

## PHENOTYPIC PLASTICITY OF EARLY MYOGENESIS AND SATELLITE CELL NUMBERS IN ATLANTIC SALMON SPAWNING IN UPLAND AND LOWLAND TRIBUTARIES OF A RIVER SYSTEM

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### Summary

Early myogenesis was studied in the offspring of Atlantic salmon (*Salmo salar* L.) spawning in a lowland (Sheeoch) and an upland (Baddoch) tributary of the River Dee System, Aberdeenshire, Scotland. Eggs from each population were incubated at the simulated natural thermal regimes of each stream, which was on average 2.8 °C cooler for the Baddoch than for the Sheeoch. Relationships between muscle cellularity variables, the density of myonuclei and responses to temperature were investigated using multivariate statistical techniques. These revealed highly significant temperature effects ( $P < 0.001$ ) at hatch (H) and first feeding (FF) and significant interactions between population and temperature ( $P < 0.001$ ), indicating that Baddoch and Sheeoch salmon responded differently to the two temperature regimes. The total cross-sectional area of white muscle (WF.ta) at the adipose fin was relatively independent of temperature at hatch and first feeding in the Sheeoch population. In contrast, for alevins of Baddoch origin, WF.ta was 18.9 % (H) and 30.5 % (FF) higher in fish incubated at Baddoch than at Sheeoch temperatures. At hatch, there were 15.6 % more white muscle fibres (WF.no) at the cooler incubation temperature in fish of Sheeoch origin and 6.0 % more in fish of Baddoch origin. However, by first feeding, the difference in WF.no between temperatures had narrowed to 7.2 % in the Sheeoch fish and increased to 17.4 % in the Baddoch population. In

contrast, at hatch, the density of myonuclei was 59.8 % higher at the warmer incubation temperature in the Sheeoch population and 23.5 % higher in the Baddoch population, but differences were less evident at first feeding. In Baddoch fish, 22.5 % of the total muscle nuclei were actively dividing at first feeding, as assessed by staining for proliferating cell nuclear antigen (PCNA). Of the PCNA-positive nuclei, 78 % were present in cells that stained for the *c-met* tyrosine kinase receptor, a marker of satellite cells and their division products. The proportion of *c-met*-positive cells staining for individual myogenic regulatory factors was 72.4 % for the myogenic transcription factor MyoD, 76.3 % for the myogenic transcription factor Myf-5, 62.1 % for myogenin and 48.7 % for the myogenic transcription factor Myf-6. For the Sheeoch population, there were 26.5 % more *c-met*-expressing ( $P < 0.01$ ) and 23.2 % more myogenic-regulatory-factor-expressing ( $P < 0.05$ ) cells at Sheeoch than at Baddoch temperatures. In contrast, incubation temperature had no significant effects on satellite cell density in the Baddoch population.

Key words: skeletal muscle, muscle growth, satellite cell, myogenic regulatory factor, temperature, fish population, Atlantic salmon, *Salmo salar*.

### Introduction

The Atlantic salmon (*Salmo salar* L.) is a migratory species that has a remarkably variable and flexible life history. Adult salmon spawn in fresh water in the autumn and winter, burying their eggs in river bed gravels (Stabell, 1984); the eggs hatch and the fry emerge the following spring. Young fish, known as parr, spend between 1 and 5 years in the river before smolting and migrating to the sea. Oceanic migrations take salmon to feeding grounds where they remain and grow for 1–4 years before becoming sexually mature and returning to their natal

streams to spawn. Some male parr mature precociously in fresh water and fertilize the eggs of adult females at spawning.

There is evidence that the homing habit of salmon, on both broad geographical scales (between-river) and narrower (within-river) scales, has resulted in local adaptation of populations (see Taylor, 1991). Tracking and radiotelemetry studies on the River Dee in Aberdeenshire, Scotland, have shown that fish entering the river in the winter and spring spawn at high altitudes, whereas summer- and autumn-running

fish return to lowland tributaries (Hawkins and Smith, 1986; Laughton and Smith, 1992; Youngson et al., 1994). Reproductive isolation of the populations is associated with genetic differentiation for allozymes, mitochondrial DNA (mtDNA) and minisatellite variation (Verspoor, 1997). Webb and McLay (1996) found that spawning occurred earliest at high-altitude sites in the upper reaches of the river and progressively later at sites farther downstream. The headwater tributaries are colder than the lowland streams except during the summer months, resulting in significantly longer development times (Shackley and Donaghy, 1992). It has been suggested that differences in spawning time within the river system are of adaptive significance in relation to the optimal timing of fry emergence (Webb and McLay, 1996). Much of the phenotypic diversity of salmon within rivers can also be related to environmental variables. For example, differences in temperature and water chemistry, related to geology and land use in the Dee catchment area, are known to influence growth rate and smolt production (Egglisshaw and Shackley, 1977; Shackley and Donaghy, 1992).

To accelerate development, salmon eggs in hatcheries are often incubated at temperatures significantly higher than those experienced in the wild. Stickland et al. (1988) made the important observation that rearing temperature influences the number and diameter of the muscle fibres present in salmon alevins at hatch. Embryos incubated at ambient temperature fluctuating around 1.6 °C had 30% more white muscle fibres per myotome at hatch than embryos reared at 10 °C. Similarly, Johnston and McLay (1997) reported that salmon reared at ambient river temperature, averaging 4.3 °C, had more muscle fibres and a lower density of muscle nuclei on hatching than fish reared at 8 °C and that there was significant inter-family variation in the response. By first feeding, however, the differences in muscle fibre number between groups were no longer significant, suggesting that the impact of temperature on the embryonic phase of myogenesis may differ from that on subsequent phases (Johnston and McLay, 1997).

Three distinct phases of myogenesis have been identified in Atlantic salmon (Higgins and Thorpe, 1990; Johnston and McLay, 1997). The first phase is entirely embryonic. Studies in zebrafish (*Danio rerio*) have shown that cells of the paraxial mesoderm become committed to a muscle fate at the end of gastrulation with the expression of the myogenic transcription factor MyoD (Weinberg et al., 1996). Embryonic red and white muscle fibre types are derived from distinct cell lineages and differentiate under the influence of different signalling systems (Devoto et al., 1996; Blagden et al., 1997). The first myotubes form adjacent to the notochord and migrate through the somite to produce the superficial red muscle layer (Devoto et al., 1996). A subset of these myotubes is mononuclear, migrating in the region of the major horizontal septum to form the muscle pioneer fibres, and these are the first to show contractile activity (Halpern et al., 1993). Similar mononuclear myotubes are present in Atlantic salmon embryos, forming at the 25–28 somite stage (Johnston et al., 1999). Embryonic myogenesis shows a rostral-to-caudal progression, with multi-nucleated

white muscle fibres forming laterally from the horizontal septum (Johnston et al., 1999). A second phase of production of white muscle fibres, from apical germinal zones, begins in the late embryo stages (Stickland et al., 1988) and continues until after hatching (Johnston and McLay, 1997). It has been suggested, although not proved, that the myoblasts needed for this second phase of myogenesis divide in, and migrate from, the adjacent mesenchymal lining (Stoiber and Sanger, 1996). The third and final phase of myogenesis, in both red and white muscles, begins before all the yolk has been internalised and continues until sexual maturity. It involves the activation of a muscle stem cell population, the division products of which provide nuclei both for fibre hypertrophy and for new fibre recruitment (see Johnston, 1999). The term ‘satellite cell’ is often used to describe both quiescent and activated myogenic cells located between the sarcolemma and basement membrane of muscle fibres (Koumans and Akster, 1995). The numbers of white muscle fibres per myotome in Atlantic salmon are approximately 5000 at hatching, 10 000 at first feeding, 180 000 in seawater-adapted smolts, 650 000 after one winter at sea and more than 1 000 000 after two winters at sea (Johnston, 1999; Johnston et al., 1999, 2000a).

In this study, we have investigated the effects of the relatively small differences in temperature that occur in spawning tributaries of a river system on muscle cellularity of Atlantic salmon at hatch and first feeding. Eggs were incubated in a hatchery under the simulated thermal regimes of an upland and a lowland stream. The main aim was to test the hypothesis that the effects of early thermal experience on myogenesis would differ between fish originating from the two tributaries, reflecting local adaptation of populations. A secondary aim was to determine whether egg incubation temperature influences the number of muscle satellite cells and, hence, has the potential to produce persistent effects on muscle growth phenotype, as has been demonstrated for Atlantic herring (*Clupea harengus* L.) (Johnston, 1993; Johnston et al., 1998).

