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Plasticity of muscle fibre number in seawater stages of Atlantic salmon in response to photoperiod manipulation

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Summary

Atlantic salmon (*Salmo salar* L.) were fed to satiety and reared from ~60 g to 5000 g at ambient seawater temperatures. The effect of photoperiod manipulation on muscle growth was investigated from the start of the first sea winter. Continuous light treatment in winter/spring (1 November to 18 June) improved growth performance in fish, resulting in a 30% increase in mean body mass relative to the ambient photoperiod fish by 12 August, but had no effect on sexual maturation. Significant increases in body mass in the continuous light groups were observed after 126 days (P<0.01). The number of fast muscle fibres per trunk cross-section was determined in a subset of the fish and was 28.5% higher in the continuous light (799×10^3) than the natural day length (644×10^3) groups after only 40 days, corresponding to the period of decreasing natural day length. Subsequent rates of fibre recruitment were similar between treatments. At the end of the fibre recruitment phase of growth (combined June and August samples), the maximum number of fast muscle fibres was 23% higher in fish from the cages receiving continuous light ($881\times10^3\pm32\times10^3$; N=19) than in the ambient photoperiod cages ($717\times10^3\pm15\times10^3$; N=20) (P<0.001). Continuous light treatment was associated with a shift in the distribution of fibre diameters, reflecting the altered patterns of fibre recruitment. However, the mean rate of fibre hypertrophy showed no consistent difference between treatments. There was a linear relationship between the myonuclear content of isolated single fibres and fibre diameter. On average, there were 27% more myonuclei in 150 μm-diameter fibres in the continuous light (3118 myonuclei cm^{-1}) than the ambient photoperiod (2448 myonuclei cm^{-1}) fish. After 40 days, continuous light treatment resulted in a transient increase in the density of myogenic progenitor cells, identified using a c-met antibody, to a level 70% above that of fish exposed to natural light. It is suggested that short days inhibited the proliferation of myogenic progenitor cells and that this was overcome by transferring fish to continuous light, causing an increase in the number of times the myogenic precursor cells divided and/or a decrease in cell cycle time. The net increase in myogenic progenitor cells resulted in proportional increases in the number and myonuclear content of fibres. The subsequent hypertrophy of these additional fibres can explain the delayed increase in body mass observed with continuous light treatment.

Key words: Atlantic salmon, *Salmo salar*, skeletal muscle, myogenesis, growth, photoperiod, myogenic precursor cell, phenotypic plasticity.

Introduction

Photoperiod is probably the major proximal cue that adjusts the seasonal timing of sexual maturation and somatic growth in salmonids (Hansen et al., 1992; Bromage et al., 1993). Decreased photoperiod in autumn is a strong stimulus for sexual maturation, although it has been shown that in some cases this can be delayed by switching fish from short to long days or to continuous light (Hansen et al., 1992). In Atlantic salmon (*Salmo salar* L.) that have spent 1 year in seawater (1-SW), exposure to continuous light from October to June was found to advance spawning, increase growth rate and decrease the proportion of fish sexually maturing relative to those in natural light (Hansen et al., 1992). Endal et al. (2000), on the other hand, found that continuous light from November to July increased maturation. Continuous light treatment was associated with elevated plasma levels of oestradiol-17B and testosterone when fish had returned to a natural photoperiod (Taranger et al., 1998), increased growth hormone concentrations (Björnsson et al., 1994) and decreased 'night-time' melatonin levels (Porter et al., 1999). Photoperiod regime during the winter and spring has also been shown to have

marked effects on swimming and feeding behaviour of salmon in sea cages (Oppedal et al., 2001). Salmon subjected to continuous light maintained a constant swimming speed in circular polarised schools, congregating in the warmer water layers, whereas fish under natural light ceased swimming at night and were found throughout the water column (Oppedal et al., 2001).

