MUSCLE GROWTH AND DEVELOPMENT IN NORMAL-SEX-RATIO AND ALL-MALE DIPLOID AND TRIPLOID ATLANTIC SALMON

IAN A. JOHNSTON1,*, GILLIAN STRUGNELL1, MARTI L. MCCrackEN2 AND RAY JOHNSTONE3

1Gatty Marine Laboratory, School of Environmental and Evolutionary Biology, 2School of Mathematics and Computational Sciences, University of St Andrews, St Andrews, Fife KY16 8LB, Scotland and 3Fisheries Research Service, Marine Laboratory, SOAEFD, Aberdeen, Scotland

*e-mail: iaj@st-and.ac.uk; http://www.st-and.ac.uk/~fmrg/

Accepted 24 April; published on WWW 7 July 1999

Summary

Muscle development and growth were investigated in diploid populations of normal-sex-ratio and all-female Atlantic salmon (Salmo salar L.) and their triploid counterparts produced by high-pressure treatment. Somites were formed at the rate of 6 h−1 in both diploids and triploids at 6 °C. The rostral-to-caudal development of myotubes, myofibrils and acetylcholinesterase staining at the myosepta was slightly more advanced in triploid than in diploid fish, although the differences were smaller than among individual families. The c-met receptor tyrosine kinase was used as a molecular marker for the satellite cells involved in postembryonic muscle growth. Satellite cell nuclei comprised 17.5 % of total myonuclei in smolts and they were 24 % more abundant in diploid than in triploid fish. Cells expressing the myogenic regulatory factor myf-6, a marker of satellite cells committed to differentiation, represented 14.8 % of total myonuclei in diploids and 12.5 % in triploids. At ambient temperatures, the number of white muscle fibres in normal-sex-ratio fish increased more than 30-fold between the alevin and smolt stages, and approximately 3.5-fold further during the first year of seawater growth. The rate of muscle fibre recruitment in seawater stages was significantly greater in diploid than in triploid fish, reaching 1162 fibres day−1 and 608 fibres day−1, respectively, for all-female fish. The probability density function of muscle fibre diameters in each fish was estimated using non-parametric smoothing techniques, and the mean densities for diploids (fD) and triploids (fT) were calculated. The peak fibre diameter was approximately 20 µm in all age classes, irrespective of ploidy. Distinct bimodal distributions of muscle fibre diameter were evident in all groups 775 days and 839 days post-hatching, reflecting seasonal cycles of fibre recruitment. fD and fT were compared using a non-parametric bootstrap technique and the reference band representing the null-hypothesis indicated that there was no difference with ploidy. Reference bands for normal-sex-ratio fish at 315 days and 470 days indicated that diploids had a higher percentage of smaller-diameter fibres and that triploid distributions had a thicker right-hand tail. Similar differences in fD and fT of muscle fibre diameters were found for all-female fish, although the statistical evidence was less strong. Reference bands indicated differences in the middle range of the distributions of muscle fibre diameter in fish 620–775 days post-hatch, with triploids having a thicker right-hand tail. Thus, a lower density of satellite cells was associated with reduced rates of fibre recruitment but a compensatory increase in muscle fibre hypertrophy in triploid compared with diploid fish.

Key words: Atlantic salmon, Salmo salar, muscle, growth, development, ploidy, sex-reversed fish.

Introduction

Polyploidy has been a significant factor in the evolution of salmonids (Allendorf and Thorgaard, 1984; Johnson et al., 1987). Low frequencies of triploid salmonids occur in natural populations (Thorgaard and Gall, 1979), and triploidy can be readily induced experimentally by either heat or pressure treatment (Benfey, 1991). There are fewer, but larger, cells in most organs and tissues of triploid fish, and it has long been suspected that this may have far-reaching consequences for their development and biology (Small and Benfey, 1987; Benfey, 1991). For example, the lower numbers of brain and sensory cells in triploids is correlated with marked changes in behaviour, including a decreased sensitivity to light and sound (Aliah et al., 1990) and reduced aggressiveness relative to diploids (Benfey, 1991). Although mitotic cell divisions proceed normally in triploids, the presence of the third chromosome means that balanced chromosome constitutions cannot be established at first meiosis. Triploids are therefore functionally sterile. In fish farming, maturation essentially...
marks the end of the useful period of rearing and, if economic yields are to be maximised, fish must be sold before the deteriorative changes associated with maturation lower their market value. Salmon farmers became interested in the use of triploid fish for sterility reasons (for a review, see Ihssen et al., 1990). Because of the potential genetic introgression threat posed by the escape of diploids from fish farms, salmonid stock managers are also interested in the sterility of triploids (for a recent review, see Youngson et al., 1997). If triploid salmon could be reared as economically as diploids, then the introgression threat to wild populations would be minimised at little cost to farmers.

Although male triploids are functionally sterile, they produce near-normal levels of circulating androgens at spawning time and therefore suffer the normal deteriorative changes associated with maturation (Lincoln and Scott, 1984). Female triploids, because of differences in the architecture of the germinal and endocrine tissues, are both functionally and hormonally sterile (Sumpter et al., 1984). Only triploid females would therefore be of use in aquaculture. Sex in fish, although determined by the presence of a Y male-inducing chromosome, is extremely susceptible to manipulation. The induction of functional testes in genetic females (XX) is therefore straightforward, involving the addition of small amounts of hormones to the feed or rearing water (Johnstone and Youngson, 1984). These ‘inverted females’ (sex-reversed males) can be used to fertilise normal eggs (XX) to produce all-female offspring from which the Y chromosome has been eliminated.

The effects of triploidy on growth are variable and probably depend on the induction method, husbandry practices and the stage of the life cycle being compared (Thorgaard, 1986). Several studies have reported that triploid fish grow more slowly than diploids when reared under communal conditions, which may be related to decreased aggressiveness and/or an increased susceptibility to stress. However, triploid Atlantic salmon often show growth performance as good as or better than that of diploids when reared separately (Carter et al., 1994; Johnstone et al., 1991).

Somatic growth is closely related to that of the muscle tissue, which comprises approximately 65% of the body mass (Weatherley et al., 1979). In Atlantic salmon, distinct germinal zones of myoblasts are present at the dorsal and ventral apices of the myotome during the yolk-sac stages (Higgins and Thorpe, 1990; Johnston and McLay, 1997). By first feeding, there are approximately 10,000 white muscle fibres per myotome, and at this stage small-diameter muscle fibres begin forming throughout the myotome on the surface of existing fibres (Higgins and Thorpe, 1990; Johnston and McLay, 1997). Surprisingly, given the importance of *Salmo salar* as a food fish, there have been no systematic studies of muscle recruitment during the seawater stages of the life cycle, although it is known that there are in excess of 1 million muscle fibres per myotome after two sea-winters of growth (Johnston, 1999).

Muscle is a post-mitotic tissue, and post-embryonic growth involves the satellite cell population, which proliferates to provide a source of nuclei for fibre recruitment and hypertrophy (Veggetti et al., 1990; Koumans et al., 1991; Johnston et al., 1995, 1998). It has been reported that the yield of mononuclear cells per gram of muscle tissue is significantly higher in primary cell cultures from diploid than it is from triploid rainbow trout (*Oncorhynchus mykiss*) (Greenlee et al., 1995), suggesting that there may be more satellite cells in diploids which, in turn, would be predicted to affect muscle growth characteristics. However, in rainbow trout greater than 3 cm in fork length, Suresh and Sheehan (1998) found that the frequency histograms of muscle fibre diameter were similar in diploid and triploid fish. These authors indirectly estimated that there were 10% fewer muscle fibres per unit cross-sectional area in triploid than in diploid individuals.

The objective of the present study was to provide a comprehensive description of muscle growth in diploid and triploid Atlantic salmon (*Salmo salar* L.) using both normal-sex-ratio and all-female populations. Specifically, we wished to test the hypotheses that ploidy influences the relative timing of embryonic myogenesis, the number of satellite cells per myotome and the rate of muscle fibre recruitment. Non-parametric smoothing techniques were applied in a novel approach to investigating the density distribution of muscle fibre diameter, and hence hypertrophic growth, at different stages of the life cycle.

Recently, the c-met tyrosine kinase receptor has been identified as a molecular marker of satellite cells in the mouse (Cornelison and Wold, 1997). The MyoD family of myogenic regulatory factors (MyoD, myogenin, myf-5 and myf-6) plays an important role in muscle differentiation and the factors are expressed in proliferating satellite cells (Grounds et al., 1992; Yablonka-Reuveni and Rivera, 1994; Cornelison and Wold, 1997). In the present study, the use of antibodies to c-met and myf-6 proteins to estimate the number of satellite cells in salmon smolts is also reported.

