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Larval programming of post-hatch muscle growth and activity in Atlantic salmon (*Salmo salar*)

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Summary

Larval muscle development in Atlantic salmon is known to be affected by temperature; however, the long term effects and possible mechanisms involved are less well understood. Therefore, the aim of this study was to evaluate the influence of egg incubation temperature on post-hatch muscle growth and fish activity.

Salmon eggs were incubated at either 10°C or 5°C from fertilization until hatching, then subsequently both groups were reared at 5°C. Fish from both groups were sampled at the eyed stage, 6 and 21 weeks after first feeding, for muscle cellularity analysis and immunocytochemistry. In addition, to try to establish a mechanism for altered growth, the activity of the fish was measured at 3, 6 and 21 weeks after first feeding.

Our results demonstrate that whereas fish incubated at 10°C grow faster, the fish incubated at 5°C show a more sustained period of muscle growth and by 21 weeks are significantly longer, heavier and have more muscle fibres than those fish incubated at a higher temperature. We also demonstrate that fish raised at 5°C show increased food seeking activity throughout development and that this may explain their sustained growth and muscle development.

These results taken together, demonstrate that egg incubation temperature up to hatching in salmon is critical for longer term muscle growth, twinned with increased activity. This is of interest to the aquaculture industry in term of the production of good quality fish protein.

Key words: *Salmo salar*, muscle cellularity, MRFs, temperature, activity.

Introduction

Global production of farmed fish has more than doubled in the past 15 years (reviewed by Naylor et al., 2000). Salmon is grown and consumed worldwide and because of increasing consumer demand efforts are made to augment the production of this species in fisheries. The anatomical part of salmon consumed in human gastronomy is mostly skeletal muscle fibres. Therefore optimising the embryonic development and growth of muscle tissue offers considerable potential for optimising both production yield and meat quality of fish (Johnston, 1999).

During embryogenesis, muscle development and growth in fish occurs as a combination of muscle hypertrophy (the increase in size of existing fibres) and muscle hyperplasia (the recruitment of new muscle fibres) (reviewed by Koumans and Akster, 1995; Rowlerson and Veggetti, 2001). This process continues throughout development into adulthood in most fish species (Greer-Walker, 1970; Stickland, 1983; Weatherley and Gill, 1984; Weatherley et al., 1988). This is in contrast to mammals in which hyperplasia stops at around the time of birth (Campion, 1984; Goldspink, 1972; Rayne and Crawford, 1975).

It has long been known that environmental temperature can affect the time of embryonic development in a variety of animal species (Krogh, 1914), including the Atlantic salmon (*Salmo salar*) (Hayes et al., 1953). In this species a warmer incubation temperature, compared to the cooler ambient temperature, produced bigger but fewer fibres with high myofibrillar content at the newly hatched stage (Stickland et al., 1988). The hatched embryos of salmon that were incubated at the cooler ambient temperature also grew better up to 3 weeks post-hatch when all juveniles were grown at ambient temperature after hatching (Nathanailides et al., 1995). Johnston et al. (Johnston et al., 2000) also showed that cooler temperature regimes up to first feeding in Atlantic salmon produced fish that grew better up to 12 weeks later. Furthermore, it was found that faster growth of salmon juveniles is achieved by increased muscle fibre hyperplasia (Higgins and Thorpe, 1990). Additionally, temperature appears to directly affect the size and the number of salmon myosatellite cells *in vitro* with differentiation and fusion occurring earlier at high temperature (Matschak and Stickland, 1995). The effects of temperature on muscle growth have also been demonstrated in many other fish species, such

as sea bass (Ayala et al., 2003; Ayala et al., 2000) and Atlantic herring (Johnston et al., 2001).

Muscle development is controlled by a family of bHLH genes called myogenic regulatory factors (MRFs), which includes MyoD and myogenin (Rescan, 2001; Rudnicki and Jaenisch, 1995; Rudnicki et al., 1993). Therefore, a change in time and level of MRF expression with developmental temperature might constitute a molecular mechanism for the observed changes in muscle phenotype (Temple et al., 2001; Wilkes et al., 2001). In rainbow trout, it was found that egg incubation at 4°C delayed and prolonged expression of MyoD and myogenin in embryos compared with those reared at 12°C (Xie et al., 2001).