Growth of the skeletal muscle involves the recruitment and subsequent hypertrophy of muscle fibres (Weatherley et al., 1988). The major mechanism for expansion of muscle bulk in postembryonic stages is mosaic hyperplasia (Rowlerson and Veggetti, 2001) involving a population(s) of proliferating myogenic progenitor cells that are scattered throughout the myotome (Johnston et al., 1995; Rowlerson et al., 1995). New myotubes form on the scaffold of existing fibres to produce a mosaic of muscle fibre diameters. Around 80% of the myogenic cells in the sub-Antarctic fish *Harpagifer bispinis* were readily labelled with bromo-deoxyuridine, indicating that they were actively dividing (Brodeur et al., 2003a). The myogenic progenitor cells are thought to undergo a limited number of divisions before exiting the cell cycle and expressing genes associated with terminal differentiation, such as myogenin (Johnston et al., 2000b) and desmin (Koumans, 1992). Myoblasts either fuse to form myotubes or are absorbed into maturing fibres as they expand in diameter (Koumans and Akster, 1995; Johnston, 2001). The embryological origin and the stage at which myoblasts become committed to particular fates is uncertain (Koumans and Akster, 1995; Stoiber and Sänger, 1996). In Atlantic salmon, mosaic hyperplasia begins around first feeding and continues throughout freshwater (Higgins and Thorpe, 1990; Johnston and McLay, 1997) and during the first part of seawater life (Johnston et al., 2000a). Significant genetic variation has been demonstrated in the duration of fibre recruitment and in the maximum number of muscle fibres (FN_{max}; Johnston et al., 2000a). FN_{max} and the density of myogenic progenitor cells also show developmental plasticity with respect to the thermal regime during the freshwater stages of the life cycle (Johnston et al., 2000b, 2003a). However, the consequence of photoperiod regime for the growth of the skeletal muscle has not previously been investigated.

The endocrine control of growth is complex and the role of hormones and growth factors regulating myogenesis such as insulin-like growth factor-I (IGF-I), myostatin (MSTN), scatter factor/hepatocyte growth factor (HGF) and the fibroblast growth factor (FGF) gene family is poorly understood (Mommsen and Moon, 2001; Johnston et al., 2003a). HGF stimulates myogenic cell proliferation (Tatsumi et al., 1998), and its receptor, c-met, is a useful marker of myogenic cells (Cornelison and Wold, 1997; Johnston et al., 1999; Brodeur et al., 2003a,b). Myogenic cells possess IGF-I (Castillo et al., 2002) and FGF receptors (Thisse et al., 1995), and IGF has been reported to stimulate both cell proliferation and muscle-specific gene expression and differentiation under certain cell culture conditions (Florini et al., 1991). Feeding is associated with an increase in IGF-I and FGF2 mRNA levels (Chauvigné et al., 2003) and an increase in the number of proliferating myogenic progenitor cells (Brodeur et al., 2003b). Growth hormone is thought to act synergistically with IGF-1 produced in the liver and locally in the skeletal muscle to stimulate muscle growth (Björnsson, 1997; Mommsen and Moon, 2001). Thus, hormones and growth factors that change in concentration in response to changing day length also influence the behaviour of myogenic cells.

In the present study, the growth of fast myotomal muscle was investigated in 1-SW Atlantic salmon subject to either natural photoperiod or continuous light from 1 November to 18 June. The aim was to test the hypothesis that enhancement of growth by continuous light treatment results from the plasticity of muscle fibre recruitment and is linked to an increase in the production of myogenic progenitor cells.

Materials and methods

Fish

The Atlantic salmon (*Salmo salar* L.) used in the study had been selectively inbred for 7–10 generations (see Johnston et al., 2003b). Eggs from 360 families were incubated at ~8.3°C until hatching and were then transferred to tanks and freshwater sea cages as described previously using standard commercial procedures (see Johnston et al., 2003b). The fish were branded, and 504 fish were individually PIT tagged (passive integrated transponder; supplied by Fish Eagle Co., Gloucester, UK) prior to seawater transfer. Smolts were stocked into four steel construction cages with a 12 mm mesh cube net of 5 m×5 m×5 m at the Loch Eil (56°49′ N 5°07′ W) trial site [Marine Harvest Scotland Ltd, Edinburgh, UK] on 17–18 April 2000. Each cage was stocked with 600 smolts, representing a random selection of the available fish. Fish were fed to satiety using an automatic feeding system (AKVAsmart Ltd, Glasgow, UK) as previously described (Johnston et al., 2003b). The diet was a standard commercial ration (Ecolife®) from BioMar Ltd (Grangemouth, UK). The diet was manufactured in five pellet sizes (3 mm, 4.5 mm, 6.5 mm, 9 mm and 12 mm) as the fish increased in size throughout the experiment. On 1 November, artificial lights were switched on in two cages until 18 June. A single 400 W submerged metal halide light (Pisces 6; Aquabeam Ltd, Grantham, UK) suspended at 2 m depth was switched on 2 h before dusk and switched off 2 h after dawn each day. A light-impermeable barrier separated the ambient photoperiod cages (1 and 2) from the lit cages (3 and 4). The minimum light levels at night in the lit cages were 10–13 lux in the corners. The distribution of fish between treatments and cages is shown in Table 1.