### Materials and methods

#### Experimental stocks

Three experimental series were conducted. In the first series, beginning in 1994, the eggs from two females taken from a fish farm were pooled and fertilised with either the milt from a normal farmed male, thus producing an all-female population (AF; for the normal sex ratio (NSR), or with the milt from a sex-inverted male, thus producing a population with a normal sex ratio (NSR and triploid AF. This series of fish was reared for a prolonged period at the Fisheries Research Service salmon-rearing facility at Aultbea, Wester Ross, Scotland, at ambient, i.e. fluctuating, temperature. Fish were weaned onto dry proprietary feed when the yolk was approximately 80%
exhausted (first feeding). S1 parr destined to become smolts after 1 year in fresh water were selected in September 1995. Fish were transferred to tanks at a uniform stocking density and fed with automatic feeders. All fish feeds were supplied by Trouw Aquaculture Ltd. Fry were initially fed unpigmented dry pellets 0.3–0.8 mm diameter at the rate of 1 % kg⁻¹ biomass day⁻¹. Pellet size was increased with growth, and feeding rates varied from 2 to 2.55 % kg⁻¹ biomass day⁻¹ during the summer months to 1 % kg⁻¹ biomass day⁻¹ in the winter. Fish were transferred to sea water in April 1996 and reared in replicated tanks at comparable stocking densities until May 1997. The temperature was recorded daily throughout the experiment. The minimum and maximum temperatures experienced by freshwater stages were 1.7 °C and 21 °C, respectively, compared with 8 °C and 14 °C for seawater fish (Fig. 1).

In the second series of experiments, started in 1995, eggs from two wild salmon were fertilized with the milt from either an NSR farmed male or a sex-inverted female to produce two families of NSR and AF offspring. Approximately half the eggs were made triploid as before, and both groups were incubated at a constant temperature of 6±0.2 °C until they hatched. In the third experimental series (in 1996), the eggs from six female wild salmon were fertilized with the milt from one of two farmed males to produce six unique families of NSR fish. Diploid and triploid (prepared by pressure treatment as described above) groups were reared until first feeding at a constant temperature of 6±0.2 °C at the SOAEPF hatchery facility at Almondbank, Perthshire, Scotland.

Using the protocol of Johnstone and Stet (1995), triploidisation success was 100 % in all three experimental series as monitored by light microscopic analysis of muscle nucleoli (diploids had one or two and triploids one, two or three per nucleus) or, in larger fish, by flow-cytometric analysis of erythrocytes.

Embryonic development

Embryos from each family were sampled daily until the end of somitogenesis and every 2 or 3 days thereafter. Eggs were dechorionated using fine forceps, and the embryos were removed without damage. From each sample, at least six live triploid and six live diploid embryos were examined from each family using a stereo microscope under both bright- and dark-field illumination. Once movements had started, the embryos were anaesthetized in a 1:5000 (m/v) solution of bicarbonate-buffered MS222 (ethyl m-aminobenzoate). The somite stage and the appearance of particular organs and tissue types were noted. Approximately 10 embryos from each group were fixed in each of the following fixatives (A) Bouin’s fluid and (B) 4 % (m/v) paraformaldehyde in 0.12 mmol l⁻¹ phosphate-buffered saline (PBS) for subsequent histological and histochemical analysis, respectively. Embryos fixed in Bouin’s fluid were embedded in wax, and 7 μm serial sagittal sections were cut and stained with haematoxylin–eosin. The most posterior somite containing myotubes and myofibrils was scored for each embryo along with the somite stage. The appearance of functional endplates at the myosepta was investigated by staining embryos for acetylcholinesterase activity. Embryos were incubated in the dark for 3–5 h at 4 °C in a solution containing (in mmol l⁻¹): copper sulphate, 3; potassium ferricyanide, 0.5; maleate buffer, 100; acetylthiocholine, 1.7. The staining reaction was stopped by rinsing several times in PBS, and embryos were mounted in glycerol under glass coverslips supported by silicone grease at each corner and examined using Nomarski differential interference (DIC) optics with a Leitz DRM Systems microscope.

Studies of muscle growth

Fish were sampled 46 and 57 days after fertilization (embryo stages), at hatching and at first feeding, and fixed in Bouin’s fluid prior to processing for wax histology. Following first feeding, muscle growth was assessed using frozen sections to avoid problems associated with shrinkage. A shrinkage correction factor was applied to fibre size measurements of the earlier stages on samples fixed in Bouin’s fluid. To quantify muscle cellularity, a cross section 3–5 mm thick was cut at the level of the pelvic fin insertions and photographed against graph paper. The cross section from one half of the body was divided into 3–10 labelled blocks depending on the size of the fish. Blocks were mounted on cork strips and frozen in 2-methyl butane cooled to near its freezing point (−159 °C) in liquid nitrogen. Samples were wrapped in tin-foil to avoid desiccation and stored in a liquid nitrogen refrigerator until they could be processed. Frozen sections, 10 μm thick, were cut, air-dried and stained with the nuclear stain Scarba Red. The outlines of muscle fibres and the total cross-sectional area of muscle tissue were digitized using an image-analysis

Salmon muscle growth 1993
system, and the equivalent muscle fibre diameters were calculated (Kontron Electronics, Basel, and ScanBeam, Denmark). The estimated value of the total number of muscle fibres per myotome was plotted against the cumulative number of fibres sampled until a stable estimate was obtained. Between 800 and 1200 muscle fibres were measured per fish, representing fields from all areas of the myotomal cross section.

**Immunohistochemistry**

Transversely cut 7 μm thick frozen sections of muscle were mounted on poly-L-lysine-coated glass slides and air-dried for approximately 1 h. Sections were fixed for 10 min in 4% (m/v) paraformaldehyde in PBS, washed three times for 3 min in PBS, blotted and placed in acetic acid for 10 min and finally air-dried for 10 min. Prior to immunohistochemistry, sections were rehydrated in 1% (v/v) Triton X-100 and 1% (m/v) bovine serum albumin (BSA) (Sigma Chemicals, Poole, UK) in PBS. Background peroxidase activity was reduced by incubating the sections in 0.5% (v/v) hydrogen peroxide for 10 min, and non-specific binding sites were blocked for 15 min with a solution containing 4% (m/v) normal goat’s serum, 1% (v/v) Triton X-100 and 1% (m/v) BSA in PBS. Sections were incubated overnight in the primary antibody at 4 °C. Primary antibodies and dilutions used were as follows: rabbit anti-m-met (Santa Cruz) at 1:100, which stains the c-met tyrosine kinase receptor (Cornelison and Wold, 1997); and rabbit anti-myf 6 at 1:100 (Santa Cruz). After three washes in PBS, sections were incubated for 1 h in the secondary antibody biotinylated goat anti-rabbit IgG (Sigma Chemicals, Poole, UK) at a dilution of 1:20 in 1% (v/v) Triton X-100, 1% (m/v) BSA in PBS. Two control incubations were carried out omitting either the primary or the secondary antibody. Sections were washed three times in PBS and incubated for 1 h in a 1:20 dilution of extraAvidin peroxidase (Sigma Chemicals, Poole, UK) in 1% (v/v) Triton X-100, 1% (m/v) BSA in PBS. Peroxidase activity was developed using 3-amino-9-ethylcarbazole, which gives a red insoluble end-product. Duplicate sections were counterstained in Mayer’s haematoxylin to visualise total myonuclei. Slides were mounted under coverslips using gelatine and stored in the dark. Counts of the number of c-met/myf-6-positive cells per nucleus were made from at least six fields of approximately 50 muscle fibres per field in each fish using an image-analysis system, and related to the total cross-sectional area of muscle.

**Electron microscopy**

Small bundles of white muscle fibres were isolated from the dorsal epaxial myotomes of NSR salmon at first feeding and fixed overnight in 2.5% (v/v) glutaraldehyde, 2.5% (m/v) paraformaldehyde in 100 mmol l⁻¹ sodium cacodylate buffer, pH 7.4 at 4 °C. Samples were processed for electron microscopy as described previously (Johnston et al., 1995). Sagittal ultrathin sections of muscle fibres were cut, stained with lead citrate and uranyl acetate, and viewed with a Philips 301 transmission electron microscope. The dimensions of satellite cells were measured from photomicrographs at a magnification of 5000 times.

**Statistics**

The relationship between developmental characters and age or somite interval was fitted by linear least-squares regression. A two-way general linear model (GLM) analysis of covariance (ANCOVA) was used to test for differences in development, with ploidy and family as fixed effects and age or somite interval as a covariate (SPSS Statistical Software, SPSS Inc., USA). The somite interval (the mean time to make a somite) was calculated by least-squares regression and is equivalent to the somite stage of the embryo up until the end of somite formation. Growth variables such as body mass, the total cross-sectional area of muscle and the number of fibres per myotome were plotted against either age post-hatch or fork length. Data were fitted by least-squares regression using a second-order polynomial and, in cases where a rate variable was required, the resulting curve was differentiated (Mathematica software, Wolfram Research Inc., USA). The hypothesis that there was a single population underlying the fitted curves was tested using multiple regression analyses (Zar, 1984). The effects of ploidy on muscle cellularity variables was tested using ANCOVA with fork length as a covariate (SPSS statistical software, SPSS Inc., USA). Satellite cell densities were compared using a one-way analysis of variance (ANOVA). The data for NSR and AF fish were analysed in separate ANCOVAs since these groups were half-siblings and therefore differed in genetic background.