## Materials and methods

### Fish

The Atlantic salmon (*Salmo salar* L.) used in the study were captured during the final stages of their spawning migration in a lowland (Sheeoch) and an upland (Baddoch) tributary of the River Dee in Aberdeenshire, Scotland, in the autumn of 1996 (Fig. 1A). Fish from the Baddoch were intercepted at a fish trap and held until the females had ovulated (30 October). Salmon from the Sheeoch were caught over a 3 week period from a 750 m stretch of the stream using electro-fishing and netting. Sheeoch fish were stripped of gametes on the 20 November. Eleven males and 11 females were obtained from each site; lengths ranged from 560 to 830 mm. Females from the Sheeoch had spent either one or two winters at sea before returning to fresh water, whereas those from the Baddoch had all spent two winters at sea. Milt and eggs (approximately 500 from each fish) were transported in plastic containers on ice to the Fisheries Research Service, Almondbank hatchery, Perthshire,

Scotland. Ova from each female were fertilised with the milt from a single male to produce 11 families from each population. Groups of eggs from each family from each population were incubated under thermal regimes designed to simulate the seasonal changes in water temperature in the Baddoch and Sheeoch. Information about the temperature variation in the two tributaries was obtained between October 1992 and June 1996 using submersible temperature recorders (Hugren, Iceland). The mean temperature over 24 h periods was calculated from hourly recordings in each stream, smoothed (Fig. 1B), and used as the input for a temperature controller supplying water to the hatchery (for further details, see H. A. McLay, I. A. Johnston, J. H. Webb and D. Robins, in preparation). The temperature in the incubation trays was recorded using Squirrel data loggers (Grant, Cambridge). The set and achieved temperatures during the experiment are illustrated in Fig. 1B. Except for brief periods, for the most part connected with essential maintenance of the cooler, the measured temperature was within 0.2 °C of the set temperature. Samples of 10 water-hardened eggs from each female were fixed in 8% (v/v) buffered formalin saline for dry mass determination.

A sample, 22 alevins from each family, under each temperature regime, was taken at 50% hatch. When approximately 95% of the yolk had been utilised and the hatchery manager deemed the fish ready to first feed, a further sample of 22 fish per family was taken. Alevins were transported live to the Gatty Marine Laboratory in vacuum flasks containing water from the experimental tanks. On arrival, they were anaesthetized in a 1:5000 (m/v) solution of bicarbonate-buffered ethyl *m*-aminobenzoate (MS222). The mass and total and standard length of each fish were recorded. In the case of the hatch sample, the yolk sac was removed and weighed, and the stage of development of the alevin was assessed by counting the number of caudal fin rays. Muscle cellularity was studied in five families at hatch ( $N=20$ ) and six families at first feeding ( $N=57$ ) for each source population. Families were selected on the basis of the mean egg dry mass of the female parent, which affects alevin size (H. A. McLay, I. A. Johnston, J. H. Webb and D. Robins, in preparation), and were representative of the range of egg sizes in the two experimental populations. Each fish was cut into three sections. The rear portion, from the adipose to the caudal fin, was fixed in Bouin's fluid for 12 h, the middle portion, between the adipose fin and last dorsal fin ray, was used to prepare frozen sections and the anterior portion was used to isolate muscle fibre bundles for electron microscopy.

#### Sample preparation

The tissue portion fixed in Bouin's fluid was embedded in wax, and 7 µm serial transverse sections were cut, mounted on glass slides and stained with haematoxylin/eosin. The whole of the middle section of the fish was mounted in a small cube of pig liver on a cork strip with the most caudal part uppermost. Blocks were frozen in 2-methyl butane cooled to near its freezing point (−159 °C) in liquid nitrogen and stored in liquid

nitrogen until sectioning. Frozen sections (−20 °C) were cut at 7 µm thickness and mounted on glass coverslips coated with poly-L-lysine. The sections were either stained with haematoxylin/eosin or used for immunohistochemical studies.

Small bundles of white muscle fibres were dissected from the anterior portion of the fish under a binocular microscope and pinned at their resting lengths to Sylgard strips and fixed for 12 h in 2.5% (v/v) glutaraldehyde, 2.5% (m/v) paraformaldehyde in 100 mmol l<sup>−1</sup> sodium cacodylate buffer at 4 °C. Samples were processed for electron microscopy as described previously (Johnston et al., 1995).

#### Immunohistochemistry

The following anti-rabbit polyclonal IgG primary antibodies were used: *m*-met, MyoD, Myf-5, myogenin and Myf-6 (all supplied by Santa Cruz Biotechnology, Inc). An anti-mouse IgG monoclonal antibody to proliferating cell nuclear antigen (PCNA) was supplied by Sigma (Poole, UK). Primary antibodies were diluted 1:20 (v/v) for *m*-met and 1:100 (v/v) for myogenic regulatory factors (MRFs) and PCNA prior to use in a solution containing 1% (v/v) Triton X-100 and 1% (m/v) bovine serum albumin (BSA) (Sigma Chemicals, Poole, UK) in phosphate-buffered saline (PBS).

Sections were fixed for 10 min in 4% (m/v) paraformaldehyde in PBS and processed for immunohistochemistry as described previously (Johnston et al., 1999). For dual labelling, *m*-met was used as the first primary antibody and PCNA, individual MRFs or mixtures of all four MRFs as the second primary antibody. Peroxidase activity was developed sequentially using 3-amino-9-ethylcarbazole and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium solution (Sigma Chemicals, Poole, UK), which give red and blue/black insoluble end products respectively. Control sections were prepared in which the various primary and secondary antibodies were omitted. Duplicate sections were counterstained in Mayer's haematoxylin to visualise total myonuclei. Counts of the numbers of cells with cytoplasm stained red for *m*-met and the number of nuclei stained blue/black for MRFs or PCNA were made from a minimum of six fields of 50 muscle fibres per fish using an image-analysis system (Kontron, Switzerland) and related to the cross-sectional area of the muscle. Nuclear counts were corrected for section thickness and the mean diameter of the nuclei determined from electron micrographs (Abercrombie, 1946).

#### Determination of muscle cellularity

The outlines of muscle fibres and the total cross-sectional area of muscle tissue were digitized using an image-analysis system, and the equivalent muscle fibre diameters were calculated (Kontron Electronik, Basel, Switzerland). A shrinkage factor was estimated from approximately 500 fibre diameters of frozen sections taken from 10 fish at hatch and 20 fish at first feeding across all families. The results were compared with equivalent measurements from Bouin-fixed material from the same fish to calculate a shrinkage factor. The