The sea cages were also stocked with Goldsinney wrasse (*Ctenolabrus rupestris*; 1 per 50 salmon) to control sea lice infestations. Additional treatments with Excis® [cypermethrin at 1% (m/v); Novartis Animal Health, Litlington, UK] for 1 h were performed on three occasions. In all treatments, the net pens were raised to a depth of 1 m and enclosed in a tarpaulin, with oxygen provided to ensure that a minimum level of 7 p.p.m. was maintained.

Table 1. *Numbers of fish by treatment and seawater cage that were repeatedly weighed to assess growth performance*

Cage	Ambient photoperiod	Cage	Photoperiod manipulated	Total
1	103	3	145	248
2	126	4	130	256
Total	229	Total	275	504

Fish were weighed on the following dates: (1) 5 April 2000, (2) 31 May, (3) 11 July, (4) 12 July, (5) 21 August, (6) 25 September, (7) 30 October, (8) 8 January 2001, (9) 5 March, (10) 30 April, (11) 17 July and (12) 12 August.

Random samples of fish (103–145 per cage) were repeatedly weighed at approximately 6-week intervals to assess growth performance (Table 1). Fork length (*FL*) and body mass (M_b) were recorded, and the condition factor (*CF*) of the fish was calculated according to the formula: $CF=[(M_b/FL^3)\times 100]$. A random sub-sample of the fish was sampled for analysis of muscle structure on the dates shown in Table 2. In all cases, fish were identified by brand and cross-referenced against PIT-tag number.

Fish that were clearly maturing as grilse were identified and removed from the cages during the weighing procedures on 17 July 2001. This enabled the percentage maturing as grilse to be determined for each cage and treatment.

Analysis of muscle cellularity

The fish were sacrificed with a sharp blow to the head, and muscle blocks prepared immediately. A 0.7 cm-thick steak was prepared at the level of the first dorsal fin ray using a sharp knife. The steak cross-section was traced onto an acetate sheet in triplicate using a fine pen to identify slow and fast myotomal muscle, the fin muscles and non-muscle components. The fast myotomal muscle component of the steak from one side of the body was divided into a series of evenly spaced blocks ranging from three per individual in the smallest fish to 12 per individual in the largest fish. Blocks were mounted on cork sheets and frozen in 2-methyl butane cooled to near its freezing point (–159°C) in liquid nitrogen. The blocks were wrapped in tin foil and stored in a liquid nitrogen refrigerator until they could be processed. The blocks were equilibrated to –20°C, and 7 μm frozen sections cut, mounted on poly-L-lysine-coated slides, air dried and either stained with Mayer's haematoxylin or used for immunohistochemistry. The outlines of 100–300 muscle fibres per block were digitised using an image analysis system (SigmaScan software, SPSS Inc., Chicago, IL, USA), and the mean fibre diameter was calculated. A minimum of 800 and a mean of 1000 muscle fibres were measured per fish and the fibre number estimated from the total cross-sectional area (Johnston et al., 1999).

Immunohistochemistry

Frozen sections (18 mm thick) were fixed in acetone for 10 min and then air dried for 10 min. Myogenic cells were identified using a c-met primary antibody (Santa Cruz Biotechnology Ltd, Santa Cruz, CA, USA) and an extravidin–Cy3 conjugated secondary antibody (Sigma, Poole, UK) as described previously (Johnston et al., 1999, 2003b). Sections were counterstained in Sytox green® (Molecular Probes Inc., Leiden, The Netherlands) to visualise all the nuclei and then mounted in a fluorescent medium (DAKO Corp., Carpinteria, CA, USA). The sections were viewed with a laser confocal microscope (BioRad Radiance 2000). The density of myonuclei (stained green with Sytox green) and c-met^{+ve} cells (stained yellow) were quantified using a sequential scanning mode in five or six fields of 0.4 mm^2 tissue section per fish using LaserPix vs. 4.0 software (BioRad, Hemel Hempstead, UK). Nuclear counts were corrected for section thickness and the mean diameter of nuclei (Abercrombie, 1946) using data previously determined from electron micrographs (Johnston et al., 2000a).