Thus, although there is some information on temperature influences of early muscle development and initial growth, we do not know how early thermal history influences longer term growth. We also do not know whether these influence the long term function or activity. The aim of the present study, therefore, was to determine the influence of egg incubation temperature on growth up to 21 weeks post-hatch, with particular reference to the cellular growth of muscle tissue, and the effect on fish activity.

Materials and methods

Embryos

1200 fertilised Atlantic salmon (*Salmo salar* L.) eggs were obtained from the Almondbank salmon hatchery station, DAFFS, Freshwater Fisheries Laboratory, Pitlochry, Scotland, UK, 24 h after fertilization. Eggs were divided into two groups; one group was kept at 5°C and the temperature for the other group was gradually raised to 10°C over 2 days. After hatching, both groups of larvae were all kept at 5°C (the 10°C group brought to 5°C over 2 days). 5°C is near the natural ambient temperature for Atlantic salmon and 10°C is towards the upper limit for incubation as, above this, hatching mortality is increased (Matschak et al., 1997).

Measurement of fish length and mass

From each temperature regime fish were taken at 6 weeks and 21 weeks after first feeding and killed by an overdose of the anaesthetic MS-222 (3-aminobenzoic acid ethyl ester; Sigma, Poole, Dorset, UK). At least 20 fish were photographed for each group and body length was measured. The fish were blotted dry with filter paper and the body mass was recorded.

Morphometry

White muscle fibres

Five fish from each temperature regime were sacrificed at eyed stage, 6 weeks after first feeding, and 21 weeks after first feeding (i.e. 30 fish in total). Complete transverse sections were cut from each fish at the level of the anal vent. The sections were fixed in 3% glutaraldehyde in 0.1 mol l^{-1} phosphate buffer (pH 7.2), washed in phosphate buffer, post-fixed in 1% osmium tetroxide, dehydrated and embedded in TAAB resin according to the method of Stickland (Stickland et al., 1988). Transverse sections of 0.5 μm thickness were obtained using a Reichert ultramicrotome and stained with 1% Toluidine Blue. Slides were examined using a Zeiss image analysis system (KS 300, Kontron, Munich, Germany). The following parameters were quantified: distribution of white fibre cross-sectional areas, total white muscle cross-sectional area and total white muscle fibre number from one half of each fish.

Red muscle area

Succinate dehydrogenase staining for oxidative muscle (Nachlas et al., 1957) was carried out on frozen transverse sections (20 μm thickness) taken at the level of the vent. Red muscle area relative to white muscle area was quantified on a half fish using a Zeiss image analysis system (KS 300, Kontron, Munich, Germany) on five specimens from each group and stage (6 and 21 weeks after first feeding).

Immunohistochemistry

Immunostaining was carried out using the avidin–biotin (Vector Laboratories, Burlingame, CA, USA) technique on frozen transverse sections (20 μm thickness) taken at the level of the vent (Xie et al., 2001). A control was performed by substituting pre-immunised 5–10% normal goat serum in PBS for primary antibody. The primary antibodies used were anti-myogenin polyclonal rabbit IgG (M-225) and anti-MyoD, polyclonal rabbit IgG (M-318) from Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA. The secondary antibody used was polyclonal goat anti-rabbit immunoglobulin from Dakocytomation, Glostrup, Denmark.