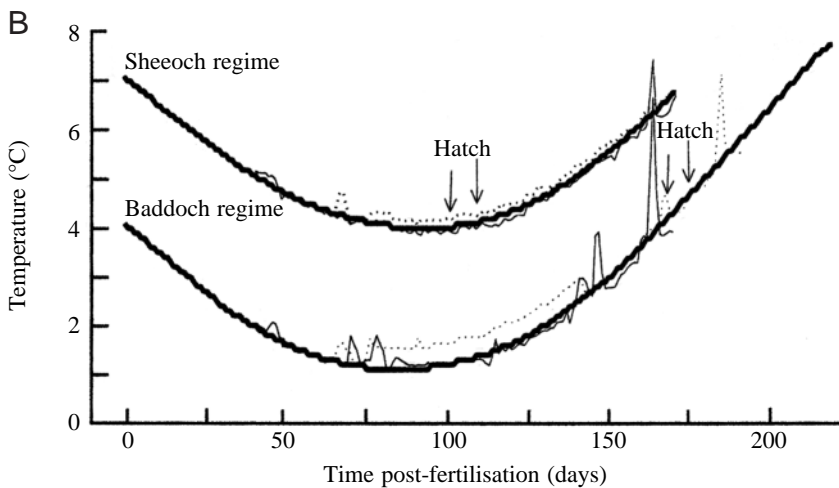
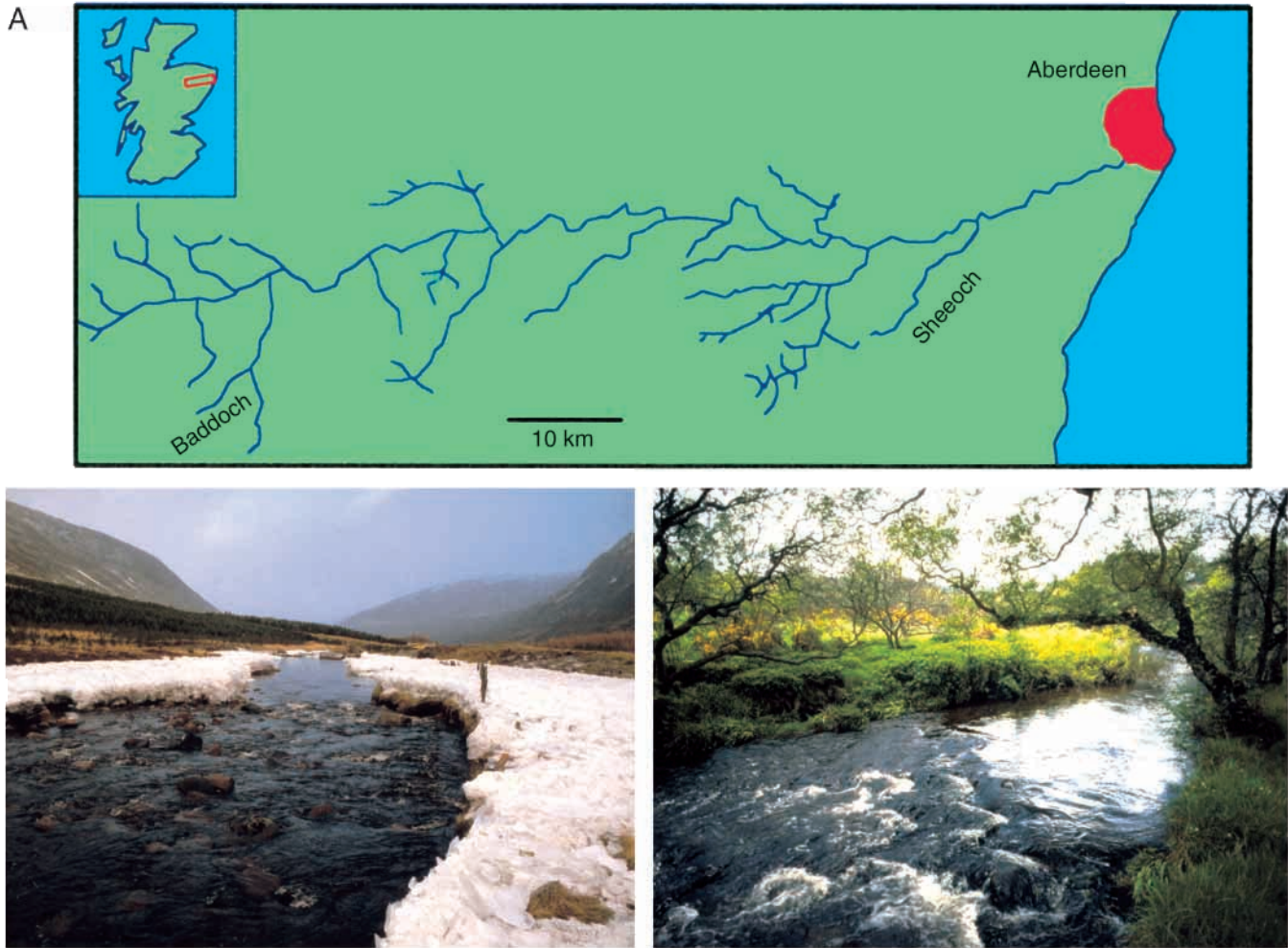


Fig. 1. (A) The River Dee system in Aberdeenshire, Scotland, illustrating the location of the lowland (Sheeoch) and upland (Baddoch) tributaries. The photographs show the spawning habitat in the two streams: Baddoch (left) and Sheeoch (right). (B) Water temperature regimes. The bold solid line shows the smoothed temperature profiles for the Baddoch and Sheeoch (the set temperatures) based on measurements made in the streams between October 1992 and June 1996. The temperatures measured in the hatchery (the achieved temperatures) are shown as fine solid lines (Baddoch eggs) and fine dotted line (Sheeoch eggs).

fibre diameters from all material fixed in Bouin's fluid were subsequently corrected using an average shrinkage factor of 5%. For the red and white muscle at hatch and the red muscle at first feeding, the number and cross-sectional areas of all the muscle fibres on one half of the fish were measured. At first feeding, all the white muscle fibres on one half of the fish were measured in 28 individuals. Since the fibre diameter

distributions were found to be similar for the dorsal and ventral quadrants of the myotomal cross section, measurements were made for the dorsal quadrant only for an additional 86 fish.

To determine the ratio of satellite cell nuclei to total myonuclei, the dimensions of satellite cells and myonuclei were measured from electron micrographs at a final magnification of 5600 $\times$ . Measurements were carried out on fry

from the Baddoch population at first feeding that had been reared at the Sheeoch temperature regime. The outlines of muscle fibres, satellite cells and myonuclei were traced onto good-quality white paper from quarter-plate negatives at the same magnification. In total, 10 fields of approximately 5–10 muscle fibres were traced, and the ratio of satellite cell nuclei to total myonuclei was calculated for each of six fish.

#### Statistical analyses

The effects of source population and thermal regime on body size at hatch and first feeding were examined by residual maximum likelihood (REML) (Robinson, 1997) using the statistical package Genstat 5. The REML algorithm estimates treatment and variance components in a mixed linear model containing fixed and random effects. Source population and thermal regime were treated as fixed effects. Two variance components, family and family $\times$ temperature, were treated as random effects. Mean egg dry mass, determined for each family, was included as a covariate (a fixed effect).

Exploratory data analysis indicated that many of the muscle cellularity variables were correlated and that the density of nuclei in white muscle was inversely related to total muscle cross-sectional area, which in turn was correlated with alevin mass. Consequently, a multivariate statistical approach was adopted to investigate responses to temperature in the progeny of Sheeoch and Baddoch salmon. The relationships between muscle cellularity variables, alevin mass and the density of myonuclei at hatch and at first feeding (stages treated separately) were examined using principal components analysis (PCA) (see Chatfield and Collins, 1980) on Genstat, using the correlation matrix. Data were log-transformed to homogenize variances. The effects of thermal regime and source population were investigated by REML on the first and second principal component scores. As before, source population and thermal regime were treated as fixed effects. Family and the variance component family $\times$ temperature were treated as random effects. The density of immunopositive cells was examined using two-way and one-way analysis of variance (ANOVA) with population source and thermal regime as fixed effects. The densities of cells expressing PCNA, MyoD, Myf-5, Myf-6 and myogenin relative to *c-met* were compared using a *t*-test or a Mann–Whitney rank sum test in cases where the data failed a test for normality.

#### Results

The range of mean egg masses was greater for the Sheeoch population (38.0–56.6 mg) than for the Baddoch (38.6–52.6 mg) population, although the average values for the means of all the families were similar, 43.4 mg and 45.8 mg respectively.

The main hatch of embryos incubated under the warmer Sheeoch regime occurred on 8–15 February (101–108 days post-fertilisation) in eggs from the Baddoch population and on 1–8 March (101–108 days post-fertilisation) in eggs from the Sheeoch population. The majority of Baddoch embryos incubated under the cooler Baddoch regime hatched between 14 and 19 April (166–171 days post-fertilisation), whereas most of the Sheeoch embryos hatched between 3 and 11 May (164–172 days post-fertilisation). The time to 50% hatch and the duration of hatching differed significantly between populations within temperatures. The detailed data have been reported elsewhere (H. A. McLay, I. A. Johnston, J. H. Webb and D. Robins, in preparation). The median numbers (range) of caudal fin rays at hatch in Baddoch and Sheeoch alevins incubated under the Sheeoch regime were 18 (14–20) and 19 (15–20), respectively. Under the Baddoch regime, the situation was reversed, Baddoch alevins having a slightly higher number of caudal fin rays 19 (15–20) compared with 18 (15–20) for Sheeoch alevins. Differences were small but significant, suggesting that alevins hatched at a slightly more advanced stage of development when reared under their native temperature regime (H. A. McLay, I. A. Johnston, J. H. Webb and D. Robins, in preparation).

#### Body size

The mean wet body mass of salmon alevins from the Baddoch and Sheeoch sampled for muscle histology was approximately 120 mg, irrespective of incubation temperature regime (Table 1). The combined adjusted mean lengths at hatch were 20.0 mm for fish from the Sheeoch and 20.5 mm for fish from the Baddoch (Table 1). Source population and thermal regime did not affect alevin size at hatch. At first feeding, alevins incubated under the Baddoch regime were slightly longer than those incubated under the Sheeoch regime ( $P<0.001$ ) (Table 1), but mass did not vary significantly between populations or with thermal regime. Inter-family variation in body mass and length was greater for fish of Sheeoch (82–225 mg; 25.3–32.5 mm) than of Baddoch

Table 1. Influence of the tributary of origin and early thermal experience on body mass and total length of Atlantic salmon (*Salmo salar* L.)