Nuclear content of isolated muscle fibres

Small bundles of fast muscle fibres were isolated from the dorsal myotome posterior to the region sampled for histology. Fibre bundles were pinned at their resting length on strips of Sylgard (RS Ltd, Corby, UK) and fixed for 6–10 h in 4% (m/v) paraformaldehyde in phosphate-buffered saline (PBS). Single muscle fibres freed from connective tissue were isolated in

Table 2. *Number of fish sampled by treatment and seawater cage to investigate muscle cellularity*

	Ambient photoperiod			Photoperiod manipulated		
Sample date	Cage 1	Cage 2	Total	Cage 3	Cage 4	Total
12 July 2000	6	6	12	0	0	0
31 Aug 2000	6	5	11	4	4	8
2 Nov 2000	*4*	*4*	*8*	*3*	*6*	*9*
10 Jan 2001	*6*	*6*	*12*	*6*	*6*	*12*
5 Mar 2001	*2*	*4*	*6*	*3*	*2*	*5*
4 June 2001	*6*	*5*	*11*	*5*	*5*	*10*
14 Aug 2001	*5*	*4*	*9*	*3*	*6*	*9*

The sample points in italic correspond to the fish used for the analysis in Table 3.

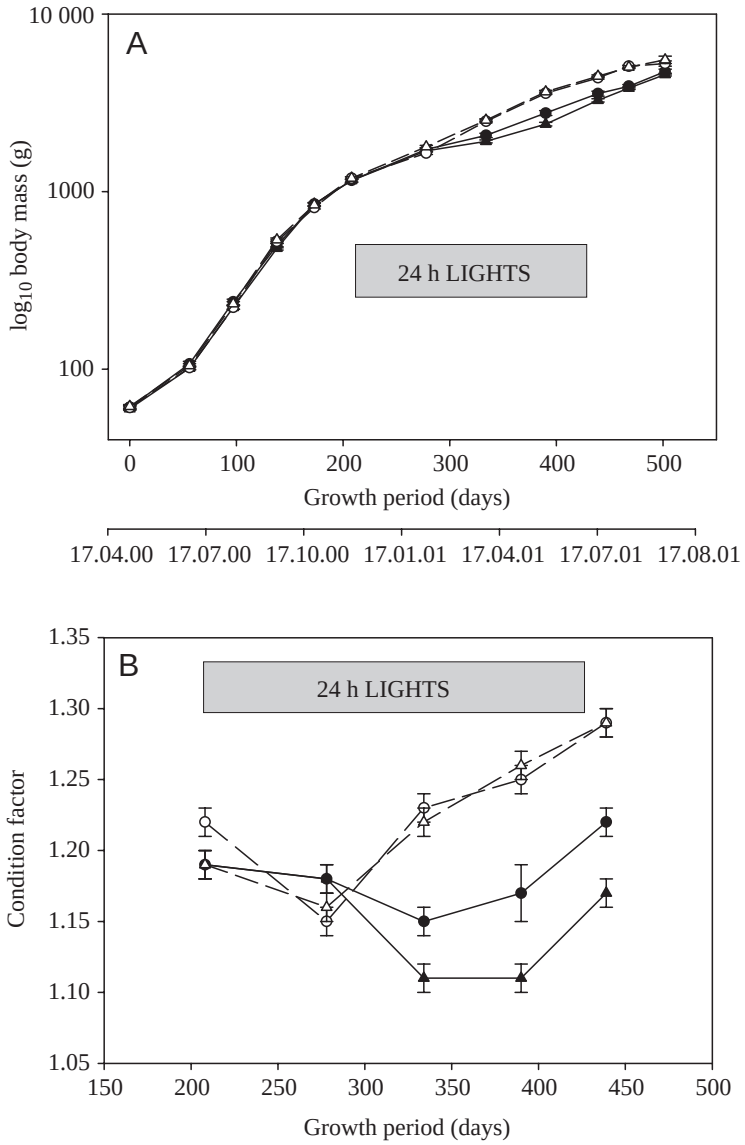

Fig. 1. (A) The relationship between $\log_{10}$ body mass and growth period in seawater for the treatment groups of PIT-tagged Atlantic salmon (*Salmo salar*) studied. Salmon were reared either under ambient photoperiod (cage 1, filled circles; cage 2, filled triangles) or were subjected to 24 h continuous lighting from 1 November 2000 to 18 June 2001 (cage 3, open circles; cage 4, open triangles). (B) The condition factor [(body mass/fork length^{-3})×100] for the treatment groups of salmon during and shortly after photoperiod manipulation. The symbols and number of fish studied are as in A. The period of continuous lighting in cages 3 and 4 is illustrated by the grey box. Values represent means ± S.E.M. The number of fish sampled from each cage and treatment is shown in Table 1.