To evaluate and compare distributions of muscle fibre diameter, smooth non-parametric estimates of the probability density functions (PDFs) were constructed using the kernel approach (Silverman, 1986) within the S-Plus computing environment utilising the sm library (Bowman and Azzalini, 1997). The kernel method uses a smooth kernel function that is itself a PDF, such as the normal curve, as the basic building block. These smooth functions are centred directly over each observation, resulting in a smooth estimate of density while preserving the value of the realisation. The variance of the kernel function is controlled by the smoothing parameter h. The kernel estimator used was of the form:

\[
\hat{f}(y) = \sum_{i=1}^{n} w(y - y_i; h),
\]

where \(\hat{f}\) is the estimated probability density function, \(y_i\) is the \(i\)th observation from the list of \(n\) and \(w\) is the kernel function.

For simplicity when comparing groups, we sampled an equal number \(n\) of fish in each group, and we sampled an equal number \(m\) of fibres within each fish. We then estimated the PDF for each fish using the normal optimal smoothing parameter (Bowman and Azzalini, 1997). Sample sizes were 800–1000 per fish, and smoothing parameters were within the range 0.13–0.19. The mean PDFs for diploid \((P^D)\) and triploid \((P^T)\) groups were then estimated using the diameters pooled.
over group. Since it is advantageous to use a common smoothing parameter when comparing densities (Bowman and Azzalina, 1997), \( f^D \) and \( f^T \) were estimated with \( h \) equal to the mean of the normal optimal smoothing parameters over fish within an age class. To restrict diameters to positive values, we estimated density functions of the natural logarithm of diameter and then transformed back to the original scale. Furthermore, we fixed the maximal diameter within an age class. To restrict diameters to positive values, we estimated density functions of the natural logarithm of diameter within an age class. To restrict diameters to positive values, we estimated density functions of the natural logarithm of diameter and then transformed back to the original scale. Since we had a small sample size of fish, we selected fibres without replacement is preferred (Davison and Hinkley, 1997). Since we had a small sample size of fish, we selected fibres without replacement and then to sample randomly, (2) ignoring original groupings, group labels were randomly assigned such that there were \( n \) fish in each group and (3) a smooth bootstrap sample of \( m \) fibres for each fish was generated. After 100 bootstrap replications, the \( P \)-value was approximated by:

\[
P = \frac{1 + \#[D^* \geq D]}{R + 1},
\]

where \( \#[D^* \geq D] \) is the number of \( D^* \geq D \), and using the results \( D_1^*, \ldots, D_R^* \) from the \( R \) bootstrap samples (Davison and Hinkley, 1997). Since the sample size of fish within a group is small, 4–5 fish, the null hypothesis will probably not be rejected if a significance level of 0.05 is required unless a large real difference exists. Since significance level and power are related, given a fixed sample size (Zar, 1996), the critical significance level was increased to 0.1 as suggested by Steel and Torrie (1980) to increase the power of the test.

This Kolmogorov–Smirnov-based bootstrap test is a global test, and a significant result strengthens the evidence that the densities are different, but the test statistic alone provides few clues to where the differences might occur. To supplement this test, the two density curves were compared graphically. First \( f^D \) and \( f^T \) were plotted. If the null hypothesis was true, it would be natural to ignore group labels and pool fibre diameters over all fish sampled in an age class when estimating the average density \( f^{D+T} \) for an age class. To assess where the differences between \( f^D \) and \( f^T \) can be attributed to a genuine difference in structure and where they can be attributed to random variation, a variability band for \( f^{D+T} \) was constructed. The region where \( f^D \) and \( f^T \) lie outside this band suggests a major difference in structure and biomass among fish.
between the densities. Since this band provides a means of evaluating the differences between $f^D$ and $f^T$, it is referred to as a reference band.

The final stage of evaluating differences between groups was to compare the values of specific percentiles of the estimated fibre densities for each fish. The Wilcoxon two-sample non-parametric test was used to test whether the median value of the specified percentile was equivalent between groups. Specified percentiles were the fifth, tenth, fiftieth, ninety-fifth and ninety-ninth percentiles. Since we were not taking into account the variance of the percentile estimate for each fish, and we were making multiple comparisons, the reported $P$-values should be interpreted with caution.

**Results**

**Embryonic development**

The unsegmented paraxial mesoderm started to segment approximately 17 days post-fertilization at 6°C, producing transient epithelial spheres (Fig. 3A) that subsequently differentiated into mesenchymal derivatives. The somites were difficult to count accurately with a binocular microscope until the following day. Mitotic bodies were relatively common in the cells of the epithelial somites (arrows in Fig. 3A). New somites were added at the rate of approximately one every 6 h until the full complement of 62–63 somites was formed approximately 35 days post-fertilization (Fig. 4). ANCOVA of six families of NSR salmon revealed significant family differences in the rate of somite formation ($F_{5,296}=2.27; P=0.047$), but no effects of ploidy.

An examination of serial sagittal sections indicated that the first myotubes formed adjacent to the notochord at the level of the horizontal septum (long arrows in Fig. 3B). Multinucleated myotubes formed in a gradient away from the horizontal septum. Myoblasts apparently exited the cell cycle following fusion since mitotic bodies were never observed within myotubes (Fig. 5A,B). A wave of myotube formation

---

**Fig. 3.** (A) A sagittal section of a normal-sex-ratio diploid salmon embryo with epithelial somites (es) sampled 22 days post-fertilization at the 28-somite stage and stained with haematoxoylin–eosin. Somites 18–23, counting from the head, are illustrated. Arrows indicate cells actively involved in mitosis. Scale bar, 25 μm. (B) Sagittal section of a normal-sex-ratio triploid salmon embryo at the 46-somite stage stained with haematoxoylin–eosin. Long arrows indicate mononuclear myotubes at the position of the horizontal septum. The short arrow indicates a mitotic body in the sclerotome associated with a non-muscle cell. Note that some nuclei have three nucleoli (tn). Scale bar, 25 μm.
Salmon muscle growth 1997

progressed down the length of the trunk, starting in the most anterior somites at approximately the 30-somite stage (Fig. 6A). ANCOVA revealed small but statistically significant effects of family \( (F_{5,66}=9.54; P<0.001) \) and ploidy \( (F_{1,66}=8.01; P=0.006) \) on myotube formation, with no significant two-way interaction \( (F_{5,66}=1.64; P=0.16) \). The relationship between the most posterior somite with myotubes and somite interval for two of the families is illustrated in Fig. 6A. One somite interval is the mean time required for the formation of one somite pair. The rostral-to-caudal sequence

![Figure 4](image)

**Fig. 4.** Somite formation at 6 °C in full-sibling normal-sex-ratio diploid (open symbols) and triploid (filled symbols) families of Atlantic salmon. Only the data from families 1 and 5 are shown for clarity. Second-order linear regressions were fitted to the data. Family 1 (circles): for diploids (line omitted for clarity), \( N=-89.22+6.40t-0.049t^2 \) \( (r^2 \text{ adjusted}=0.96; P<0.001) \); for triploids (dashed line), \( N=-137.13+11.10t-0.156t^2 \) \( (r^2 \text{ adjusted}=0.92; P<0.001) \). Family 5 (triangles): for diploids (dotted line), \( N=-154.29+11.84t-0.161t^2 \) \( (r^2 \text{ adjusted}=0.96; P<0.001) \); for triploids (solid line), \( N=-139.28+10.82t-0.144t^2 \) \( (r^2 \text{ adjusted}=0.96; P<0.001) \), where \( N \) is the number of somites and \( t \) is age in days.

![Figure 5](image)

**Fig. 5.** Sagittal sections of normal-sex-ratio salmon embryos stained with haematoxylin–eosin. (A) Diploid embryo sampled 25 days post-fertilization at the 42-somite stage. Somite 18 is illustrated. Arrowheads indicate multinucleated myotubes. Note that the nuclei have a maximum of two nucleoli. Scale bar, 15 μm. (B) Triploid embryo sampled 42 days post-fertilization at the end of somitogenesis. Somites 31 and 32 are illustrated. Arrows indicate cells in the myosepta. Note that some of the nuclei have three nucleoli (tn). Scale bar, 25 μm. ms, myoseptum.
of myotube formation was somewhat more advanced with respect to somite interval in the majority of triploid than of diploid families. The adjusted means for families 1–6 were somite 38.3, 27.3, 24.3, 28.1, 32.9 and 29.1, respectively, whereas the adjusted means for ploidy were somite 28.06 for diploid fish and 31.91 for triploid fish.