		Sheeoch population		Baddoch population	
		Sheeoch regime	Baddoch regime	Sheeoch regime	Baddoch regime
Body mass (mg)	H	120.6 $\pm$ 7.9	119.0 $\pm$ 8.2	119.8 $\pm$ 3.2	121.2 $\pm$ 3.3
	FF	164.9 $\pm$ 5.5	164.8 $\pm$ 6.9	173.7 $\pm$ 3.7	182.6 $\pm$ 4.0
Total length (mm)	H	20.3 $\pm$ 0.2	19.7 $\pm$ 0.4	20.5 $\pm$ 0.2	20.5 $\pm$ 0.1
	FF	28.9 $\pm$ 0.3	29.8 $\pm$ 0.29	29.2 $\pm$ 0.2	29.8 $\pm$ 0.1

Values are means  $\pm$  S.E.M. for 40 fish at hatch (H) and 114 fish at first feeding (FF), representing six families per population.

(142–220 mg; 27.6–30.7 mm) origin. Mean egg dry mass, included as a covariate, had highly significant effects on alevin size ( $P < 0.001$  for length and mass at both stages). Comparison of the significance of random terms in models including and excluding egg dry mass indicated that much of the inter-family variation in mass at hatch was explicable in terms of egg mass. There was also evidence that families differed in their responses to temperature; the variance component family $\times$ temperature had significant effects on mass at hatch ( $P < 0.01$ ) and on length ( $P < 0.05$ ) and mass ( $P < 0.001$ ) at first feeding. It should be noted that these findings relate to the subset of the offspring sampled for the analysis of muscle characteristics. Analysis of data from all the families in the study indicated small but significant differences in alevin size at hatch and that alevins reared under the cooler Baddoch regime were both longer and heavier than fish reared under the Sheeoch regime when sampled at first feeding (for details, see H. A. McLay, I. A. Johnston, J. H. Webb and D. Robins, in preparation).

#### *Muscle cellularity at hatch and first feeding*

Descriptive statistics (means  $\pm$  S.E.M.) for red and white muscle cellularity variables determined in Sheeoch and Baddoch families reared under the two thermal regimes are summarised in Table 2, and inter-family variation in white muscle fibre variables at first feeding is illustrated in Figs 2A–D, 3A,B.

Since preliminary data analysis indicated that many variables were significantly correlated, relationships and responses to temperature were investigated using principal

components analysis. The data were adequately described by the first two principal components, which explained 81 % and 85.4 % of the variation at hatch and first feeding, respectively (Figs 4A, 5A). On the first axis, the component loadings revealed positive correlations between fibre number, mean fibre diameter, total cross-sectional area of muscle and alevin body mass, and negative correlations between these variables and the number of muscle nuclei per mm<sup>3</sup> white muscle, at both hatch and first feeding (Figs 4A, 5A). The second principal component revealed contrasting relationships between fibre number and cross-sectional area, alevin mass and mean fibre diameter at hatch, indicating that higher fibre numbers were associated with lower mean fibre diameters. Similarly, at first feeding, there were contrasts between red fibre number and mean diameter and cross-sectional area, and between white fibre number and mean diameter, but the number of white fibres, the total cross-sectional area of the white muscle and alevin mass were positively correlated (Figs 4A, 5A).

Component scores denoted according to incubation temperature regime and population showed a clear temperature effect at hatch (Fig. 4B), reflecting the higher fibre numbers and muscle cross-sectional area and the lower density of nuclei in alevins reared under the cooler Baddoch regime. REML on the first principal component scores revealed highly significant temperature effects (Wald statistic 28.2, d.f.=1;  $P < 0.001$ ), but no indication that populations responded differently to the two temperature regimes. The variance component associated with family was significant in the random part of the model ( $P < 0.01$ ). Analysis of the second principal component revealed

Table 2. *Influence of the tributary of origin and early thermal experience on the amount and cellularity of red and white muscle from the myotomes of Atlantic salmon (Salmo salar L.)*

		Sheeoch population		Baddoch population	
		Sheeoch regime	Baddoch regime	Sheeoch regime	Baddoch regime
<b>Red muscle</b>					
Cross-sectional area muscle (mm <sup>2</sup> )	H	0.038 $\pm$ 0.0020	0.044 $\pm$ 0.0021	0.039 $\pm$ 0.0018	0.049 $\pm$ 0.0021
	FF	0.127 $\pm$ 0.0040	0.124 $\pm$ 0.0047	0.154 $\pm$ 0.0049	0.143 $\pm$ 0.0030
Number of fibres per myotome	H	251 $\pm$ 10	292 $\pm$ 6	262 $\pm$ 4	283 $\pm$ 3
	FF	491 $\pm$ 8	477 $\pm$ 10	483 $\pm$ 8	504 $\pm$ 9
Mean fibre diameter ( $\mu$ m <sup>2</sup> )	H	14.14 $\pm$ 0.20	13.31 $\pm$ 0.28	13.94 $\pm$ 0.31	14.43 $\pm$ 0.29
	FF	16.45 $\pm$ 0.22	16.51 $\pm$ 0.25	17.26 $\pm$ 0.27	17.50 $\pm$ 0.18
<b>White muscle</b>					
Cross-sectional area muscle (mm <sup>2</sup> )	H	0.86 $\pm$ 0.049	0.95 $\pm$ 0.059	0.90 $\pm$ 0.048	1.07 $\pm$ 0.031
	FF	2.74 $\pm$ 0.11	2.87 $\pm$ 0.14	3.05 $\pm$ 0.10	3.98 $\pm$ 0.11
Number of fibres per myotome	H	4110 $\pm$ 102	4752 $\pm$ 96	4269 $\pm$ 103	4526 $\pm$ 79
	FF	6187 $\pm$ 97	6634 $\pm$ 105	6336 $\pm$ 74	7436 $\pm$ 114
Average fibre diameter ( $\mu$ m <sup>2</sup> )	H	16.38 $\pm$ 0.29	14.85 $\pm$ 0.41	16.5 $\pm$ 0.23	16.39 $\pm$ 0.22
	FF	20.95 $\pm$ 0.27	20.37 $\pm$ 0.38	21.68 $\pm$ 0.30	22.97 $\pm$ 0.22
Nuclear density (mm <sup>-3</sup> )	H	429 884 $\pm$ 14 445	269 014 $\pm$ 12 478	311 070 $\pm$ 8921	251 880 $\pm$ 5405
	FF	167 804 $\pm$ 2839	164 146 $\pm$ 6467	153 466 $\pm$ 3642	141 600 $\pm$ 1947

Values are means  $\pm$  S.E.M. for 40 fish at hatch (H) and 112 fish at first feeding (FF), representing six families per population.

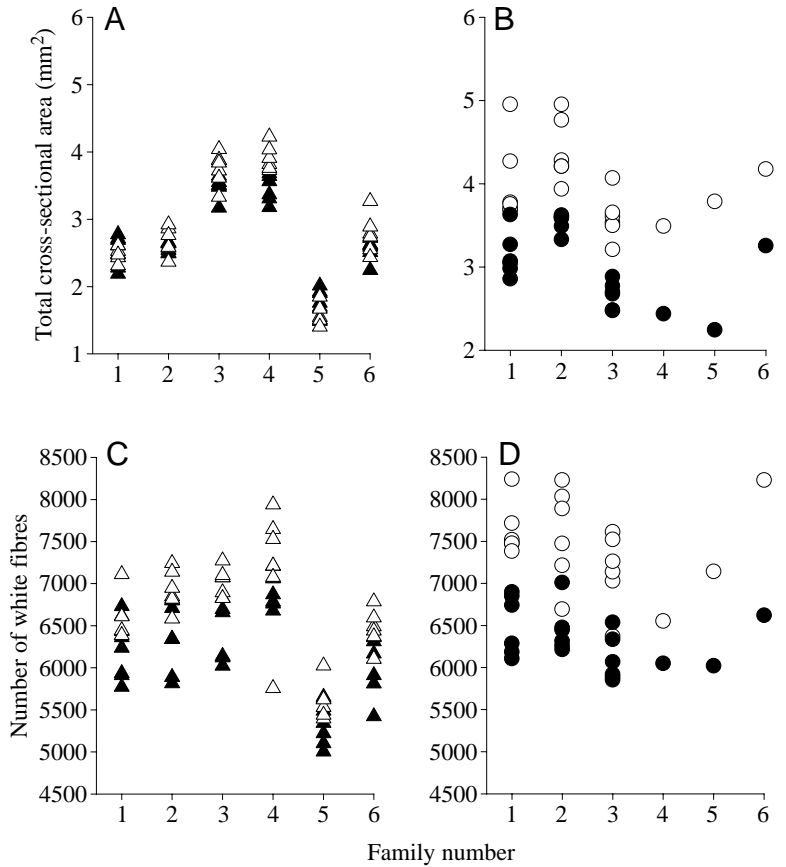


Fig. 2. Family variation in total cross-sectional area of white muscle (mm<sup>2</sup>) (A,B) and number of white muscle fibres per myotome at the level of the pelvic fin (C,D) at first feeding in Sheeoch (triangles) and Baddoch (circles) salmon reared under Sheeoch (filled symbols) and Baddoch (open symbols) thermal regimes. Each data point represents an individual fish.