PBS solution using a binocular microscope fitted with dark-field illumination. Fibres were suspended in 1% (m/v) saponin in PBS for 3 h, washed three times in PBS and treated with 2 μg ml^{-1} units RNase (Sigma). Following further washes in PBS, the nuclei were stained with 30 μmol l^{-1} Sytox green in PBS for 5 min in the dark. Fibres were mounted on glass slides using fluorescent mounting medium (DAKO Corp.) and viewed with a laser confocal microscope (BioRad Radiance 2000). The density of fluorescent myonuclei was quantified in fibre segments 0.3–0.6 mm long using a *z*-series of 1 μm optical thick sections and LaserPix vs. 4.0 software.

Statistical analysis

The effects of growth performance [M_b, *FL* and *CF*] and muscle cellularity [fibre number and fibre density (fibre number/muscle cross-sectional area)] were investigated with a General Linear Model analysis of co-variance (ANCOVA) with a normal error structure using sequential sums of squares (Minitab^{TM} statistical software; Minitab Inc., State College, USA). *Post-hoc* testing was by Tukey's multiple comparison tests. Plots of residuals *versus* fitted values, the normal probability of residuals and histograms of residuals were routinely examined to ensure the data fulfilled the assumptions of the ANOVA.

Nonparametric statistical techniques were used to fit smoothed probability density functions (pdfs) to the measured diameters using a kernel function as described in Bowman and Azzalini (1997). The application of these methods to the analysis of muscle fibre diameters has been described in detail previously (Johnston et al., 1999). Values for the smoothing parameter h (Bowman and Azzalini, 1997) were in the range 0.084 to 0.209, with no systematic variation between samples and/or treatments. Bootstrap techniques were used to distinguish underlying structure in the distributions from random variation (Bowman and Azzalini, 1997; Davison and Hinkley, 1997; Johnston et al., 1999). The Kolmogorov–Smirnov two-sample test statistic was used to test the null hypothesis that the probability density functions of groups were equal over all diameters. To supplement this test, density curves for each treatment were compared graphically by constructing a variability band around the density estimate for the combined populations estimated by pooling fish over the age-class and using the mean smoothing parameter. Any region where the individual pdfs fell outside of this 'reference' band provided evidence for a major difference between the densities.

Results

Growth performance

The growth performance of the fish is illustrated in Fig. 1A. The mean body mass of salmon smolts at seawater transfer was 60.8 g for the ambient photoperiod cages (cages 1 and 2) and 61.8 g for the continuous light cages (cages 3 and 4). All cages were exposed to ambient photoperiod until 31 October 2000 (weighing 6) by which time the mean body mass of fish was 1161 g and 1202 g in the ambient and photoperiod-manipulated treatments, respectively. An ANOVA model was

Table 3. *Analysis of variance (General Linear Model) with the number of fast muscle fibres (*FN*) as a dependent variable using the method of sequential sums of squares for tests*

Source	d.f.	Seq. SS	Seq. MS	*F*	*P*
Model A					
Treatment	1	3.177×10^{11}	3.177×10^{11}	23.19	0.042
Growth period (*GP*)	1	3.093×10^{11}	3.093×10^{11}	34.39	0.0001
Treatment × *GP*	1	5.842×10^{10}	5.842×10^{10}	6.49	0.013
Cage (treatment)	2	2.731×10^{10}	1.365×10^{10}	1.52	0.225
Error	86	7.727×10^{10}	8.985×10^{9}		
Total	91	1.485×10^{12}			
Model B					
Treatment	1	3.177×10^{11}	3.177×10^{11}	26.25	0.037
TCA	1	4.181×10^{11}	4.181×10^{11}	51.91	0.0001
TCA × treatment	1	3.439×10^{10}	3.439×10^{10}	4.27	0.042
Cage (treatment)	2	2.411×10^{10}	1.205×10^{10}	1.50	0.229
Error	86	6.911×10^{11}	8.037×10^{9}		
Total	91	1.485×10^{12}			

d.f., degrees of freedom; Seq. SS, sequential sums of squares; Seq. MS, sequential mean squares; *F*, variance ratio; *P*, probability.