Fish activity

Fish activity was measured using the Linton (AM10530) activity monitor (Linton Instruments, Diss, UK). The instrument fits over the fish tank and has two levels of infrared beams which, when broken by movement of fish, register activity units using the Amonlite software (Linton Instruments; Fig. 1). Fifteen fish from each incubation temperature at three different stages (3 weeks after first feeding, 6 weeks after first feeding and 21 weeks after first feeding) were used to measure

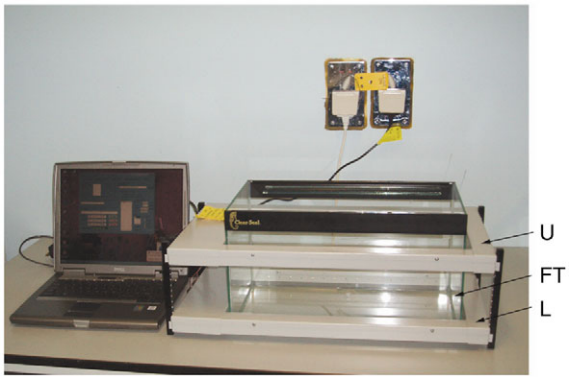

Fig. 1. Activity monitor used in the present study. U, upper level infrared beam source; L, lower level infrared beam source; FT, fish tank.

the fish activity. The activity of fish was initiated by adding a pinch of fish pellets to the tank exactly at time 0 and 5 min into the 10 min experiment for all stages examined. The Amonlite software measures the total activity, i.e. the total number of beam breaks. The software was set up such that the total number of beam breaks were recorded every 10 s, therefore a total of 60 individual measurements were made within the 10 min experiment.

Statistical analysis

All statistical analyses were performed using the SPSS 14.0 for Windows software (Chicago, IL, USA). The data was initially checked for normal distribution using the 'explore' function of the software and no modifications were necessary. Differences between the two temperatures regimes (namely 5°C and 10°C groups) were analysed using the unpaired Student's *t*-test combined with the Levene's test for homogeneity of variances to determine whether equal variances should be assumed. Results were considered statistically significant when $P<0.05$.

Results

Embryos incubated at 10°C hatched 45 days after fertilization, whereas embryos incubated at 5°C hatched 98 days after fertilization. Thus, at the higher temperature embryos hatched twice as fast as those incubated at 5°C.

Wet mass and length

The average mass of the fish incubated at 10°C was significantly greater than those incubated at 5°C at 6 weeks after first feeding. By contrast, at 21 weeks after first feeding, the average mass of the fish incubated at 5°C was significantly heavier ($P<0.001$; Fig. 2A). At 5°C the fish were significantly longer ($P<0.001$) than those incubated at 10°C at both 6 and 21 weeks after first feeding (Fig. 2B).

Morphometry

Total white muscle fibre numbers of the embryos and fish incubated at 5°C was slightly but not significantly greater at eyed stage and 6 weeks after first feeding (Fig. 3), but when muscle fibre size was investigated the 5°C group had significantly more small muscle fibres than the 10°C group at 6 and 21 weeks after first feeding (Fig. 4). The number of white muscle fibres at 21 weeks after first feeding in the 5°C group was almost twice that in the high temperature group ($P<0.001$; Fig. 3). Fig. 5 shows more fibres in the small size class at 21 weeks after first feeding in the 5°C group.

At 6 weeks after first feeding the total cross-sectional area of the white muscle was higher in the fish incubated at 10°C ($P<0.05$), whereas at 21 weeks after first feeding, the cross sectional area of the white muscle in fish incubated at 5°C was almost twice that of the 10°C group ($P<0.001$; Fig. 6A).

There was no difference in the relative area of red muscle between the two groups of fish at either 6 ($P<0.638$) or 21 ($P<0.330$) weeks after first feeding (Fig. 6B).

Immunohistochemistry

Both MyoD and myogenin immunostaining was localized in the myosepta, in agreement with the findings of Xie et al. (Xie et al., 2001). At 6 and 21 weeks after first feeding, more positive staining of MyoD and myogenin was found in fish