Myofibrils started to form at the 30- to 40-somite stage (Fig. 6B). The rostral-to-caudal sequence of myofibril assembly is illustrated for two of the families in Fig. 6B. ANCOVA revealed significant differences for main effects between families \( (F_{5,67}=6.68; P<0.001) \) and with ploidy \( (F_{1,67}=7.76; P=0.007) \), with no significant family-by-ploidy interaction \( (F_{5,67}=0.35; P=0.88) \). Myotubes containing myofibrils are illustrated in Fig. 5B. Myofibril assembly coincided with the appearance of cells closely associated with the myosepta (arrows in Fig. 5B). Nuclei within myotubes from diploid fish contained one or two nucleoli (Fig. 5A), compared with one, two or three nucleoli in triploids (Fig. 5A,B). In the majority of families, myofibrillar assembly had reached more posterior somites at any given somite interval in triploid than diploid embryos. The adjusted means for families 1–6 were somite 31.5, 24.0, 19.8, 24.7, 29.4 and 23.4, and the adjusted means for ploidy were somite 23.51 for diploid fish and 15.90 for triploid fish.

Acetylcholinesterase (AChE) staining was concentrated at the myosepta (Fig. 7A) and in the cell bodies and axons of motor neurones within the spinal cord (Fig. 7B). The relationship between the most posterior somite with the character of interest, \( N \), namely myotubes (A), myofibrils (B) and acetylcholinesterase (AChE) staining at the myosepta (C) and somite interval at 6°C, in full-sibling normal-sex-ratio diploid (open symbols, dotted lines) and triploid (filled symbols, solid lines) families of Atlantic salmon (Salmo salar L.) reared at 6°C. Somites were numbered starting from the head. One somite interval (SI) is the mean time taken for the formation of one somite pair, and its use allows the embryo to be staged beyond the period of somitogenesis. Only the data from family 1 (circles) and family 5 (triangles) are illustrated for clarity. First-order linear regressions were fitted to the data. (A) \( N= \) myotubes. Family 1: diploids, \( N=14.07+0.85SI \) \( (r^2=0.97; P<0.001); \) triploids, \( N=-15.11+0.96SI \) \( (r^2=0.93; P<0.001) \). Family 5: diploids, \( N=-11.94+0.74SI \) \( (r^2=0.96; P<0.001); \) triploids, \( N=-8.36+0.65SI \) \( (r^2=0.98; P<0.001) \). (B) \( N= \) myofibrils. Family 1: diploids, \( N=-8.39+0.58SI \) \( (r^2=0.96; P<0.001); \) triploids, \( N=-12.31+0.68SI \) \( (r^2=0.93; P<0.001) \). Family 5: diploids, \( N=-33.48+0.99SI \) \( (r^2=0.97; P<0.001); \) triploids, \( N=-30.64+0.96SI \) \( (r^2=0.92; P<0.001) \). (C) \( N= \) AChE. Family 1: diploids, \( N=-14.07+0.85SI \) \( (r^2=0.93; P<0.001); \) triploids, \( N=-46.32+1.00SI \) \( (r^2=0.94; P<0.001) \). Family 5: diploids, \( N=-10.04+0.62SI \) \( (r^2=0.86; P<0.01); \) triploids, \( N=-15.90+0.70SI \) \( (r^2=0.88; P<0.01) \).

Organogenesis was investigated in relation to both somite stage and age for eight NSR (six from the 1995 series and two from the 1996 series) and two AF (1995 series) families of triploid and diploid fish (Table 1). Although there were small family differences in the somite stage at which some morphological characters were first visible using DIC optics, no consistent differences between diploid and triploid fish
Salmon muscle growth

could be detected with the frequency and density of sampling undertaken. We therefore conclude that the triploidisation procedure produces at most only very minor changes in the timing of organogenesis. Hatching started 81 days post-fertilization at 6 °C for both groups and continued for 10–12 days.

**Somatic growth**

The relationship between fork length and age post-hatch was described by second-order polynomials and was not significantly different between diploids and triploids in NSR ($F_{3.80}=2.88; P=0.082$) (Fig. 8A) and AF ($F_{3.77}=2.55; P=0.12$) fish (Fig. 8B). However, diploids had a higher body mass for a given fork length than triploids for both NSR ($F_{3.39}=7.17; P<0.02$) (Fig. 8C) and AF ($F_{3.46}=18.44; P<0.001$) fish (Fig. 8D). For example, at 40 cm fork length in NSR groups, the mean body mass calculated from the regression equations in Fig. 8C was 7.6% greater for diploid (832.9 g) than for triploid (747.2 g) fish. There was a low (<5%) incidence of skeletal deformities in triploid groups, with the most common abnormalities being pronounced lower jaw growth, craniomegaly and twisted caudal vertebrae.

**Muscle growth in freshwater stages**

Muscle growth is a complex process involving the recruitment and hypertrophy of successive cohorts of muscle fibres. In NSR diploids, the mean number of muscle fibres per myotome at the level of the pelvic fin insertions had increased from 2786 in embryos 46 days post-fertilization to 5374 following hatching (85 days post-fertilization) and had almost doubled to 9852 by the time more than 95% of the yolk had been reabsorbed and the fish were ready to feed (Fig. 9). At hatching, the production of white muscle fibres was restricted to germinal zones at the ventral and dorsal (arrowheads in

Fig. 7. Acetylcholinesterase staining in whole-mount Atlantic salmon (*Salmo salar* L.) embryos. (A) A 45-somite stage normal-sex-ratio triploid embryo 39 days post-fertilization. Note that the staining is largely confined to the myosepta (arrows). Scale bar, 100 μm. (B) Motor neurone cell bodies in the spinal cord of a diploid Atlantic salmon embryo sampled 27 days post-fertilization at the 45-somite stage. The position of the spinal cord is marked by large arrows. Small arrows indicate primary motor neurone cell bodies, and pairs of arrowheads illustrate the cell bodies of secondary motor neurones. Scale bar, 25 μm. a, anus; ff, primary fin-fold; g, gut; sc, spinal cord.
Fig. 10A) apices of the myotomes. By first feeding, newly recruited very small-diameter white muscle fibres were evident scattered throughout the myotome in both diploid (arrows in Fig. 10B) and triploid (not illustrated) fish, consistent with the activation of the satellite cell population. A general linear model ANCOVA was run for yolk-sac stages with age and ploidy as main effects, fish length as covariate and fibre number and diameter as dependent variables. For NSR fish, the number of muscle fibres per myotome increased with age ($F_{3,36}=12.02; P<0.001$) and was significantly greater in diploids than triploids ($F_{1,36}=5.85; P=0.022$), with no significant age-by-ploidy interaction. The differences in muscle fibre number were relatively small for embryos, 7.0% at 46 days post-fertilization and 7.3% at hatching (Fig. 9C). In contrast, there were 31% more white muscle fibres in diploid than in triploid fish at first feeding (Fig. 9D). There was still a significant main effect of ploidy when the ANCOVA was re-run with only the embryos ($F_{1,25}=13.03; P=0.001$), with weighted mean numbers of fibres per myotome of 3894 for diploid fish and 3599 for triploid fish, i.e. 7.6% higher in

Eight families of normal-sex-ratio diploid and triploid fish and two families of all-female fish were examined.