used to measure growth performance (M_b, *FL* and *CF* as dependent variables) with treatment, growth period following seawater transfer (*GP*) and a treatment × *GP* interaction as fixed factors. Cage was nested in treatment, with cage as a random factor and *GP* as a covariate. Weighings 6–10 were used in the analysis, corresponding to the onset of artificial lighting in cages 3 and 4 (1 November) until 17 days after the lights were switched off (18th June). For body mass, all fixed factors were significant: treatment ($F_{1,2514}$=26.54; P<0.05), treatment × *GP* ($F_{1,2514}$=225.47; P<0.001) and cage nested within treatment ($F_{2,2514}$=13.19; P<0.001). The cage effect was smaller than the treatment effect and largely due to variation between cages 1 and 2 at weighings 8–10 (Fig. 1A). The data from replicate cages were combined, and no significant difference between treatments was observed until 126 days after the lights were switched on at weighing 8 (5 March 2001). At this point, the M_b of the fish receiving 24 h light was 26% higher (2534 g) than that of the ambient photoperiod groups (2009 g) (P<0.01; Tukey's test). By weighing 10, the mean M_b of the photoperiod-manipulated groups was 4425 g, or 29% greater than the ambient treatment groups (3417 g) (P<0.01; Tukey's test; Fig. 1A). At the final weighing (11) of all the PIT-tagged fish, ~1 month after the lights were switched off, the photoperiod-manipulated fish remained 30.5% heavier (5057 g) than the ambient fish (3873 g) (Fig. 1A). The feed conversion ratio (FCR) was in the range of 0.8–1.2 and showed no consistent differences between cages/treatments (not illustrated).

Fork length (not illustrated) demonstrated a similar response to body mass, with significant effects of treatment ($F_{1,2514}$=46.35; P=0.02), treatment × *GP* ($F_{1,2524}$=98.40; P<0.001) and cage nested within treatment ($F_{2,2514}$=4.76; P<0.01). Condition factor demonstrated the largest cage effect of the parameters investigated, and this was particularly marked for the ambient photoperiod cages (Fig. 1B). All the fixed factors tested were significant for condition factor; treatment ($F_{1,2514}$=20.49; P<0.05), treatment × *GP* ($F_{1,2514}$=97.96; P<0.001) and cage nested within treatment ($F_{2,2514}$=8.72; P<0.01). Combining the data for replicate cages, condition factor was significantly higher in the continuous light than in the ambient photoperiod cages for weighings 8–10 (P<0.01; Tukey's test).

Maturity

The percentage identified as maturing as grilse was 42% and 33% for the ambient photoperiod cages and 41% and 31% for the cages subjected to continuous light over the winter.

Muscle cellularity

Changes in the number of fast muscle fibres per myotomal cross-section with growth and following photoperiod manipulation are illustrated in Fig. 2. This figure also illustrates how temperature and natural day length varied throughout the experiment. The effect of photoperiod regime on muscle fibre number was analysed using two ANOVA models for the sample points shown in Table 3. For Model A, with growth period as covariate, there were significant effects of treatment and a significant treatment × growth period interaction but no significant cage effect on fibre number (Table 3). On 2 November, 24–30 h after the lights were switched on, there were 637×10^3 fibres in the ambient fish and 621×10^3 fibres in the photoperiod-manipulated fish sampled (Fig. 2). At the next sample on 10 January, 40 days after the start of the experiment, fibre number had increased 28.5% in the fish subject to continuous lighting (~799×10^3) (P<0.01; Tukey's test) but was unchanged in the fish on ambient photoperiod (~644×10^3). The appropriate reference for muscle growth is the total cross-sectional area of the fibres (TCA) (Fig. 3A). For Model B, with fibre number as dependent variable and TCA as covariate, there were significant effects