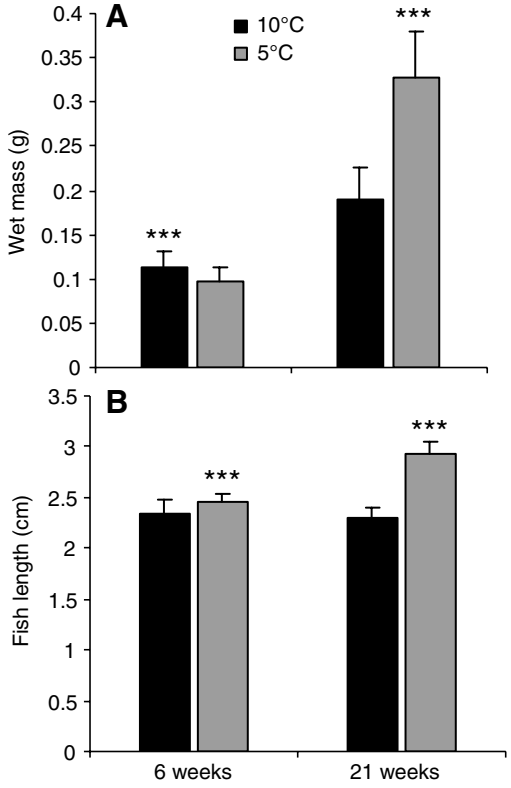

Fig. 2. Effect of incubation at either 5°C or 10°C on (A) total wet mass and (B) body length of fish at two different stages, 6 weeks and 21 weeks after first feeding. Error bars represent standard deviation of the means. A significant difference was found between the two incubation temperature groups at both stages; ***$P<0.001$, $N=20$ for both graphs.

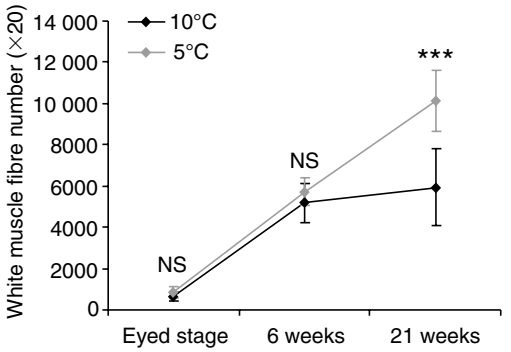

Fig. 3. Total number of white muscle fibres at different stages of growth in each incubation temperature. No significant differences were found between 10°C and 5°C fish at eyed stage and 6 weeks after first feeding (NS). However, a significant difference was found at 21 weeks after first feeding; ***$P<0.001$, $N=5$.

incubated at 5°C (Fig. 7). In control samples (with no primary antibody), no positive staining was found in the myosepta.

Fish activity

Total activity of the fish from each temperature regime was measured at three different stages (3 weeks after first feeding, 6 weeks after first feeding and 21 weeks after first feeding) using the Linton activity monitor. Fish reared at 5°C throughout were at least twice as active at 3 weeks, four times more active at 6 weeks and eight times more active at 21 weeks after first feeding than the 10°C group (P<0.001 in all cases). Moreover, the activity of the 5°C group increased twofold from 3 weeks to 6 weeks (P<0.01) and again doubled from 6 weeks to 21 weeks (P<0.001; Fig. 8).

