are imprinted within an ‘optimum thermal range’, whereas colder conditions and heating experiments lead to reduced densities (Johnston et al., 2000; Johnston et al., 2003).

Pearlfish are clearly best conditioned for post-hatching growth if the embryos are imprinted at moderate (medium, non-extreme) temperatures (in this case 13°C) with respect to the species’ range of embryonic thermal tolerance and natural spawning site conditions (Kainz and Gollmann, 1997; Siligato and Gumpinger, 2005). Only the second best result is achieved from cold rearing at 8.5°C, a temperature not sustainable during immediate post-hatching development (Kainz and Gollmann, 1997). By contrast, fish imprinted at 16°C, the value previously regarded as favourable for efficient rearing of the species (Kainz and Gollmann, 1997), exhibit the lowest muscle growth potential. This provides support for a type of response to thermal imprinting that may indeed be more widespread among teleost species than judged on first impression. This is because some of the earlier studies suggested that, for example, enhanced hyperplasia of the dominant fast muscle tissue

in the larval and/or juvenile period is promoted by either cold (Nathanilides et al., 1995; Johnston et al., 2003; Albokhadaim et al., 2007) or warm (Martell and Kieffer, 2007; Galloway et al., 1999) rearing during the embryonic period. However, more recent studies agree with the present pearlfish data. In zebrafish adults that had been imprinted at 22, 26 and 31°C, the final fast fibre numbers were optimum in the 26°C-group (Johnston et al., 2009), and in Atlantic salmon imprinted at 2, 5, 8 and 10°C, fast fibre numbers were highest in the 5°C-group at the onset of smoltification (Macqueen et al., 2008).

In a more general sense, the present study reveals that the degree of variation between pearlfish groups in response to thermal imprinting is much lower than in Atlantic salmon, as demonstrated by the study of Albokhadaim et al., which reports some of the strongest imprinting effects measured to date (Albokhadaim et al., 2007). This raises the question of whether the responsiveness of teleost embryos to thermal imprinting may simply vary between species or even populations (cf. Johnston et al., 2000), or whether the responses may also (and perhaps strongly) depend on the thermal conditions chosen for post-imprinting development. If the latter is true, then equilibration to moderate temperature levels with respect to the species' range of thermal tolerance (as in the present study) may allow for only limited patterns of modulation. These responses may then differ from the much more vigorous ones achieved when warm-imprinted embryos are cooled down to the temperature of the cold-reared batch (Albokhadaim et al., 2007). Further research will be required to examine how exactly the imprinting response of the teleost muscle depends upon the nature of the post-imprinting thermal environment. Testing of species (or even populations) in this respect would appear to be useful and necessary in the economic, phylogenetic and, not least, in the climatic context.

LIST OF ABBREVIATIONS

c.s.a.	cross-sectional area
DM	dermomyotome
d.p.f.	days post-fertilisation
d.p.h.	days post-hatching
H3P	phosphohistone H3
MPC	muscle precursor cell
Myog	myogenin
Pax7	paired box transcription factor 7
SL	standard length

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