Table 1. Embryological development in Atlantic salmon (Salmo salar L.) reared at 6 °C

<table>
<thead>
<tr>
<th>Time post-fertilization (days)</th>
<th>Somite stage</th>
<th>Developmental features</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>0</td>
<td>Embryos still in gastrulation stages</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>Neural tube formation; 20–30% epiboly</td>
</tr>
<tr>
<td>17</td>
<td>6</td>
<td>50% epiboly; germ ring and embryo distinct; some somites visible</td>
</tr>
<tr>
<td>18</td>
<td>9–11</td>
<td>Formation of brain and eye vesicles; 60–90% epiboly</td>
</tr>
<tr>
<td>19</td>
<td>15–16</td>
<td>Development of eyes (visible laterally)</td>
</tr>
<tr>
<td>20</td>
<td>18–21</td>
<td>Otic vesicle formed; 90–100% epiboly</td>
</tr>
<tr>
<td>21</td>
<td>22–24</td>
<td>Start of segmentation of hindbrain into five rhombomeres; development of branchial chamber; cardiac tube in pericardial chamber</td>
</tr>
<tr>
<td>22</td>
<td>27–28</td>
<td>Forebrain/midbrain boundary appearing; optic lens visible</td>
</tr>
<tr>
<td>23</td>
<td>31–34</td>
<td>Gut and stomach primordia visible; myotomes acquiring chevron shape; midbrain/hindbrain boundary apparent; one branchial arch visible; heart tube bending forwards</td>
</tr>
<tr>
<td>24</td>
<td>36–37</td>
<td>Choroid fissure complete; lumen in stomach and gut; operculum visible</td>
</tr>
<tr>
<td>25</td>
<td>38–40</td>
<td>Hindgut formation; tail detached from yolk at most caudal somites; otoliths present in otic vesicles</td>
</tr>
<tr>
<td>26</td>
<td>41–44</td>
<td>Separation of ventral mid-body from yolk; heart tube moved to the left; slow heart beat apparent; posterior end of pronephros level with end of gut; hindgut turned ventrally towards future anus</td>
</tr>
<tr>
<td>27</td>
<td>45–47</td>
<td>Blood flow in dorsal aorta; two branchial arches; pectoral fin buds at somites 2–4</td>
</tr>
<tr>
<td>28</td>
<td>47–50</td>
<td>Weak sporadic contractions of the trunk</td>
</tr>
<tr>
<td>29</td>
<td>49–52</td>
<td>Two-chambered heart with double beat and faster heart rate; movement in response to dechorionation; lens separate from optic cup; rhombomere divisions starting to disappear; anus formed at level of somites 37–38</td>
</tr>
<tr>
<td>30</td>
<td>53–55</td>
<td>Circulation visible along gut; rhombomere divisions almost disappeared; jaw developing; mesenchyme in fin-fold</td>
</tr>
<tr>
<td>31</td>
<td>62–64</td>
<td>Rhythmic movements observed in situ; strong movements in response to dechorionation; olfactory organ present; hatching glands apparent; dorsal and ventral fin-folds present; rhombomeres no longer distinct; three branchial arches; pectoral fin as high as it is wide</td>
</tr>
<tr>
<td>32</td>
<td>63–64</td>
<td>Thickening of fin-folds</td>
</tr>
<tr>
<td>33</td>
<td>63–64</td>
<td>Rudimentary cloaca; liver primordium; vitelline vein functioning; cerebral circulation apparent</td>
</tr>
<tr>
<td>34</td>
<td>63–64</td>
<td>Pigmentation over part circumference of eye; gill slit in anterior branchial chamber; heart valves present</td>
</tr>
<tr>
<td>35</td>
<td>63–64</td>
<td>Four branchial arches; operculum covers base of first branchial arch; caudal end of notochord curves upwards</td>
</tr>
<tr>
<td>36</td>
<td>63–64</td>
<td>Pigmentation of whole of circumference of eye; haemoglobin present; eyes visible through chorion; intestinal arteries and gut capillaries functional</td>
</tr>
<tr>
<td>37</td>
<td>63–64</td>
<td>Approximately two-thirds of yolk sac vascularised; liver lobulated and vascularised</td>
</tr>
<tr>
<td>38</td>
<td>63–64</td>
<td>Superior semicircular canal complete; lateral head movements – lower jaw free of yolk sac</td>
</tr>
<tr>
<td>39</td>
<td>63–64</td>
<td>Complete vascularisation of yolk; circulation in dorsal aorta extends to tail; early caudal fin rays apparent; circulation in branchial arches and olfactory pits; all semicircular canals complete</td>
</tr>
<tr>
<td>40</td>
<td>63–64</td>
<td>Branchial arch blood vessels complete; blood flow in network of fine vessels across brain; fin-fold shaped inwards at anus; formation of dorsal and anal fin rays; skin pigmentation apparent on dorsal surface of head and trunk</td>
</tr>
<tr>
<td>41</td>
<td>63–64</td>
<td>Spontaneous flapping movement of pectoral fins; jaw movements; formation of gill filaments</td>
</tr>
<tr>
<td>42</td>
<td>63–64</td>
<td>Elongation of head and snout; pelvic fin buds apparent; operculum covers 3–4 of the gill arches; elongation of yolk sac; skin pigmentation visible through chorion; pectoral fin rays present</td>
</tr>
<tr>
<td>43</td>
<td>63–64</td>
<td>Hatching begins</td>
</tr>
</tbody>
</table>

I. A. JOHNSTON AND OTHERS

Fig. 10A) apices of the myotomes. By first feeding, newly recruited very small-diameter white muscle fibres were evident scattered throughout the myotome in both diploid (arrows in Fig. 10B) and triploid (not illustrated) fish, consistent with the activation of the satellite cell population. A general linear model ANCOVA was run for yolk-sac stages with age and ploidy as main effects, fish length as covariate and fibre number and diameter as dependent variables. For NSR fish, the number of muscle fibres per myotome increased with age ($F_{3,36}=12.02; P<0.001$) and was significantly greater in diploids than triploids ($F_{1,36}=5.85; P=0.022$), with no significant age-by-ploidy interaction. The differences in muscle fibre number were relatively small for embryos, 7.0% at 46 days post-fertilization and 7.3% at hatching (Fig. 9C). In contrast, there were 31% more white muscle fibres in diploid than in triploid fish at first feeding (Fig. 9D). There was still a significant main effect of ploidy when the ANCOVA was re-run with only the embryos and newly hatched alevins ($F_{1,25}=13.03; P=0.001$), with weighted mean numbers of fibres per myotome of 3894 for diploid fish and 3599 for triploid fish, i.e. 7.6% higher in
Salmon muscle growth

In contrast, for AF fish, the main effect of ploidy on the number of muscle fibres per myotome was not significantly different for similar stages (Fig. 9A–C) \((F_{1,26}=3.63; \ P=0.068)\).

The mean diameter (\(\mu m\)) of white muscle fibres was significantly greater in triploids than in diploids for both NSR \((F_{1,29}=9.81; \ P=0.004)\) and AF \((F_{1,26}=76.9; \ P<0.001)\) fish, with
adjusted mean values of 7.76 μm (triploid) and 6.44 μm (diploid) and 5.37 μm (triploid) and 4.50 μm (diploid), respectively (Fig. 11) (all stages). The number of white muscle fibres increased approximately 30-fold in NSR salmon between the alevin and smolt stages (Table 2).

Satellite cell densities in smolts

The number of satellite cells was estimated in 7 μm thick frozen sections using antibodies against c-met and myf-6. The c-met receptor tyrosine kinase is located on the cell surface, whereas myf-6 is expressed in the nucleus. The dimensions of satellite cells were investigated using transmission electron microscopy in sagittal sections of white muscle from diploid fish. Satellite cells are spindle-shaped cells approximately 2–5 μm in diameter and 7–14 μm long with a separate membrane from the adjacent muscle fibres, containing a prominent nucleus (Fig. 12A,B). At first feeding, the satellite cells were relatively abundant, and most were located between the muscle fibres (Fig. 12A). In sagittal section, the nucleus occupied approximately 0.40±0.14 (mean ± s.d.) of the length of the cell (measurements from 12 cells). Ultrathin (60 nm) transverse sections at the level of the satellite cell nucleus revealed a relatively sparse cytoplasm with occasional rough endoplasmic reticulum (Fig. 12A).

Frozen sections were stained with antibodies to c-met (Fig. 13A,B) and myf-6 (Fig. 13C). In estimating the number of nuclei in c-met-positive cells, a correction factor was applied to account for the greater probability of encountering the cytoplasm than the nucleus in transverse sections of satellite cells. Control sections with either the primary or secondary antibodies omitted were completely unstained. The density of c-met-positive cells was 24 % higher in diploid than in triploid smolts (F₁,₈=5.98; P=0.040) and the density of myf-6-positive cells was 34 % higher (F₁,₈=8.10; P=0.022) (Table 3). The ratio of nuclei in c-met-positive cells to total myonuclei was approximately 0.175, irrespective of ploidy. In smolts, 22 cm mean length, there were 23 % more myofibre nuclei of all classes per unit volume in diploids than in triploids (F₁,₈=10.4; P=0.012) (Table 3).

Muscle growth in seawater stages

The total cross-sectional area of white muscle in NSR diploids increased more than 3100-fold between the alevin stage and the end of first year of seawater growth to 42 cm fork length (Fig. 14A,B). In smolts, 18 cm fork length, the total cross-sectional area of white muscle was similar in NSR diploids (511 mm²) and NSR triploids (478 mm²) (Fig. 14A). However, the hypothesis that the total cross-sectional area of white muscle fibres was the same in diploid and triploid fish was rejected for both NSR (F₃,₇₈=7.48; P<0.001) (Fig. 14A) and AF (F₃,₇₆=4.18; P=0.017) fish (Fig. 14B) because of

Table 3. Density of c-met- and myf-6-positive nuclei and total myofibre nuclei in the fast muscle of normal-sex-ratio diploid and triploid salmon smolts

<table>
<thead>
<tr>
<th>Antibody/or other stain</th>
<th>Number of nuclei per mm³ muscle cross-sectional area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diploids</td>
</tr>
<tr>
<td>c-met</td>
<td>12 197±2423</td>
</tr>
<tr>
<td>myf-6</td>
<td>10 200±2329</td>
</tr>
<tr>
<td>Meyer’s haematoxylin (total myofibre nuclei)</td>
<td>69 087±6943</td>
</tr>
</tbody>
</table>

A correction was made for section thickness and the relative probability of encountering the cytoplasm or nucleus of a mononuclear cell stained with c-met (see text for details).