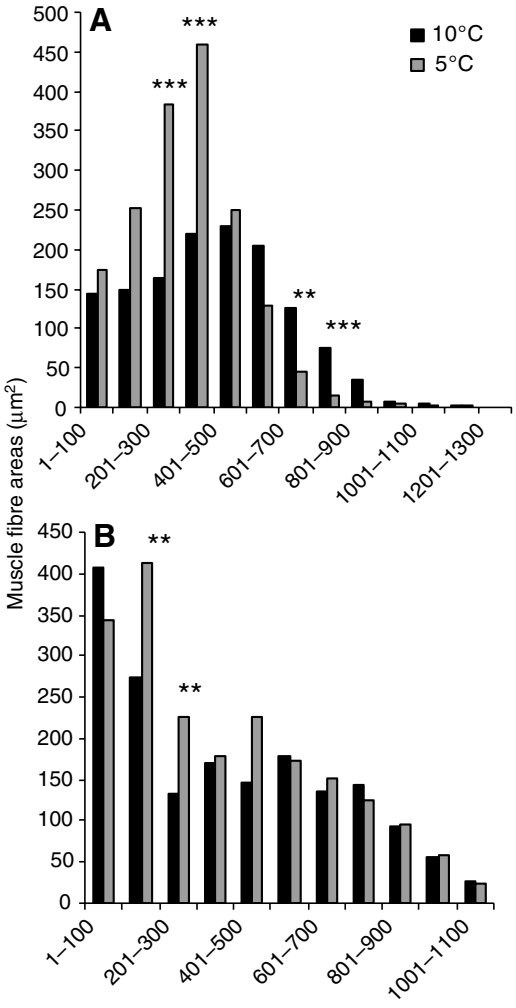

Fig. 4. Effect of incubation at either 5°C or 10°C on white muscle fibre cross-sectional areas of salmon fish at two different stages: (A) 6 weeks and (B) 21 weeks after first feeding. Significant differences in the fibre sizes were found between different regimes: **P<0.01, ***P<0.005, N=5.

Discussion

Temperature plays an essential role in salmonid muscle development (Stickland et al., 1988). Muscle growth of

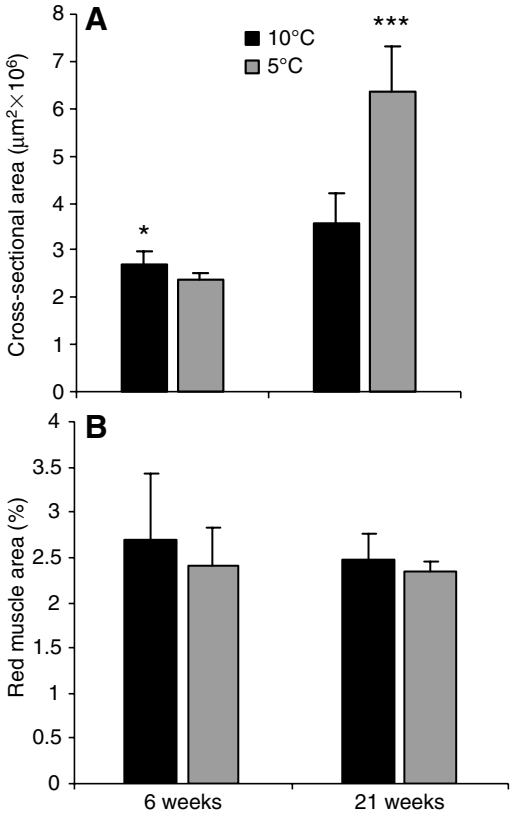

Fig. 6. (A) Total cross-sectional area, of muscle, at, 6 weeks and 21 weeks after first feeding. Significant differences were found between 10°C- and 5°C-incubated fish at both stages; *P<0.05, ***P<0.001, N=5. (B) Total red muscle area in relation to the white muscle area for fish incubated at 5°C or 10°C, at 6 weeks and 21 weeks after first feeding. No significant differences were seen between the two temperature groups, N=5.

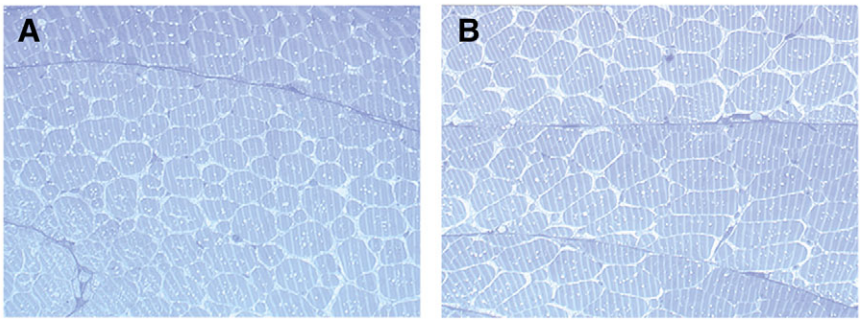

Fig. 5. Micrographs of cross sections of muscle from fish incubated at (A) 5°C and (B) 10°C, 21 weeks after first feeding, stained with 1% Toluidine Blue showing mosaic hyperplasia (MH), which was more frequently observed in fish incubated at 5°C than in fish incubated at 10°C.

