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Temperature until the 'eyed stage' of embryogenesis programmes the growth trajectory and muscle phenotype of adult Atlantic salmon

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We investigated how adult growth in Atlantic salmon (*Salmo salar* L.) was affected by changing embryonic temperature from fertilization until the completion of eye pigmentation. Fertilized eggs from several hundred families were divided between four temperature treatments (2, 5, 8 or 10°C) and subsequently reared in identical conditions in replicated tanks. Fish exposed to 2 and 5°C treatments were significantly smaller at smoltification than groups at higher temperatures, but showed substantial compensatory catch-up growth. Remarkably, temperature during this short window of embryogenesis dictated adult myogenic phenotype three years later with significant treatment effects on the muscle fibre final number (FFN), maximum diameter, nuclear density and size distribution. FFN was highest for the 5°C treatment and was reduced at higher and lower treatment temperatures. Our results require direct temperature effects on embryonic tissues, such as the stem cell-containing external cell layer, in order to produce persistent effects on juvenile and adult growth.

Keywords: embryonic temperature; developmental plasticity; myogenesis; ectotherms

1. INTRODUCTION

Sub-lethal temperature stress during embryogenesis can strongly modify developmental outcomes in teleosts (Hempel & Blaxter 1961; Johnston 2006). An established example is the muscle fibre phenotype that is sensitive to early environmental temperature (Johnston 2006). In teleosts, the myotubes that give rise to fast muscle are produced during embryonic, larval and adult growth stages. Two principal embryonic fast-twitch muscle progenitor cell (MPC) populations are recognized. Cells in the posterior epithelial somite generate the embryonic myotome (Hollway *et al.* 2007), marked in salmon by the expression of myogenic regulatory factors (MRFs; Macqueen *et al.* 2007). Cells in the anterior somite express Pax7 and migrate laterally to establish the 'external cell layer' (ECL), which generates new muscle fibres during late embryonic and larval stages, and MPCs, which migrate into the myotome to reside next to existing muscle fibres (Hollway *et al.* 2007). The most significant source of fast fibre production during juvenile and adult stages is mosaic hyperplasia, involving widespread formation of myotubes from MPCs on the surface of existing fibres, a process that continues until approximately 45% of the maximum body length (Johnston 2006). MPCs also provide nuclei for fibre expansion in length and diameter during growth and for nuclear turnover at all stages in the life cycle.

Here we investigated the growth trajectories and muscle structure of adult Atlantic salmon reared at four embryonic temperatures chosen to encompass the range for normal development.

2. MATERIALS AND METHODS

(a) *The experiment*

Fertilized eggs from 60 female and 10 male *Salmo salar* (Salmobreed A/S, strain) were incubated at AKVAFORSK (Sunndalsora, Norway) at 2, 5, 8 and 10°C in replicated trays (approx. 1000 eggs per tray) from fertilization (3 November 2003) until the relative age of 165 Ts as defined by Gorodilov (1996), which is just subsequent to the period when the eye becomes completely pigmented. Subsequently, embryos were maintained at a constant 8°C until first feeding, at which time juveniles were transferred to replicated tanks and the temperature increased to 12°C over 3 days. Fry were transferred to EWOS Innovation (Lonningdal, Norway) and the treatment groups reared at ambient temperature (2.7–15.0°C) in separate freshwater tanks to minimize size-biased feeding hierarchies. Fish were fed a commercial diet (EWOS, micro range) and provided with continuous light until January 2005, and then 12 L : 12 D cycle until smoltification. One-hundred fish from each treatment were passive integrated transponder (PIT) tagged (Fish Eagle, Gloucestershire) and randomly transferred to three replicate seawater tanks (May 2005). Fish were reared during seawater stages under continuous light and fed a commercial diet (EWOS pyramid range).

(b) *Samples*

Fish were weighed and identified by their PIT tag five times from the point of seawater transfer until the end of the experiment (May and November 2005, March, June and November 2006). At the final sample, 18 fish (six per tank) were randomly sampled per treatment and a further random subsample (2°C, $n=13$; 5°C, $n=13$; 8°C, $n=12$ and 10°C, $n=12$ 7) was selected for the analysis of muscle structure. Muscle morphometric parameters were determined from a half-myotomal cross section at the level of the first dorsal fin ray involving 1000 measurements of fibre cross-sectional area from six blocks per fish as previously described (Johnston *et al.* 1999). Maximum fibre diameter (Dmax) was estimated by averaging the 20 largest fibre diameters of each fish. Myonuclear density was estimated from three fields of 0.4 mm^2 per section from six blocks per fish.