Results are presented as ±s.d. for five fish per group.
differences in the larger fish. This may in part reflect differences in body shape between diploids and triploids as well as a small difference in body mass for a given fork length. In 42 cm fish, the cross-sectional area of white muscle was 2995 mm² in NSR diploids compared with 2533 mm² in NSR triploids, i.e. 14.8% greater. At this body length, the cross-sectional area of white muscle was approximately 4.8% greater in AF diploids than in triploids.

The number of white muscle fibres increased approximately 3.5-fold during the first year of seawater growth. Muscle recruitment was investigated by fitting a second-order polynomial to the estimates of the number of white muscle fibres per myotome in the different age classes (Fig. 15A,B). ANCOVA with fish length as covariate showed significant main effects of age class and ploidy on the number of white muscle fibres per myotome for both NSR and AF populations (P<0.001). Tukey post-hoc tests revealed significant differences in muscle fibre number between diploids and triploids for all the seawater stages of the life-cycle (P<0.05). After 800 days, the mean number of fast muscle fibres calculated from these relationships was 540 051 for NSR diploids, 363 596 for NSR triploids, 525 361 for AF diploids and 369 891 for AF triploids. The mean rates of muscle fibre recruitment were found by differentiating the second-order regressions in Fig. 15A,B. Rates of recruitment 800 days post-hatch were 931 and 659 fibres day⁻¹ for diploids and triploids, respectively, in the NSR groups (Fig. 15C) and 1162 and 608 fibres day⁻¹ in diploids and triploids, respectively, in the AF fish (Fig. 15D).

The relationship between the number of muscle fibres per myotome at the level of the pelvic fin insertions and fork length is illustrated in Fig. 16A,B. For 42 cm fork-length fish, there were approximately one-third more muscle fibres per myotome in diploid than in triploid groups, 649, 878 and 413 619, respectively.
respectively, for all-female fish. The major differences in muscle cellularity arising from the higher rates of fibre recruitment and lower rates of hypertrophic growth in diploid than in triploid groups are illustrated in Fig. 16C,D by plotting the number of fibres against the total cross-sectional area of white muscle. In general, for the seawater stages of the life cycle, there were approximately one-third more muscle fibres per unit of muscle cross-sectional area in diploid than in triploid fish in both groups.

The distribution of muscle fibre size: estimating hypertrophic growth

The estimated probability density function (PDF) for each fish in the age classes sampled and the mean PDF for all fish are shown for the NSR (Fig. 17) and AF groups (Fig. 18). Variation between individual fish generally increased with age (Figs 17, 18). At first feeding in NSR diploid salmon, the peak PDF corresponded to a muscle fibre diameter of approximately 20 μm. For individual NSR diploids, several peaks in the PDF were apparent at 315 days, 470 days and 620 days post-hatch, indicating cycles of new muscle fibre recruitment (Fig. 17). Variability bands for the mean PDF for these ages (Fig. 19) provided no evidence for a multimodal distribution. Although the PDF was broader in these age classes, the peak still corresponded to a diameter of 20 μm. The maximum muscle fibre diameter in NSR fish increased from 45 μm at first feeding to 120 μm at 315 days post-hatch and was approximately 200 μm in the 775 day and 839 day age classes.

Distinct bimodal distributions of muscle fibre diameter were apparent and supported by the variance bands (Figs 17–20) in

Fig. 11. The mean diameter of fast muscle fibres in the embryonic and yolk-sac stages of normal-sex-ratio (NSR) and all-female diploid (open columns) and triploid (filled columns) Atlantic salmon reared at ambient temperatures. (A) 46-day embryos, (B) 57-day embryos, (C) 1-day-old alevins (Hatch) and (D) fish at first feeding (all-female fish not sampled). Values are means ± s.d. for five or six fish per sample point.

Fig. 12. Electron micrographs of sagittally sectioned white muscle fibres from diploid normal-sex-ratio salmon at first feeding illustrating (A,B) satellite cells and (C) myofibre nuclei. The arrowhead indicates rough endoplasmic reticulum and the double arrowheads indicate the muscle fibre sarcolemma. mt, mitochondrion; mf, myofibril; n, myofibre nucleus; s, satellite cell; sn, satellite cell nucleus; z, Z-line. The scale bars represent 1 μm in A, 2 μm in B and 3 μm in C.
both the NSR and AF groups sampled 775 days and 839 days post-hatch, reflecting seasonal cycles of seawater growth. The density of muscle fibres was compared using a non-parametric bootstrap test and reference band, which represents the null hypothesis that there was no difference between the groups. Regions of the curves where the density estimates (broken line, diploids; solid line, triploids) were out of the range of the 100 bootstrapped density estimates indicated a difference between diploid and triploid fish (Figs 21, 22). Test results between groups with age class are shown in Table 4. For NSR fish, the probability density curves were not significantly different between diploids and triploids at first feeding (101 days post-hatch) \( P = 0.32 \), but there was evidence for significant differences for all other age classes \( P = 0.07 \) (Table 4).

For AF fish, the density curves were significant for 315 days, 775 days and 839 days \( P = 0.07 \), but not significant for 470 days and 620 days (Table 4). For both these age classes, there is one fish whose estimated PDF is distinctively different from that of the other fishes in the group. When the Kolmogorov–Smirnov test statistics were significant, the reference bands were used to help determine where the differences occurred. Reference bands for NSR fish at 315 days and 470 days indicate that the diploids had a higher percentage of smaller fibres and the triploids had a thicker right-hand tail. For 620, 775 and 839 days, the reference bands indicated major differences in the middle ranges of the distributions, with triploids having a thicker right-hand tail. Similar patterns existed for the AF fish at 315 days, 775 days and 839 days, but the evidence was not as strong. The fifth, tenth, fiftieth, ninety-fifth and ninety-ninth percentiles were calculated for the mean probability density function of each age class. Since the data were found to have non-constant variance and non-normal errors, a non-parametric Wilcoxon test was used to give an indication of differences between diploids and triploids in

<table>
<thead>
<tr>
<th>Age post-hatch (days)</th>
<th>Approximate ( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal-sex-ratio fish</td>
<td></td>
</tr>
<tr>
<td>101</td>
<td>0.32</td>
</tr>
<tr>
<td>315</td>
<td>0.05</td>
</tr>
<tr>
<td>470</td>
<td>0.07</td>
</tr>
<tr>
<td>620</td>
<td>0</td>
</tr>
<tr>
<td>775</td>
<td>0.04</td>
</tr>
<tr>
<td>839</td>
<td>0.07</td>
</tr>
<tr>
<td>All-female fish</td>
<td></td>
</tr>
<tr>
<td>315</td>
<td>0.06</td>
</tr>
<tr>
<td>470</td>
<td>0.32</td>
</tr>
<tr>
<td>620</td>
<td>0.18</td>
</tr>
<tr>
<td>775</td>
<td>0.03</td>
</tr>
<tr>
<td>839</td>
<td>0.07</td>
</tr>
</tbody>
</table>

\( P \)-values of approximately 0.1 were considered to indicate significant differences in the overall distribution.
cases where the Kolmogorov–Smirnov test statistic was significant (Tables 5, 6). For NSR fish (Table 5), the fiftieth, ninety-fifth and ninety-ninth percentiles of the mean PDF for the triploids were consistently higher than the percentiles for diploids except at 839 days, where the differences were not significant. This relationship appears to hold at 315 days and 775 days for the AF fish (Table 6), but the evidence is not as strong. The fiftieth, ninety-fifth and ninety-ninth percentiles of the mean PDF in AF fish at 839 days were similar for diploids and triploids, as for the NSR groups.

### Discussion

Myogenesis is a complex process involving the proliferation of mesodermal stem cells, commitment to a specific muscle-fibre lineage, exit from the cell cycle and terminal differentiation (Stockdale, 1992). The majority of embryonic muscle fibres in fish are formed by the fusion of several myoblasts to form multi-nucleated myotubes that subsequently express contractile proteins (Waterman, 1969; Van Raamsdonk et al., 1974; Johnston et al., 1995). Increased heterozygosity and lower numbers of cells would be expected to decrease, and larger cell size to increase, rates of development in triploid compared with diploid fish (Leary et al., 1985). In the present study, we found evidence that myogenesis and neuromuscular development occurred at slightly earlier somite stages in triploids than in diploids, although the differences were minor and smaller than the variation among different families. A small decrease in the time between fertilization and hatching was noted in triploid rainbow trout (*Oncorhyncus mykiss*) (Happe et al., 1988; Quillet et al., 1988). However, salmonids can hatch over a range of developmental stages (Killeen et al., 1999), and we found no evidence that triploidisation significantly affected the timing of organ formation relative to somite stage (Table 1).