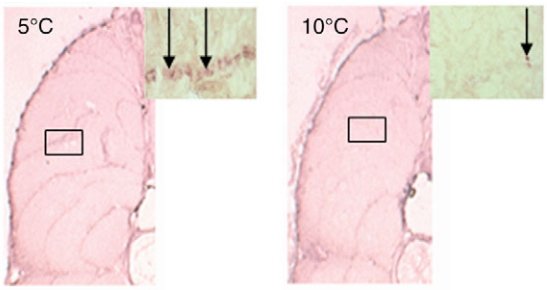

Fig. 7. Immunostaining for myogenin on transverse cross sections (21 weeks after first feeding). Boxes indicate the regions shown at higher magnification on the right. Staining shows abundant protein in the myosepta at 5°C with weaker staining in the myosepta at 10°C. Arrows highlight nuclear localization of myogenin staining.

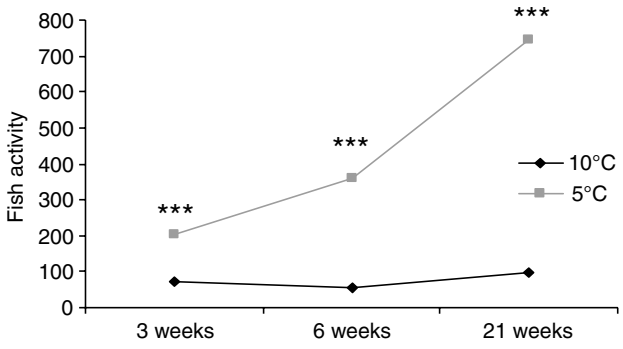

Fig. 8. Effect of incubation temperature (5°C and 10°C) on total activity of the salmon at different stages (3 weeks, 6 weeks and 21 weeks after first feeding). Significant differences was found between the 5°C and 10°C groups; ***P<0.001. Significant differences were also found between the different stages for the 5°C group; ***P<0.001, N=15.

Atlantic salmon occurs as a consequence of both hyperplasia and hypertrophy of muscle fibres. Both mechanisms continue throughout growth in salmonids (Higgins and Thorpe, 1990; Stickland, 1983). The present study has provided new information on the longer term growth and activity up to 21 weeks after first feeding of salmon reared at different incubation temperatures up to hatching.

In Atlantic salmon, high temperature is known to accelerate the rate of development and growth (Hayes et al., 1953; Marr, 1966). In our study also, embryos incubated at 10°C hatched twice as fast as those incubated at 5°C. At 6 weeks after first feeding, the fish that were incubated at 10°C tended to be shorter but heavier than the 5°C group. This shortening in the fish incubated at 10°C may be due to a decrease in the number of vertebrae formed (Matschak et al., 1995; Pavlov, 1984). The small difference between the two groups in the length of fish at 6 weeks became more pronounced by 21 weeks. At 21 weeks fish incubated at 5°C were also heavier than those incubated at 10°C. This may be because of larval transformation and shifting from endogenous to exogenous feeding which appears to significantly change the body mass to length ratio in the cooler-reared group (Nathanailides et al., 1995). At the eyed stage, the number of the white fibres was the same in embryos reared at both temperatures, this is in agreement with previous reports (Usher et al., 1994).

At 6 weeks after first feeding, the total white muscle cross-sectional area was significantly greater in the 10°C group fish, but there was no difference in the total number of white muscle fibres. One possible explanation is that the muscle growth of Atlantic salmon and other salmonids in young stages is dominated by hypertrophy of existing fibres (Higgins and Thorpe, 1990; Kiessling et al., 1991; Weatherley et al., 1980). However at 21 weeks after first feeding, the total white muscle cross-sectional area and the number of the white muscle fibres in the fish incubated at 5°C were almost double that of the 10°C group. At both 6 weeks and 21 weeks after first feeding, there were more fibres in the small size class in the 5°C incubation group compared with the 10°C group. Small fibres are almost certainly an indication of new muscle fibre formation (Stickland, 1983), which would appear to be taking place predominantly in the 5°C group between 6 and 21 weeks after first feeding. Fig. 5 shows that these small fibres are located throughout the myotome. The small fibres might contribute to the greater growth of the fish incubated at 5°C.