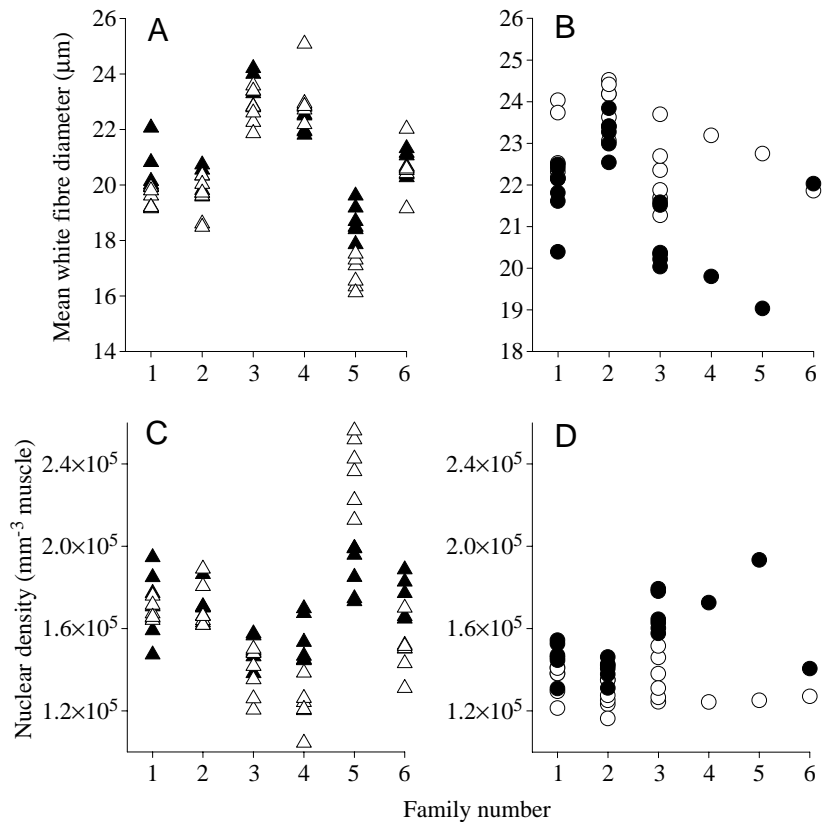


Fig. 3. Family variation in the mean diameter of white muscle fibres (µm) (A,B) and the number of nuclei per mm<sup>3</sup> of white muscle (C,D) at first feeding in Sheeoch (triangles) and Baddoch (circles) salmon reared under Sheeoch (filled symbols) and Baddoch (open symbols) thermal regimes. Each data point represents an individual fish. The diameter of a minimum of 1000 fibres per fish was measured.

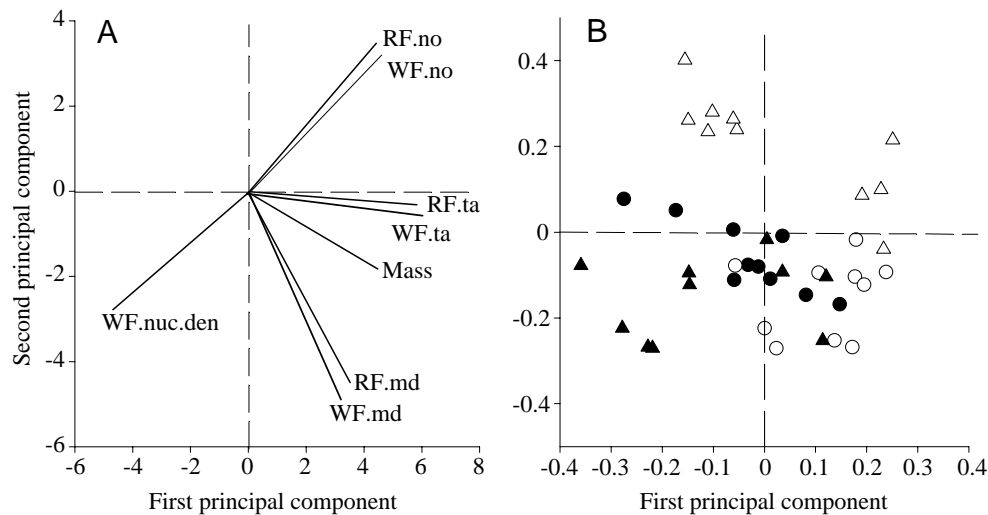


Fig. 4. Principal components analysis of muscle cellularity variables determined in Atlantic salmon (*Salmo salar* L.) from the Baddoch (circles) and Sheeoch (triangles) source population at hatching. The eggs were incubated under the simulated natural temperature regime of the Baddoch (open symbols) and the Sheeoch (filled symbols) tributaries. (A) Component loadings for the number of red muscle fibres (RF.no), total red muscle cross-sectional area (RF.ta), mean red muscle fibre diameter (RF.md), the number of white muscle fibres (WF.no), total white muscle cross-sectional area (WF.ta), mean white muscle fibre diameter (WF.md), the density of white muscle myonuclei (WF.nuc.den) and alevin body mass (Mass). (B) Component scores separated according to source population and egg incubation regime.

further temperature effects (Wald statistic 38.6, d.f.=1;  $P<0.001$ ) and population $\times$ temperature interactions (Wald statistic 15.9, d.f.=1;  $P<0.001$ ), these findings reflecting the different effects of temperature on the relationship between fibre number and mean fibre diameter in Sheeoch and Baddoch alevins. At first feeding, component scores were less clearly delineated according to temperature (Fig. 5B,C). However, analysis of the first principal component scores revealed temperature effects (Wald statistic 8.0 d.f.=1;  $P<0.01$ ) and population $\times$ temperature interactions (Wald statistic 10.2, d.f.=1;  $P<0.001$ ), indicating that the populations responded differently to the two temperature regimes. Temperature effects were more evident in Baddoch than in Sheeoch fish (Fig. 5B,C). The variance components family and family $\times$ temperature were also highly significant ( $P<0.001$ ) in the fitted model. REML on the second principal component revealed no significant effects.

The descriptive statistics (Table 2) were used to explore further the effects of temperature on muscle cellularity variables and responses in Baddoch and Sheeoch salmon. In general, there were only minor differences in the amount and cellularity of red muscle either between populations or thermal regimes (Table 2). At hatch (H), the total cross-sectional area of red muscle at the level of the adipose fin was 15.8% higher (Sheeoch fish) and 25.6% higher (Baddoch fish) in embryos incubated under the cooler Baddoch regime (Table 2). However, there was less evidence of temperature effects at first feeding (FF) (Table 2).

The total cross-sectional area of white muscle (WF.ta) at the level of the adipose fin was relatively independent of temperature at hatch and first feeding in the Sheeoch population (Table 2). In contrast, for the Baddoch population,

WF.ta was 18.9% and 30.5% higher at hatch and first feeding, respectively, at Baddoch than at Sheeoch temperatures. At hatch, there were 15.6% more white muscle fibres (WF.no) at the cooler incubation regime in fish of Sheeoch origin and 6.0% more in fish of Baddoch origin. However, by first feeding, the difference in WF.no between temperature groups had narrowed to 7.2% in the Sheeoch population and increased to 17.4% in the Baddoch population (Table 2). These differences are consistent with the results of our analysis of the principal component scores, which showed that Baddoch and Sheeoch salmon responded differently to the incubation temperatures, with temperature effects being greatest in Baddoch salmon at first feeding.

Compared at the respective thermal regimes of their natal streams, the cross-sectional area of white muscle was 45% greater in the Baddoch than in the Sheeoch fish, reflecting both the higher number and the greater diameter of white muscle fibres (Table 2). In general, differences in mean muscle fibre diameter between groups were much less than for fibre number (Table 2). For the Baddoch population, the mean diameter of white fibres was 6.1% (H) and 5.6% (FF) higher at the cooler temperature regime, but fibre diameter was relatively unaffected by temperature in the Sheeoch fish (Table 2).

The mean numbers of white muscle fibres recruited between hatch and first feeding for the Sheeoch population were 2077 for fish reared at Baddoch temperatures and 1882 for fish reared at Sheeoch temperatures. The corresponding figures for the Baddoch population were 2910 and 2067 respectively. Thus, Baddoch fish reared under their native thermal regime recruited 40.1–54.6% more muscle fibres over the period between hatch and first feeding than did the other groups.