(c) *Statistics*

Growth in seawater was analysed in a mixed-model framework using the nlme library (Pinheiro & Bates 2000) in R (R Development Core Team 2007) with fish mass as a dependent variable. Sample date and embryonic temperature were fixed factorial treatments, whereas tank and individuals were treated as random effects with individuals nested within tanks. The data were log transformed to remove data heterogeneity.

Muscle fibre morphometry statistics were analysed in MINITAB v. 13 (Minitab, Inc.). Data for each measured parameter (mass, fork length, mean fast-twitch steak cross-sectional area (FSCA), mean fibre area, mean fibre diameter, mean fibre final number (FFN), Dmax and myonuclear density (nuclei per mm^2)) were continuous, normally distributed and homogenous. Variation between measured parameters was modelled in a GLM ANOVA using sequential sum of squares and considering temperature, tank and tank×temperature as fixed factors. Fisher's least comparison test was used *post hoc* to establish the source of significant differences. To test the null hypothesis

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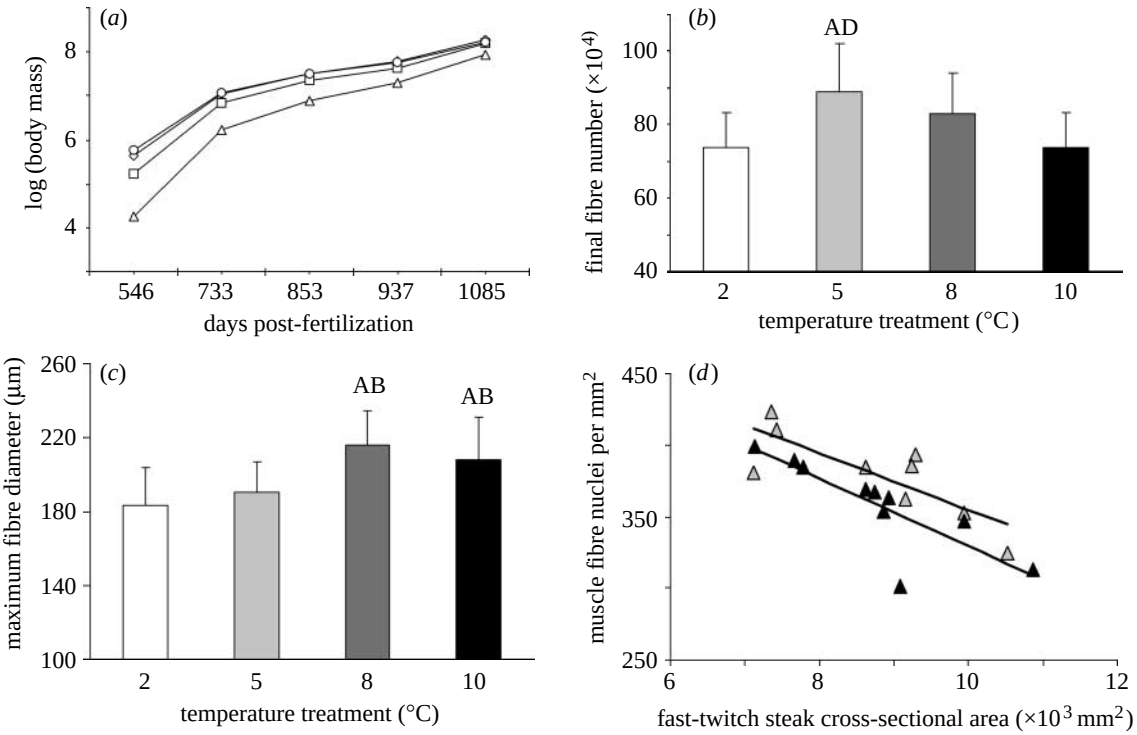

Figure 1. Embryonic temperature significantly altered the growth trajectory and final muscle phenotype of adult Atlantic salmon. (*a*) The change in log (body mass) of salmon derived from four embryonic temperature treatments during 18 months of seawater growth in identical conditions. Triangles, 2°C; squares, 5°C; diamonds, 8°C and circles, 10°C. (*b*) The change in mean final muscle fibre number; A and D indicate a significant difference ($p<0.001$) to 2 and 10°C, respectively. (*c*) The change in Dmax; A and B indicate a significant difference ($p<0.001$) to 2 and 5°C, respectively. (*d*) Scatterplot of nuclear density versus FSCA at 5 and 10°C. The number of muscle fibre nuclei per unit area was significantly different between 5 and 10°C (see text). Grey triangles, 5°C and black triangles, 10°C. Values with error bars represent mean ± s.d.

that the myonuclear density was unaffected by treatment, a GLM ANOVA was used using sequential sum of squares and considering FSCA, temperature and FSCA×temperature as fixed factors.