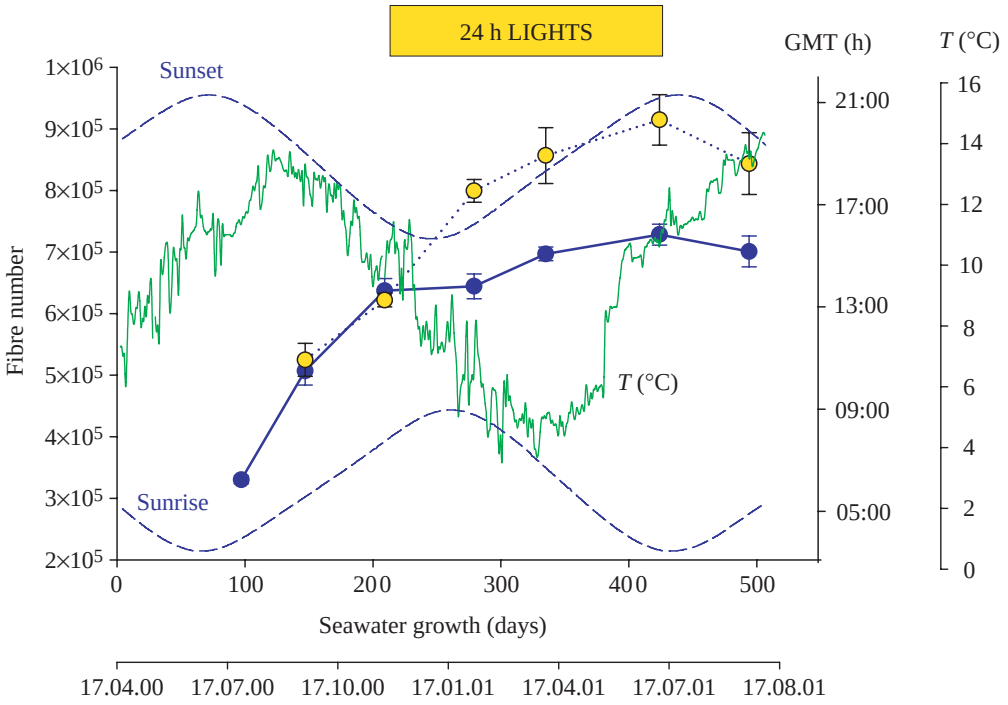

Fig. 2. The number of fast muscle fibres per trunk cross-section at the level of the first dorsal fin ray in a subset of Atlantic salmon (*Salmo salar* L.) reared under conditions of extended winter day length (yellow symbols, dashed line; period of continuous lighting shown by yellow box) or at ambient photoperiod (blue symbols, solid line). The broken blue line shows sunrise and sunset (Greenwich Mean Time, GMT) at Fort William, and the green line illustrates daily recordings of sea temperature. The results are means ± S.E.M. The number of fish sampled from each cage is shown in Table 2.

of treatment, with a significant treatment × TCA interaction term but no significant cage effect (Table 3). TCA was rather similar between treatments until the final sample in August 2001 (Fig. 3A). TCA for the final sample, 70 days after the lights were switched off, was 12.4% greater (10 952 mm^2 of muscle) in the continuous light than in the ambient photoperiod groups (9743 mm^2 of muscle) (P<0.05; Tukey's test). An examination of the distributions of muscle fibre diameters in the June and August 2001 samples indicated that fibre recruitment had ceased. There were ~1% of fibres in the range 5–10 μm in June and no fibres less than 10 μm diameter in the August sample. These samples were therefore combined to provide an estimate of the maximum number of fibres (FN_{max}). FN_{max} was 22.9% higher in the continuous light (mean ± S.E.M., $881\times10^3\pm32\times10^3$; N=19) than in the ambient photoperiod ($717\times10^3\pm15\times10^3$; N=20) groups (one-way ANOVA, $F_{1,37}$=22.0; P<0.001).

Fibre density is a composite parameter reflecting changes in fibre number and size. The density of fast muscle fibres declined with growth in seawater as the expansion of muscle fibres outpaced the recruitment of new muscle fibres (Fig. 3B). An ANOVA revealed a significant treatment effect ($F_{1,127}$=21.73; P<0.05), whereas a treatment × growth period interaction term and cage nested within treatment were not significant. In the sample 70 days after the lights were switched on, the fibre density was 28% higher in the continuous light than in the ambient treatment groups (P<0.05; Tukey's test), reflecting the higher rate of fibre recruitment. There was a tendency for the fibre density to become more similar between groups as the experiment progressed, and in the final sample 70 days after the lights were switched off the treatment effect was no longer significant (Tukey's test).

The smooth distributions of muscle fibre diameter were calculated for all the sample points. In November, the peak probability density (*PD*) of fibre diameter comprised a broad peak with a plateau at 50–100 μm for both treatments (not shown). For the ambient photoperiod group, the peak *PD* of fibre diameter had increased to 110 μm by June 2001 (solid line in Fig. 4) and reached 140–150 μm in August (not shown). In the June sample, the left-hand tail of the distribution of fibre diameters had higher values of *PD* in fish subject to continuous light (dashed line) than in the fish at ambient photoperiod (solid line), reflecting the higher fibre number (Fig. 4). One hundred bootstrap estimates of the combined population of fibres from both treatments were calculated. For the January, March (not shown) and June (Fig. 4) samples, portions of the left-hand and right-hand tail of the *PD* were shifted to respectively higher and lower values in the lit than ambient groups, and significant differences were found between the groups in nonparametric Kolmogorov–Smirnov tests (P<0.01). These differences in fibre size were most pronounced for the June sample (Fig. 4). By contrast, in the final sample, the overall distributions of fibre diameter were not significantly different between treatments (not shown). The maximum fibre size was ~220 μm diameter in both treatments.