Studies in zebrafish have shown that the myoblasts that give rise to embryonic red muscle fibres originate from rows of large cuboidal cells adjacent to the notochord that can be identified by their expression of MyoD and snail 1 prior to somite formation (Weinberg et al., 1996). These adaxial cells commit to the red muscle lineage under the influence of the glycoprotein Sonic hedgehog, secreted from the notochord, and migrate through the somite to a superficial position just underneath the skin (Devoto et al., 1996; Blagden et al., 1997). A subset of these cells elongate to form mononuclear myotubes.
Salmon muscle growth 2007

Fig. 14. The relationship between the total cross-sectional area (CA) of white muscle fibres in myotomes adjacent to the pelvic fin insertions and fork length (FL) for (A) normal-sex-ratio (NSR) and (B) all-female Atlantic salmon (Salmo salar L.). Diploids, open symbols; triploids, filled symbols. The lines (dotted for diploids and solid for triploid fish) were fitted with a series of second-order linear regression equations with the following formulae: for normal-sex-ratio diploids, $CA = -12.89 - 2.74FL + 1.77FL^2$ ($r^2$ adjusted=0.95; $P<0.001$); for normal-sex-ratio triploids, $CA = 9.86 + 0.43FL + 1.42FL^2$ ($r^2$ adjusted=0.97; $P<0.001$); for all-female diploids, $CA = 16.62 - 0.83FL + 1.58FL^2$ ($r^2$ adjusted=0.98; $P<0.001$); for all-female triploids, $CA = 20.70 - 1.96FL + 1.46FL^2$ ($r^2$ adjusted=0.97; $P<0.001$).

Fig. 15. (A,B) The relationship between the number of white muscle fibres in myotomes adjacent to the pelvic fin insertions and age post-hatch (days) for (A) normal-sex-ratio (NSR) and (B) all-female Atlantic salmon (Salmo salar L.). Diploids, open symbols; triploids, filled symbols. The lines (dotted for diploids and solid for triploid fish) were fitted with a series of second-order linear regression equations with the following formulae: for normal-sex-ratio diploids, $n = -5869.5 + 432.8t + 0.31t^2$ ($r^2$ adjusted=0.92; $P<0.001$) (where $n$ is the number of muscle fibres); for normal-sex-ratio triploids, $n = -5564.3 + 339.9t + 0.15t^2$ ($r^2$ adjusted=0.89; $P<0.001$); for all-female diploids, $n = 17481.13 + 52.4t + 0.73t^2$ ($r^2$ adjusted=0.90; $P<0.001$); for all-female triploids, $n = 1013.2 + 319.6t + 0.18t^2$ ($r^2$ adjusted=0.87; $P<0.001$). (C,D) Muscle fibre recruitment in (C) normal-sex-ratio and (D) all-female Atlantic salmon (Salmo salar L.). The data were obtained by differentiating the equations in A and B using the Mathematica computing environment. The equations obtained were as follows: for normal-sex-ratio diploids (dotted line), $r = 439.8 + 0.614t$; for normal-sex-ratio triploids (solid line), $r = 308.6 + 0.437t$; for all-female diploids (dotted line), $r = 90.85 + 1.339t$; for all-female triploids (solid line), $r = 319.6 + 0.360t$, where $r$ is the rate of fibre recruitment (fibres per day).

and migrate through the myotome to form the muscle pioneer fibres (Devoto et al., 1996) that are thought to have a role in guiding the growth cones of the primary motor neurones (Kimmel et al., 1991). Similar mononuclear myotubes were
observed at the horizontal septum in Atlantic salmon (Fig. 3B). In zebrafish, the white muscle fibres were shown to originate from a distinct population of myoblasts in the lateral segmental plate mesoderm (Devoto et al., 1996) that do not require Sonic hedgehog for commitment (Blagden et al., 1997). It has been reported that myotomes of rainbow trout contain only white muscle fibres at the yolk-sac stage, with the red muscle layer consisting of undifferentiated myoblasts (Nag and Nursall, 1972). In contrast, a single superficial layer of red muscle fibres containing myofibrils is already present in Atlantic salmon alevins at hatching (Fig. 10A). In their natal streams, the yolk-sac stages of both salmon and trout fry remain buried in the gravel and are not free-swimming.

Undifferentiated myoblasts which may contribute to the satellite cell population were observed on the surface of the embryonic muscle fibres as early as 42 days post-fertilization in Atlantic salmon (Fig. 5B). It has been suggested, on the basis of ultrastructural observations on two species of freshwater fish, that satellite cells originate from the adjacent mesenchymal tissue lining and migrate into the muscle tissue via the myosepta (Stoiber and Sanger, 1996). Although we observed cells in the myosepta at approximately 42 days post-fertilization (Fig. 5B), these could just as conceivably have been cells associated with the synthesis of structural elements such as collagen fibres.

Foetal and neonatal myoblasts that are involved in the formation of primary and secondary muscle fibres, respectively, have been identified in mammals (Wilson et al., 1992; Stockdale, 1992). The full complement of muscle fibres is present at approximately the time of birth, and subsequent growth involves fibre hypertrophy only (Rowe and Goldspink, 1969). In the muscles of adult mammals, another population of myoblasts can be identified that are referred to as satellite cells because of their anatomical position beneath the basal lamina of muscle fibres (Mauro, 1961). Satellite cells represent approximately 24% of the total myonuclei in the biceps brachii of rapidly growing newborn mice (Brown and Stickland, 1993). The proportion of satellite cells to total myofibre nuclei falls dramatically with age in mice, reaching less than 1% at 24 months (Gibson and Schultz, 1983). In
Salmon muscle growth

Adult mammals, satellite cells are usually quiescent, becoming activated and entering the cell cycle in response to muscle damage, which indicates that they have a role in repair and regeneration (Schultz and McCormick, 1994). In mice, approximately 80% of satellite cells divide with a cell cycle time of 32 h (Schultz, 1996). The remaining 20% of satellite cells cycle more slowly and are thought to represent a reserve population of muscle stem cells. Muscle stem cells are thought to undergo an asymmetric division resulting in a daughter stem cell and an activated stem cell that can undergo a limited number of further divisions prior to terminal differentiation (Schultz, 1996).

Genetic analyses of knock-out mice have shown that the four members of the MyoD gene family (MyoD, myogenin, myf-5 and myf-6) play distinct but overlapping roles in myogenic determination and differentiation (for reviews, see Olson and Klein, 1994; Yun and Wold, 1996). Activated satellite cells accumulate members of the MyoD and myocyte enhancer binding factor-2 (MEF2) gene families of muscle transcription factors, whereas myogenic regulatory factor (MRF) proteins are not expressed in quiescent satellite cells (Grounds et al., 1992; Smith et al., 1994; Yablonka-Reuveni and Rivera, 1994; Cornelison and Wold, 1997). myf-5 and MyoD appear at relatively early stages of the myogenic pathway in mammals, whereas myogenin and myf-6 are expressed at later stages (Smith et al., 1994). A proportion of MRF-positive cells also express m-cadherin (Cornelison and Wold, 1997) and Bcl-2, a protein involved in the delay or suppression of apoptosis (Dominov et al., 1998). Cell cycle withdrawal and myogenic differentiation are also associated with the up-regulation of

Fig. 17. Probability density functions of muscle fibre diameter in normal-sex-ratio Atlantic salmon 101–839 days post-hatch. Dotted lines illustrate the functions for individual fish and the solid line is the calculated mean value.
expression of the general cyclin-dependent kinase inhibitor p21 (Walsh and Perlman, 1997). Studies on muscle satellite cells have been handicapped by the absence of suitable molecular markers. Recently, Cornelison and Wold (1997) have shown that quiescent and activated mouse satellite cells express the c-met receptor tyrosine kinase, which was not detected on muscle-derived fibroblasts or other mononuclear cells. c-met is a membrane-bound disulphide-linked heterodimer with an intracellular tyrosine kinase domain (Gonzatti-Haces et al., 1988) activated by the ligand hepatocyte growth factor/scatter factor (HGF/SF) (Matsumoto and Nakamura, 1996). HGF/SC is present in adult mammalian muscle and is capable of activating satellite cells in tissue culture (Tatsumi et al., 1998).