To compare the distribution of muscle fibre size, a non-parametric method was used to fit smoothed probability density functions (PDFs) to approximately 1000 measurements of fibre diameter per fish using a kernel function (smoothing coefficient $h=0.18$; Bowman & Azzalini 1997). Bootstrapping was used to distinguish random variation in diameter distribution from treatment differences. The Kolmogorov–Smirnov two-sample test statistic was used to test the null hypothesis that PDFs of muscle fibre diameter in 5 and 10°C treatments were identical.

3. RESULTS

(a) *Embryonic temperature and adult growth rate*

The embryonic eye was completely pigmented (the 'eyed stage') after 94 days at 2°C, 53 days at 5°C, 33 days at 8°C and 25 days at 10°C, which encompassed 67, 62, 55 and 47% of the period to hatching, respectively. There were significant effects on subsequent growth with treatment and there was a treatment×sampling date interaction ($p<0.0001$; table 1; figure 1*a*). At the point of seawater transfer, 10 and 8°C fish were significantly heavier than 5 and 2°C animals (by approx. 32–42% and 68–78%, respectively). However, 5 and 2°C treatments grew faster than those at higher temperatures (figure 1*a*) and by the final sample date 5, 8 and 10°C fish were of an equivalent size, and each group was significantly heavier than 2°C fish.

Table 1. Summary of mixed-model ANOVA parameters used to distinguish variation in post-smolt body mass.

variable	d.f.	*F*	*p*
(intercept)	1	973 110.8	<0.0001
sample date	4	40 256.2	<0.0001
temperature	3	536.6	<0.0001
sample date×temperature	12	358.3	<0.0001

(b) *Embryonic temperature and muscle fibre characteristics*

For all measurements, variation between tanks and from temperature–tank interactions was not significant (n.s.; table 2). The mean mass of the subsample closely reflected that of each fish population (2°C = 2540 ± 591 g, 5°C = 3832 ± 895 g, 8°C = 4403 ± 916 g and 10°C = 4081 ± 964 g; mean ± s.e.), and there were significant treatment effects for FSCA, average fibre diameter, Dmax ($p\leq0.001$) and fibre number ($p=0.017$; figure 1*b–d*; table 2). The absence of the smallest size class of fibres (5–10 µm) in each treatment group indicated that the FFN had been reached. FFN exhibited an optimum at 5°C (89.1×10^4) with approximately 17 and 14% fewer fibres in the 10 and 2°C treatments, respectively (figure 1*b*; $p<0.001$). Furthermore, Dmax was significantly greater ($p<0.001$) at 8 and 10°C than at 5 or 2°C, although differences between 8 and 10°C or 5 and 2°C treatments were non-significant (figure 1*c*). Treatment differences in mean

Table 2. Summary of GLM ANOVA parameters used to distinguish variation in muscle fibre characteristics.

variable	d.f.	Seq SS	Seq MS	*F*	*p*
mass					
temperature	3	26 020 217	8 673 406	11.78	<0.001
tank	2	433 439	216 719	0.29	0.747
temperature–tank	6	4 629 072	771 512	1.05	0.410
fork length					
temperature	3	94 511	31 504	13.68	<0.001
tank	2	738	280	0.12	0.886
temperature–tank	6	9726	1621	0.70	0.648
mean fast-twitch steak cross-sectional area (*FSCA*)					
temperature	3	72 520 817	24 173 606	10.63	<0.001
tank	2	325 867	162 934	0.07	0.931
temperature–tank	6	17 354 994	2 892 499	1.27	0.293
mean fibre area					
temperature	3	102 209 598	34 069 866	9.30	<0.001
tank	2	1 929 389	964 694	0.26	0.770
temperature–tank	6	6 610 203	2 768 367	0.76	0.609
mean fibre diameter					
temperature	3	4050.6	1350.2	12.02	<0.001
tank	2	114.4	57.2	0.51	0.605
temperature–tank	6	459.4	76.6	0.68	0.665
mean final fibre number (*FFN*)					
temperature	3	1.5842E+11	5.2806E+10	3.87	0.017
tank	2	3.8770E+10	1.9385E+10	1.42	0.254
temperature–tank	6	1.1720E+11	1.9533E+10	1.43	0.229
max fibre diameter (*Dmax*)					
temperature	3	8299.9	2766.6	6.84	0.001
tank	2	1071.4	535.7	1.32	0.278
temperature–tank	6	1508.6	251.4	0.62	0.712
myonuclear density					
steak area	1	10578.6	10578.6	28.51	<0.001
temperature	1	1976.5	1976.5	5.33	0.036
steak area–temperature	1	83.2	83.2	0.22	0.643