The response of nuclear density (nuclei per mm<sup>3</sup> white



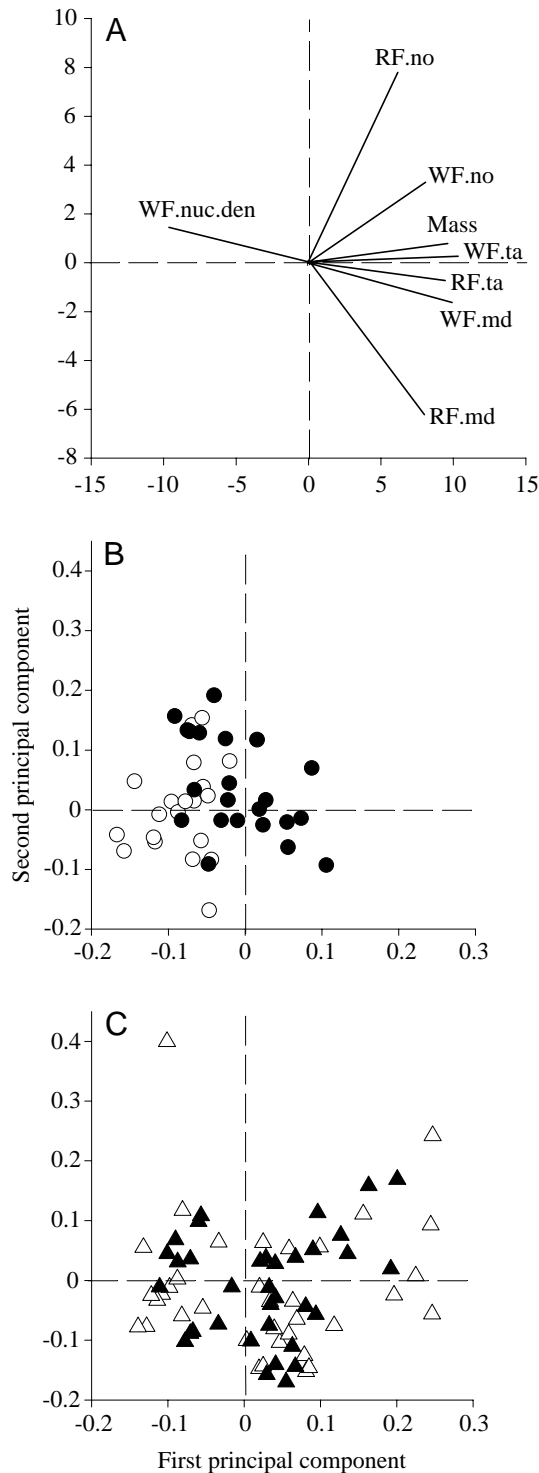


Fig. 5. Principal components analysis of muscle cellularity variables determined in Atlantic salmon (*Salmo salar* L.) from the Baddoch (circles) and Sheeoch (triangles) source population at first feeding. The eggs were incubated under the simulated natural temperature regime of the Baddoch (open symbols) and the Sheeoch (filled symbols) tributaries. (A) Component loadings for the number of red muscle fibres (RF.no), total red muscle cross-sectional area (RF.ta), mean red muscle fibre diameter (RF.md), the number of white muscle fibres (WF.no), total white muscle cross-sectional area (WF.ta), mean white muscle fibre diameter (WF.md), the density of white muscle myonuclei (WF.nuc.den) and alevin body mass (Mass). (B) Component scores for the Baddoch source population. (C) Component scores for the Sheeoch source population.

#### Muscle ultrastructure at first feeding

At first feeding, new fibres were being formed throughout the myotome, signalling the start of the final satellite cell phase of myogenesis. Muscle fibre recruitment was investigated at the ultrastructural level for 10 fish from each of the population/temperature groups. In sagittal sections, a wide range of nuclear morphology was observed (Fig. 6A–D). The early stages of myotube formation are illustrated in Fig. 6A. The arrowheads indicate one of a number of mononuclear cells (myoblasts) that appear to be fusing to form a multi-nucleated myotube on the surface of an existing fibre. Fig. 6B shows a somewhat later stage in which the cell membranes between individual myoblasts have disappeared but myofibril synthesis has not yet begun. The arrowhead indicates what appears to be a remnant of cell membrane from one of the constituent myoblasts (Fig. 6B). At this stage, the myotube is little more than the width of a nucleus, and the early stages of mitochondrial biogenesis are evident. Spindle-shaped mononuclear cells were relatively common at first feeding (Fig. 6A,B). The immunohistochemical results suggest that the majority of these cells correspond to muscle satellite cells. In some cases, a presumptive satellite cell nucleus was observed that appeared to be fusing with a mature muscle (Fig. 6C). At first feeding, the myosepta were still relatively poorly developed but contained nuclei that were presumably actively involved in the synthesis of collagen fibres (Fig. 6D). The ratio of mononuclear cell nuclei to myonuclei was determined from transverse sections for fish of Baddoch origin reared under the Sheeoch regime. The mononuclear cell nuclei represented  $24.5 \pm 3.2\%$  of the total muscle nuclei (mean  $\pm$  S.E.M.,  $N=10$  fish).

#### Immunocytochemical studies of satellite cells at first feeding

Muscle is a differentiated post-mitotic tissue and therefore only non-myonuclei can divide. Candidates for proliferating cells include the satellite cells, and their division products fated to differentiate, as well as fibroblasts and cells associated with myosepta and capillary endothelial cells. The various types of nuclei were characterised in fish of Baddoch origin reared at the Sheeoch thermal regime. The total density of nuclei was determined in sections stained with Mayer's haematoxylin and was  $135\,812 \pm 6022\text{ mm}^{-3}$  muscle (mean  $\pm$  S.E.M.,  $N=10$ ). Proliferating cell nuclear antigen (PCNA) was used as a

muscle) to temperature was different from that of the other cellularity variables. There were 59.8% and 23.5% more nuclei in the warmer than in the cooler rearing regimes in the Sheeoch and Baddoch embryos respectively (Table 2). There was considerable inter-family variation in myonuclear density (Fig. 3C,D) and in response to temperature. The Sheeoch family (no. 5) that had the smallest mean dry egg mass responded quite differently to temperature compared with other families and had a higher density of nuclei (Fig. 3C).