In Atlantic salmon, c-met is present in mononuclear cells that also express proliferating cell nuclear antigen, an auxiliary protein to DNA polymerase (results not illustrated), indicating that they also represent an actively dividing population of cells. The majority of c-met-containing cells (84 % in diploids and 72 % in triploids) also expressed myf-6, which is consistent with them representing a subset of proliferating cells committed to differentiation. On the basis of these markers, putative satellite cell nuclei in salmon smolts represent approximately 17.5 % of the total myofibre nuclei (Table 3), reflecting the rapid rate of recruitment and hypertrophy of muscle fibres during the early saltwater stages. c-met transduces migratory, mitogenic and morphogenic signals in a number of non-muscle tissues during development and wound-healing as well as in satellite cells (Matsumoto and Kamura, 1996). Before c-met can be definitively accepted as a molecular marker of satellite cells in salmon, further studies on its localisation at an ultrastructural level are required to determine whether it can be expressed in any other cell types.

We estimated that NSR diploids were recruiting more than 1000 fibres myotome^{-1} day^{-1} during the second summer after seawater transfer (Fig. 15A,C). Using ultrastructural criteria, Koumans et al. (1991) determined that, in the common carp (Cyprinus carpio), the percentage of satellite cell nuclei was approximately 8 % in rapidly growing juveniles approximately 5 cm in standard length. The proportion of satellite cell nuclei

---

**Fig. 18.** Probability density functions of muscle fibre diameter in all-female Atlantic salmon 315–839 days post-hatch. Dotted lines illustrate the functions for individual fish and the solid line is the calculated mean value.
Salmon muscle growth fell to less than 2% of the total in fish 15–20 cm long (Koumans et al., 1991), a value significantly lower than that in Atlantic salmon, which have significantly faster growth rates at this length.

The hypothesis that diploid fish would have a higher density of satellite cells, leading to correspondingly faster rates of fibre recruitment, was sustained. However, muscle fibres in triploid fish showed greater rates of hypertrophic growth than in diploids, although the patterns observed were complex and age-dependent. In the present study, we have applied non-parametric statistical techniques to compare the distribution of fibre size in diploid and triploid groups to provide a more quantitative description of the growth history of the successive cohorts of muscle fibres recruited. The majority of previous studies of muscle growth in fish have been essentially descriptive, involving the measurement of the diameters or cross-sectional areas of 50–200 muscle fibres in different age classes and plotting the results as histograms with only a limited attempt at statistical analysis (Carpenè and Veggetti, 1981; Stickland, 1983; Weatherley and Gill, 1985; Rowlerson et al., 1995). For example, Keissling et al. (1991) used the Kolmogorov–Smirnov test statistic to provide a general indication of differences in the frequency histograms of muscle fibre cross-sectional area with growth in rainbow trout.

Fig. 19. Mean value (red) and variability band (shaded blue area) of the probability density functions of muscle fibre diameter in normal-sex-ratio Atlantic salmon 101–839 days post-hatch.
However, when the detailed shape of the density function of a continuous variable, such as muscle fibre diameter, is of interest, non-parametric smoothing techniques have distinct advantages over methods dependent on a histogram of the realisations. The basic construction of a histogram involves dividing the sample space into intervals and then representing each realisation in an interval by an equal-sized box that is stacked within an interval. The result is that the function is not smooth and the value of the realisation is lost. The kernel method has the advantage of preserving the value of the realisation and providing smooth density estimates that make comparisons among different groups easier using graphical techniques.

To compare the distribution of fibre size between groups, we first estimated the mean PDF for each group using the kernel method. To evaluate the evidence of a structural characteristic, we graphically created a variability band using the bootstrap technique. If the characteristic was apparent in the variability band, we concluded that there was evidence that the characteristic was genuine.

The next step was to compare the densities for diploids and triploids within each age class. First, we used a Kolmogorov–Smirnov-based bootstrap test to test the null hypothesis of no difference. Since the Kolmogorov–Smirnov test provides few clues to where differences might occur, we compared the two estimated densities graphically if the test proved significant. First, we plotted the individual densities and then we plotted the estimated density under the null hypothesis and placed a reference band around this estimate. The region where one or both of the group densities lie outside the reference band suggests where there is a major difference between the two densities.

Next, we compared the values of specific percentiles of the estimated fibre densities for each fish. The Wilcoxon
two-sample non-parametric test was used to test whether the median value of the specified percentile was equivalent between groups. Although we made multiple comparisons for all age classes, this stage of multiple comparisons should not be scrutinised unless the Kolmogorov–Smirnov test statistic was significant. We have included reference bands and the results from the Wilcoxon test for the age classes where there was no significant difference between diploids and triploids to demonstrate the consistency in results between the steps.

Comparing distributions between diploids and triploids by using estimated smooth density curves alongside non-parametric tests provided us with a clearer picture of the differences and similarities between diploids and triploids. For NSR fish, there were approximately one-third more muscle fibres in diploid than in triploid groups at first feeding, although
the overall shapes of the probability density functions of fibre diameter were similar (\(P=0.32\), Kolmogorov–Smirnov statistic; Table 4). The average (not illustrated) or median fibre diameter (Table 5) provides a poor index of hypertrophic growth, e.g. in diploids it was relatively constant between 315 days and 620 days post-hatch (Table 5) during which period the mean fork length increased from 14 to 25 cm (Fig. 8). The peak fibre diameter remains close to 20 \(\mu\)m in all age classes of Atlantic salmon studied because the hypertrophy of existing fibres was counterbalanced by new myotube formation. For NSR fish, the fiftieth percentiles of fibre diameter were 12.0 %, 24.5 %, 34.2 %, 20.9 % and 14.7 % greater in triploid than in diploid fish at 315 days, 470 days, 620 days, 775 days and 839 days, respectively (Table 5). In contrast, Suresh and Sheehan (1998) found that there was no difference in mean fibre diameter in diploid and triploid rainbow trout over the size range 3–30 cm. We found evidence for a bimodal distribution of fibre diameter 620 days, 775 days and 839 days post-hatch, consistent with seasonal cycles of recruitment and expansion of the muscle fibres (Fig. 17). Carpenè and Veggetti (1981) also found evidence for seasonal cycles of recruitment in Mugilidae. From an analysis of fibre size distribution in the rainbow trout, Kiessling et al. (1991) inferred an increased importance of hypertrophy during periods of rapid growth. In the present study, the number of muscle fibres in Atlantic salmon was found to increase approximately 30-fold during the freshwater phase of the life cycle, reaching 150,000–180,000 in seawater-adapted smolts. The hypertrophy of these fibres is responsible for a significant proportion of early seawater growth. The left-hand peak of fibre diameters in fish sampled during March and May 1997 probably represents fibres recruited during the first winter in sea water. Muscle fibre number increased a further 3.5-fold after the first year of seawater growth (Fig. 15A,B).

Muscle fibre densities in diploid and triploid Atlantic salmon became much more similar 839 days post-hatch (Tables 4–6; Figs 21, 22). The largest muscle fibres found in salmon were in the region of 200 \(\mu\)m diameter, which may represent the upper limit for diffusion of metabolites from the capillaries. Similar findings have been reported for rainbow trout, in which almost identical frequency histograms of muscle fibre diameter were found in diploid and triploid fish greater than 30 cm in fork length (Suresh and Sheehan, 1998). In the present study, similar results were obtained for AF and for NSR groups, but the statistical evidence was less strong. Further studies with a larger number of families are required to determine whether there are differences in the muscle growth patterns in NSR and AF fish. Our general conclusion from this study is that the lower density of satellite cells in triploid than in diploid salmon was associated with a proportional decrease in the rate of muscle fibre recruitment. Interestingly, although fibre recruitment was compromised in triploids, there was a significant compensatory increase in muscle fibre hypertrophy. Presumably, the lower density of satellite cell nuclei in triploids was compensated for by an increase in mean nuclear size so that the nuclear-to-cytoplasmic volume ratio remained relatively constant, and this may be a critical factor in hypertrophic growth. Ploidy manipulation promises to be an important tool for dissecting the cellular and molecular mechanisms underlying hypertrophic and hyperplastic growth processes in fish.

The distribution of muscle fibre size and associated changes in connective tissue matrix are thought to be major determinants of texture and processing characteristics in fish and, as such, are important determinants of flesh quality (Hurling et al., 1996; Johnston, 1998). From a commercial standpoint, it is interesting to note that, for these particular fish, as size increases towards the commercial slaughter body mass of 2.5–4.0 kg, the distribution of muscle fibre sizes became more similar in diploids and in triploids, which argues against any major differences in textural characteristics.

This study is part of a comprehensive evaluation of the use of sterile triploid Atlantic salmon supported by a FAIR contract (AIR 3CT94 2216) from the European Commission. We are grateful for the skilled assistance of Jane Godolphin and Irvine Davidson and to Phillip McLachlan and Mike Miles and their staff at Aultbea and Almondbank for fish husbandry.

References


Salmon muscle growth 2015