myonuclear density were non-significant (not shown). However, there was a significant interaction between FSCA and mean myonuclear density (table 2), with fish from 5°C treatments having more myonuclei than those from the 10°C treatment ($p=0.036$; figure 1*d*). Removing the outlying point on the plot (figure 1*d*; 10°C group) did not alter this result ($p=0.042$; not shown).

There were significant differences in the PDFs of muscle fibre diameters for a subset of the 5 and 10°C treatment groups ($n=11$ per treatment) size matched within 4 cm to minimize confounding scaling effects (figure 2*a*; $p<0.01$). The 5°C treatment had a higher proportion of fibre diameters up to approximately 75 µm (left-hand tail of the distributions) than the 10°C treatment, whereas the latter had more fibre diameters in the largest size classes (right-hand tail of the distributions; figure 2*b*).

4. DISCUSSION

Previously, we found that Atlantic salmon reared in ambient or heated water during freshwater stages showed different growth characteristics following seawater transfer and exhibited significant differences in FFN and myonuclear density (Johnston *et al.* 2003). The main finding in this study was that the temperature until the eyed stage of embryogenesis was sufficient to produce similar changes in growth and muscle phenotypes. Furthermore, we found that FFN was optimal at an embryonic temperature of 5°C and reduced for 2, 8 and 10°C treatments (figure 1*b*). Fish in the 2 and 5°C treatments were much smaller than at 8 and 10°C, but subsequently showed compensatory growth such that at the end of the experiment there was no significant difference between 5, 8 and 10°C treatments (figure 1*a*,*b*). The current experiment underlies the importance of establishing a reaction norm when studying developmental plasticity of phenotype. For example, if we had investigated only the 2 and 10°C treatments, an incorrect conclusion would have been made that temperature strongly modified growth with little effect on the final muscle phenotype.

The developmental period of our temperature treatment encompasses the time when the somitic ECL is formed in zebrafish. The ECL provides new myofibres and MPCs during late embryonic stages (Hollway *et al.* 2007). Additionally, undifferentiated, self-renewing MPCs remain in the ECL and probably contribute to post-embryonic hyperplasia and the absorption of MPC nuclei during fibre hypertrophy (Hollway *et al.* 2007). The myogenic potential of the ECL is almost certainly conserved in