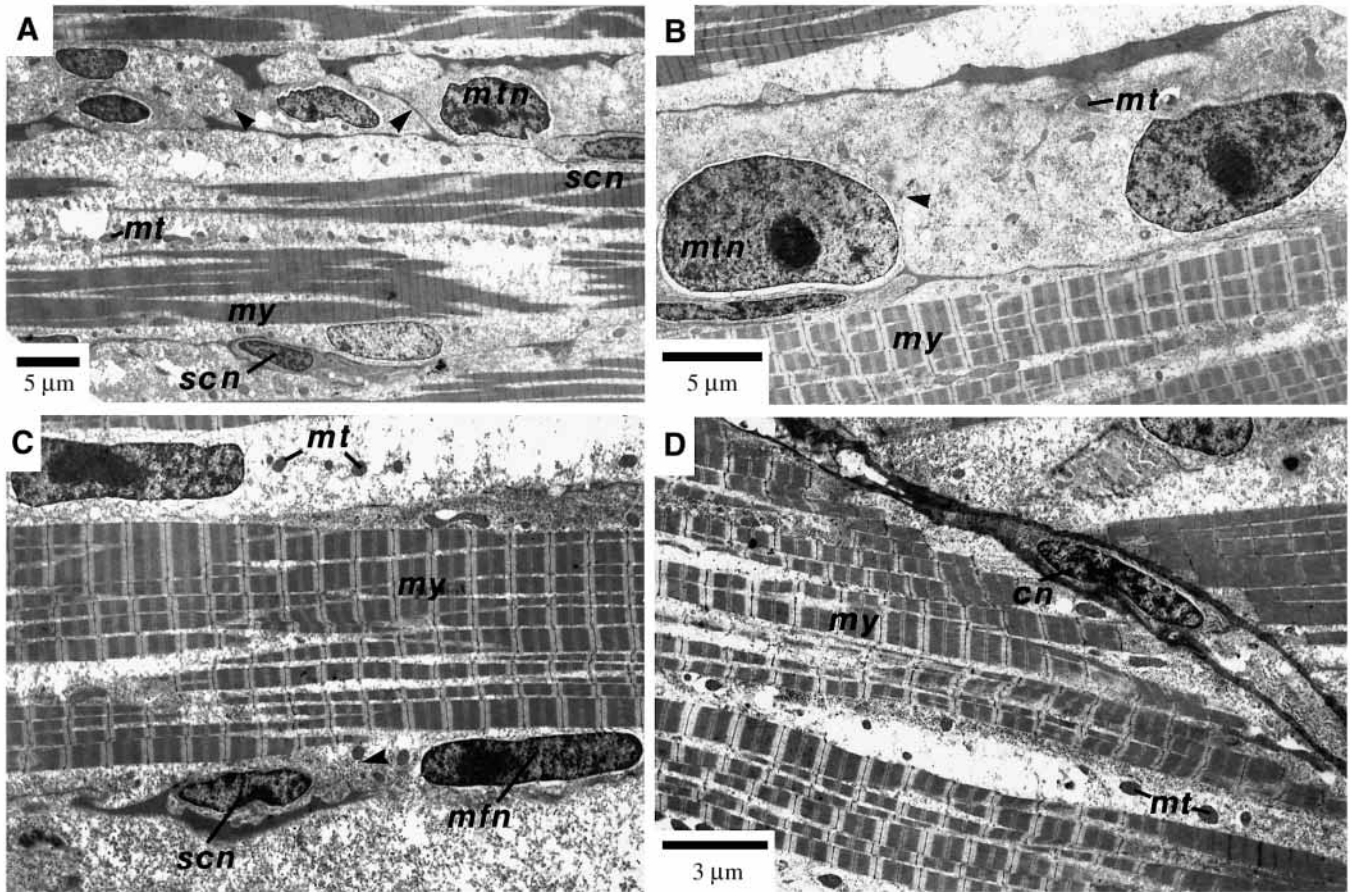


Fig. 6. Electron micrographs of sagittal sections of white muscle from Baddoch salmon at first feeding. (A) The formation of a myotube on the surface of a white muscle fibre. The arrowheads indicate the cell membrane of a myoblast prior to fusion with the myotube on the left-hand side of the micrograph. Note the wide range of nuclear morphology. (B) A mature myotube in which only a few remnants of the myoblast cell membranes are present (arrowhead). At this stage, there are no myofibrils and only small mitochondria (*mt*), which may represent an early stage of their biogenesis. (C) A segment of a white muscle fibre showing two elliptical myonuclei (*mfn*). Also illustrated is a mononuclear cell that appears to be fusing with the muscle fibre membrane (arrowhead). (D) Section through a myoseptum containing a nucleus. *cn*, connective tissue cell nucleus; *mtm*, myotube nucleus; *scn*, satellite cell nucleus; *my*, myofibril.

marker of cell division. Nuclei immunopositive for PCNA ( $30\,183 \pm 2198\text{ mm}^{-3}$  muscle; mean  $\pm$  S.E.M.,  $N=10$ ) represented  $22.5 \pm 1.8\%$  of the total nuclei. This is similar to the percentage of mononuclear cell nuclei identified in the same group of fish using electron microscopy (24.5%). An *m-met* antibody that reacts with the *c-met* tyrosine kinase receptor was used as a marker for muscle satellite cells. The density of *c-met*-positive cells was equivalent to  $17.3 \pm 3.5\%$  ( $23\,449 \pm 922\text{ mm}^{-3}$  muscle; mean  $\pm$  S.E.M.,  $N=10$ ) of the total myonuclei. There was a significant difference in the density of *c-met*- and PCNA-positive cells ( $P=0.006$ ; Mann-Whitney rank sum test). Most *c-met*-positive cells (78%) expressed PCNA, indicating that the majority were actively dividing. Assuming that *c-met* stains all satellite cells, it is likely that approximately 20% of the dividing cells in these sections corresponded to other cell types (fibroblasts, connective tissue, endothelial cells, etc.). Proliferating division products of satellite cells committed to differentiation express one or more myogenic regulatory factor. The densities of cells/nuclei immunopositive for *c-met*,

PCNA and members of the MyoD gene family in Atlantic salmon at first feeding are summarised in Fig. 7. The percentage of *c-met*-positive cells that were immunopositive for individual MRFs were 72.4% for MyoD ( $P=0.0009$ ), 76.3% for Myf-5 ( $P=0.018$ ), 62.1% for myogenin ( $P<0.001$ ) and 48.7% for Myf-6 ( $P<0.001$ ) (*t*-tests).

#### Quantification of satellite cell numbers

The total number of satellite cells was estimated by counting the number of *c-met*-immunopositive cells (Table 3). There were 26.5% more *c-met*-positive cells in Sheeoch fish from eggs incubated under the Sheeoch than the Baddoch thermal regime (Table 3) ( $P<0.01$ ; *t*-test). The division products of the satellite cells were quantified by counting the number of nuclei that were immunopositive for a mixture of all four MRFs. There were 23.2% more MRF-expressing cells in the warmer than in the cooler rearing regime for the Sheeoch population ( $P<0.05$ ). The difference between the number of *c-met*- and MRF-expressing cells provides a measure of the muscle stem

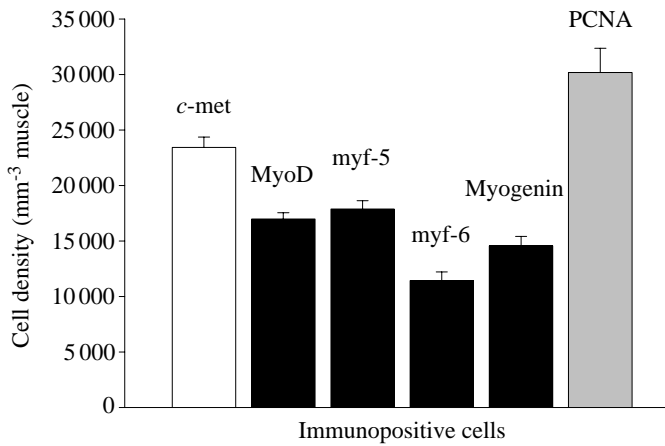


Fig. 7. Mononuclear cells in white muscle from Baddoch salmon at first feeding reared under the Sheechoch thermal regime. The density of cells immunopositive for *c-met*, MyoD, myf-5, myf-6, myogenin and proliferating cell nuclear antigen (PCNA). The columns represent mean values and standard errors,  $N=10$  fish per stain.

cell population, and the average difference was also higher for Sheechoch fish reared under the Sheechoch than under the Baddoch temperature regime. Quantitative conclusions about the relative size of the stem cell population should, however, be treated with caution because of the high variance of the estimate (Table 3). There was evidence that the density of both *c-met*- ( $F_{1,20}=8.89$ ;  $P=0.007$ ) and MRF- ( $F_{1,20}=5.07$ ;  $P=0.036$ ) expressing cells differed between populations (ANOVA). The higher values in the Baddoch than in the Sheechoch fish were largely due to the reduced densities of immunopositive cells for these antigens in Sheechoch fish incubated at the cooler temperature regime. In contrast to the situation with fish originating from the Sheechoch population, the density of satellite cells (*c-met*-positive) and their division products (MRF-positive) was independent of thermal regime in the Baddoch population (Table 3).

### Discussion

This is the first study to investigate the effects of temperature on muscle development in Atlantic salmon in a strong ecological context. Even relatively small differences in the

simulated natural temperature regime, averaging 2.8 °C, were found to be sufficient to affect muscle cellularity in salmon alevins (Table 2). We found that responses to temperature differed for the offspring of Atlantic salmon spawning in highland and lowland tributaries, consistent with our first hypothesis. Furthermore, the response to temperature regimes was different at hatch and first feeding and much more pronounced in white than in red muscle (Table 2).

At hatch, the number of white muscle fibres was 6.0% (Baddoch salmon) and 15.6% (Sheechoch salmon) higher at the cooler temperature regime; with similar differences for the red muscle (Table 2). Fish of Baddoch origin also had a 6.1% greater mean fibre diameter at Baddoch than at Sheechoch temperatures. In contrast, for Sheechoch salmon, the mean diameters of red and white muscles were similar at both temperature regimes. The differences in fibre number at hatch are of a similar magnitude to those reported previously for salmon incubated at either fluctuating ambient temperature averaging 4.3 °C or at a constant 8 °C (Johnston and McLay, 1997). In contrast, in studies involving differences of more than 8 °C between groups, white fibre number has been reported to be 30–50% greater, and mean fibre diameter significantly reduced, at the lower temperature (Stickland et al., 1998; Usher et al., 1994). Precocial hatching often occurs in salmonids reared at elevated temperatures (Heming, 1982; Gorodilov, 1983), and this may account for some of the variability in muscle fibre number reported in the literature. However, it is not sufficient to explain the differences between temperature groups observed in the present study, since embryos hatched at broadly equivalent stages of development.