It has been reported that cold adaptation increases the proportion of red muscle in post-larval fish such as goldfish (Johnston and Lucking, 1978). However, in salmon it has been reported that temperature had no effect on red muscle relative area in early embryonic development (Usher et al., 1994). In our study, incubation temperature appeared to have no influence on the proportion of red to white muscle areas at later stages.

MyoD and myogenin in fish are key regulatory factors of muscle formation (Rescan et al., 1994). Activity of myogenic precursor cells is important for muscle development and muscle growth (Grounds, 1991). New muscle fibre hyperplasia in the post-larval growth of fish requires myosatellite cells (Johnston et al., 1995; Johnston et al., 1998; Rowlerson et al., 1985; Veggetti et al., 1990). These cells are usually located between the sarcolemma and the basal lamina of muscle fibres until they become activated (reviewed by Koumans and Akster, 1995). Our results showed staining of MyoD and myogenin in myosepta at 6 weeks and 21 weeks after first feeding, with apparently more of these protein in fish incubated at 5°C. This is in agreement with studies on rainbow trout at earlier stages (Xie et al., 2001). The presence of the MRFs in myosepta is consistent with the high density of myogenic precursor cells in this region described by Stoiber and Sanger (Stoiber and Sanger, 1996).

It is known that salmon spend much of their time hidden in gravel and are almost completely inactive before the yolk sac is depleted (Bams, 1967; Hansen and Moller, 1985). In this experiment salmon larvae incubated at high temperature continued to remain on the gravel after the yolk was depleted, whereas salmon larvae incubated at 5°C were more often seen

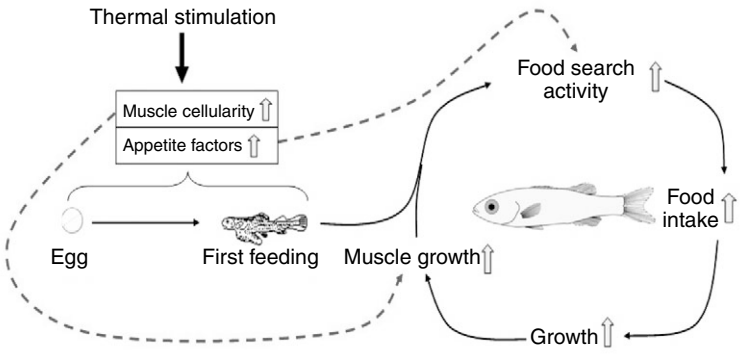

Fig. 9. A schematic to demonstrate how larval programming by thermal stimulation of muscle cellularity and appetite regulation factors may influence post-hatch growth and activity.

swimming up in search of food. We therefore decided to quantify this activity at various time points.

The results showed that incubation temperature had a very significant effect on the activity of salmon larvae at later stages of growth. Fish incubated at 5°C were found to be more active than those incubated at 10°C when food was offered at 3, 6 and 21 weeks after first feeding. Furthermore, the activity increased incrementally in the fish raised at 5°C as they aged, whereas the activity in the 10°C fish remained more constant.

One possible explanation for this phenomenon is that, during embryonic development temperature has an effect on appetite and therefore food search activity and food intake (Fig. 9). Appetite in fish is driven by a number of factors including neuropeptide Y which is widely distributed in the central nervous system in a variety of fish species, including Atlantic salmon (Vecino and Ekstrom, 1992). Neuropeptide Y plays a critical role in the control of food intake and body mass in salmonids (Aldegunde and Mancebo, 2006; Silverstein et al., 1998), but there are also other factors such as galanin and grhelin (Lin et al., 2000; Volkoff et al., 2005). We can speculate that incubation temperature of 5°C upregulates appetite-stimulating factors (compared to 10°C incubation), which then drives greater activity in the search for food. Both the muscle exercise and increased food intake would also drive faster growth (Fig. 9).

In conclusion, our results show that incubation temperature up to hatching influences post-hatch growth rate and the cellular mechanism of muscle growth up to 21 weeks after first feeding. In addition, incubation temperature has a very marked effect on post-hatch activity, increasingly so up to 21 weeks post-hatch.

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