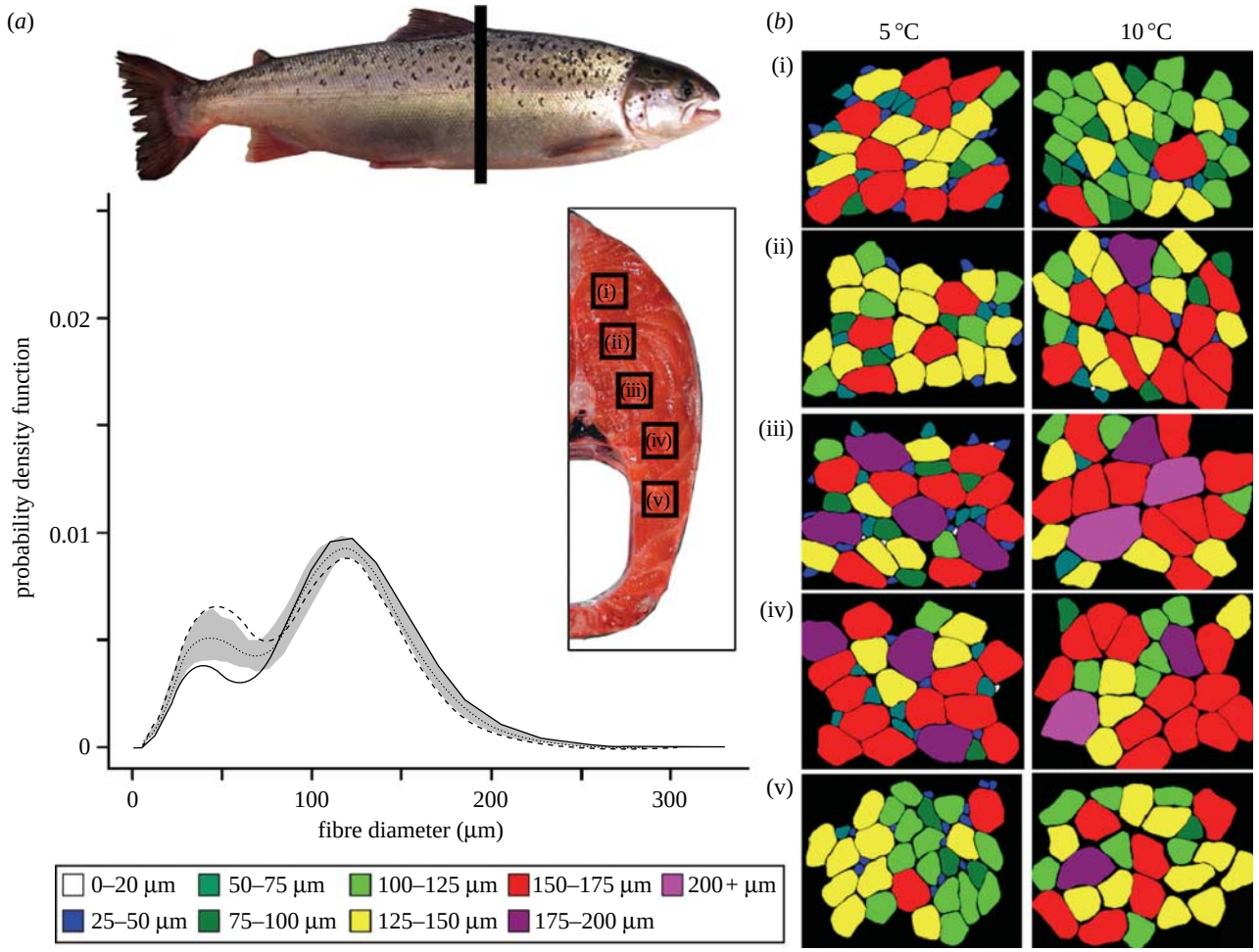

Figure 2. Embryonic temperature affected the adult muscle fibre size distribution. (*a*) Probability density functions (PDFs) of muscle fibre diameter in length matched 5 and 10°C fish ($n=11$ per treatment). The dashed (5°C) and solid lines (10°C) show the average PDFs, respectively. The dotted line central to the shaded area is the average PDF for combined groups. The shaded area shows 100 bootstrap estimates from combined populations of fibre diameter. ((*b*)(i–v)) Three randomly selected fields (at 10× magnification) were sampled from each block for the three largest (approx. size matched) fish in the 5 and 10°C treatments. Each muscle fibre was colour coded based on variation in diameter (shown to l.h.s. of *b*). Images shown are representative of other fish.

Atlantic salmon, evidenced by a similar embryonic *pax7* expression domain (Macqueen & Johnston 2008). An inverse relationship exists between the protein titres of Pax7 and MRFs in the zebrafish embryo. When MRFs were ablated by morpholino antisense RNA, the number of Pax7 expressing cells was increased (Hammond *et al.* 2007). Recently, we examined salmon embryos from the 2, 5 and 8°C treatments of this experiment and observed that the expression of MRFs was temperature dependent at equivalent developmental stages (Macqueen *et al.* 2007). Based on these results, alterations in the timing of expression of MRFs could feasibly affect the number of Pax7 expressing ECL cells. Since the ECL MPCs are self-renewing (Hollway *et al.* 2007), an increase in their embryonic number, or proliferative capacity, could influence the final MPC population available for mosaic hyperplasia affecting the intensity and/or duration of myotube production as well as nuclear accretion associated with fibre hypertrophy. In any future test of this hypothesis, it would be important to establish the exact contribution of the ECL MPCs to post-embryonic muscle growth.

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