By first feeding, the difference in white fibre number between temperature groups had narrowed to 7.2% in Sheechoch fish, but had increased to 17.4% in fish of Baddoch origin (Table 2). Thus, muscle fibre recruitment was substantially higher for Baddoch fish reared at the temperature of their natal stream than it was for the other groups (Table 2). The mean diameter of white muscle fibres at first feeding also continued to be greater at Baddoch than Sheechoch temperatures in the Baddoch population (Table 2). These findings are consistent with differences in alevin size and length/mass relationships at first feeding reported by H. A. McLay, I. A. Johnston, J. H. Webb and D. Robins (in preparation). Analysis of the data from all families included in the study indicated that Baddoch

Table 3. The density of immunopositive mononuclear cells in the white muscle of Atlantic salmon (*Salmo salar*) of Sheechoch and Baddoch origin reared to first feeding (FF) under the simulated temperature regime of the Sheechoch and Baddoch tributaries

Antigen	Sheechoch population cell density (mm <sup>-3</sup> muscle)		Baddoch population cell density (mm <sup>-3</sup> muscle)	
	Sheechoch regime	Baddoch regime	Sheechoch regime	Baddoch regime
<i>c-met</i>	29 003±740**	22 924±1535	29 571±3118	34 103±1668
Myogenic regulatory factors	24 909±873*	20 214±1667	24 223±3579	29 378±2190

Values are means ± S.E.M., six fish per group.

Significant differences between regimes: \* $P<0.05$ ; \*\* $P<0.01$ .

alevins were both longer and heavier in relation to length when reared under the thermal regime of their stream of origin. There were significant differences in patterns of growth in response to temperature between the two populations, and yolk conversion efficiency between hatch and first feeding was more efficient in Baddoch fish (H. A. McLay, I. A. Johnston, J. H. Webb and D. Robins, in preparation).

Our results are consistent with the growing body of evidence for the existence of a genetic basis for differences among salmon populations both between and within river systems. It is well established that segregation of the time and place of spawning in salmonids is associated with discrete stocks that differ in a wide range of phenotypic characteristics (see Taylor, 1991). Body mass in spawning Atlantic salmon, for example, decreases with the size of the river system and mean annual water discharge rates (Jonsson et al., 1991), and there is considerable variation among stocks with respect to age and mass at maturity. In chum salmon (*Oncorhynchus keta*) in British Columbia, fish returning to large rivers have larger heads, thicker caudal peduncles and larger fins than those spawning in smaller rivers (Beacham and Murray, 1987). Early-spawning stocks of chum salmon (Beacham and Murray, 1987) and Atlantic salmon (Webb and McLay, 1996) produce larger eggs, and the fry emerge later, than in later-spawning stocks. Fish from different areas also exhibit differences in morphological characters (Riddell and Leggett, 1981), physiological performance (Nicieza et al., 1994), hatch timing and pH tolerance (Donaghy and Verspoor, 1997) when reared in a common environment. Riddell and Leggett (1981) found that differences in body morphology between Atlantic salmon spawning in tributaries with different flow characteristics were heritable. Fish from the faster-flowing Rocky Brook tributary of the Miramichi River, New Brunswick, Canada, had more fusiform bodies and larger fins than fish from the slower-flowing Sabbies tributary. In chum salmon reared at constant temperature, fry survival was greater and yolk-to-tissue conversion efficiency was greater at 4°C in northern than in southern populations. Although plausible explanations for the adaptive relevance of all these characteristics can be advanced, none has been explicitly tested. However, given the growing evidence of a genetic basis for differences between populations, it is likely that, at least in some cases, they represent adaptations to particular spawning and freshwater environments. In the present study, the superior growth performance of Baddoch fish under the cooler temperature regime of their natal stream, if perpetuated beyond hatch and emergence, would serve to compensate for the longer development time and shorter duration of the growing season and thus help to maximise growth opportunity in the first year. To test rigorously the hypothesis that differences in muscle fibre recruitment and growth are of adaptive significance, it would be necessary to demonstrate a competitive fitness advantage by releasing marked fish into the wild.

Factors regulating muscle fibre number in fish embryos are very poorly understood. Stickland et al. (1988) found that a difference in the number of white muscle fibres between

temperature groups was only apparent in the late embryo, although it was not stated whether this corresponded to the appearance of germinal zones. Recent evidence suggests that differences in hypertrophic phenotype may involve the IGF-1 and/or calcineurin signalling pathways (Musarò et al., 1999; Semsarian et al., 1999). Calcineurin is a Ca<sup>2+</sup>-activated calmodulin-dependent serine phosphatase. A variety of studies with post-mitotic myocyte cell lines of rat origin suggest that muscle IGF-1 isoforms act to mobilise intracellular stores, which activate calcineurin, and induce the nuclear translocation of the transcription factor NF-Atc1 (nuclear factor of activated T cells) (Musarò et al., 1999; Semsarian et al., 1999). IGF-1 and/or activated calcineurin have also been implicated in the expression of another transcription factor, GATA-2 (Musarò et al., 1999). In cell culture, NF-Atc1 and GATA-2 have been shown to accumulate in a subset of myocyte nuclei (Abbott et al., 1998; Musarò et al., 1999) and are thought to be involved in the up-regulation of a whole range of genes involved in fibre hypertrophy (Musarò et al., 1999; Semsarian et al., 1999).

A range of molecular markers for muscle satellite cells have been identified in mammals, including *c-met* (Cornelison and Wold, 1997), myocyte nuclear factor- $\beta$  (Yang et al., 1997) and extracellular-signal-regulated kinases 1 and 2 (Yablonka-Reuveni et al., 1999). The *c-met* tyrosine kinase receptor is the receptor for hepatocyte growth factor/scatter factor (HGF/SF), which has been shown to stimulate adult satellite cells to enter the cell cycle (Tatsumi et al., 1998). *c-met* stains both muscle stem cells and their division products committed to terminal differentiation (Cornelison and Wold, 1997). In the present study, the total number of mononuclear cells was quantified from electron micrographs from one of the salmon groups at first feeding. Of the total muscle nuclei, 24.5% were mononuclear and not included in muscle fibres. This was a very similar percentage of cells to that staining for PCNA, an accessory protein of DNA polymerase that shows maximal activity during the S-phase of the cell cycle (Bravo et al., 1987). However, only 78% of the PCNA-positive cells also stained for *c-met* (Fig. 7). These results are consistent with *c-met* being a specific marker of a subset of the mononuclear cells, most probably the satellite cells. The remaining 20% of the mononuclear cells probably correspond to fibroblasts, capillary endothelial cells and macrophages. In Atlantic salmon fry, the majority of *c-met*-positive cells also expressed one or more MRFs, indicating their commitment to differentiation (Fig. 7; Table 3). Studies with mammals have shown that the primary MRFs, MyoD and Myf-5, are involved in myogenic determination, whereas the secondary MRFs, myogenin and Myf-6, are expressed later and are involved in differentiation (Megeney and Rudnicki, 1995). The variable proportions of individual MRFs expressed in salmon satellite cells are probably a reflection of their different roles in myogenesis and of temporal patterns of expression in relation to the cell cycle.

In the present study, for the Sheeoch population, the densities of *c-met*-positive and MRF-expressing cells were

both significantly higher in fish reared at the warmer temperature regime of their natal stream (Table 3). In contrast, the density of *c*-met-positive and MRF-expressing cells was independent of rearing temperature in the Baddoch population (Table 3), which may reflect the operation of additional regulatory mechanism(s) to keep cell numbers constant. Egg incubation temperature was also found to influence the number of satellite cells per mm<sup>2</sup> muscle cross-sectional area in spring-spawning Atlantic herring (*Clupea harengus* L.), with higher densities present at 5 °C than at 8 °C (Johnston, 1993). In subsequent experiments, herring were reared at either 5 °C or 8 °C until first feeding and then transferred to ambient temperature in triplicate tanks and fed *ad libitum*. Fish initially reared at 5 °C recruited more muscle fibres and showed greater rates of fibre hypertrophy over a given period than fish initially reared at 8 °C, correlating with the higher density of satellite cells (Johnston et al., 1998). In the companion paper (Johnston et al., 2000b), we test the hypothesis that temperature-related differences in the densities of muscle satellite cells in Baddoch and Sheeoch fish have persistent effects on patterns of fibre recruitment in white muscle.

Evidence for local adaptation of salmon populations within different parts of a single river system has important consequences for the management and conservation of genetic diversity. In particular, it indicates that a cautious approach should be taken to the restocking of rivers using either fish from different source populations or fish from the same source population reared under unnatural temperature regimes in a hatchery.

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