The mean fibre diameter and scaled plots of fibre diameter distribution reflect a combination of fibre recruitment and hypertrophy, which tend to decrease and increase fibre size, respectively. We therefore used the mean values of fibre number per group to estimate the numbers of fibres recruited between successive sample points. The 800–1000 fibres measured at each sample were ranked by diameter, and then the estimated proportion of fibres recruited since the last sample was subtracted and the mean diameter of the remaining

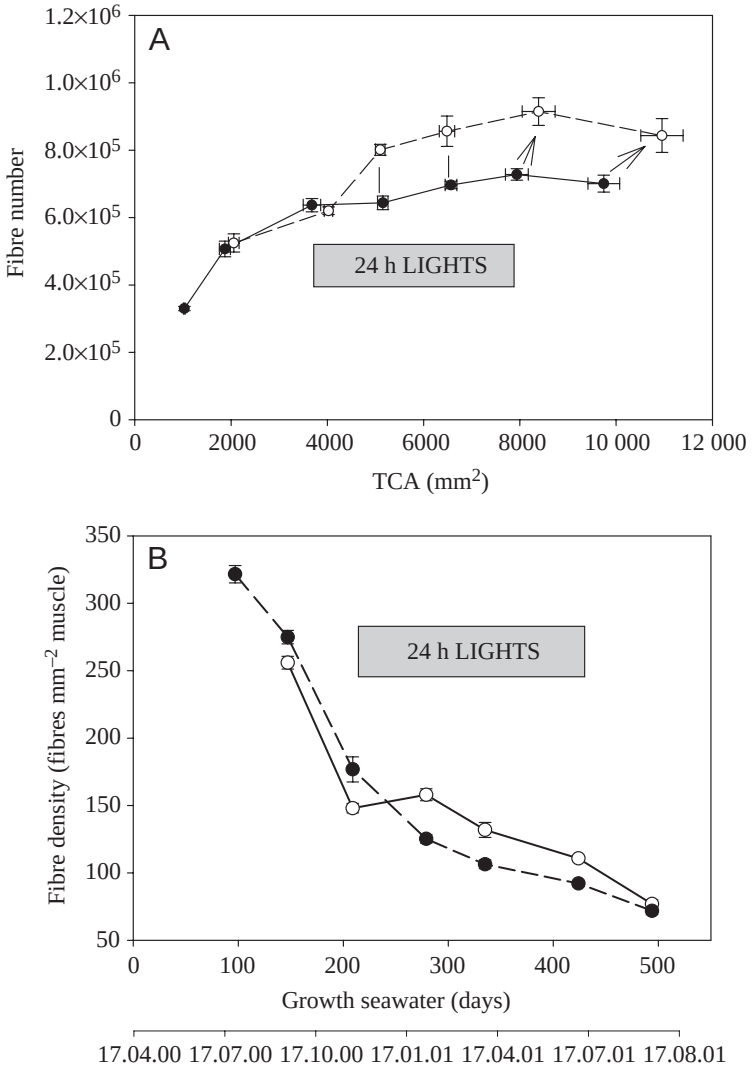

Fig. 3. Atlantic salmon (*Salmo salar* L.) reared under conditions of ambient photoperiod (closed circles, solid line; combined cages 1 and 2) or continuous lighting from 1 November 2000 to 18 June 2001 (open circles, broken line; combined cages 3 and 4). (A) The relationship between the number of fibres and the total cross-sectional area (TCA) of fast myotomal muscle at the level of the first dorsal fin ray. The arrows join common sample points. (B) The relationship between the densities of fast muscle fibres (fibres mm^{-2} cross-sectional area) and the growth period in seawater. The results are means ± S.E.M. The number of fish sampled from each cage is shown in Table 2.

samples were calculated. The mean rate of fibre hypertrophy over each growth period showed no consistent difference with treatment (Fig. 5).

Myonuclei content of isolated fibres

The myonuclear content of isolated single fibre segments was determined for the June 2001 sample (Fig. 6). An ANOVA with treatment as a fixed factor and fibre diameter as covariate revealed a significant difference between treatments ($F_{1,390}$=318.1; P<0.001). First-order linear regression equations were fitted to the data. r^2 values were significantly lower for the continuous lit (0.34) than for the ambient photoperiod (0.80) groups (Fig. 6). For fibres of 150-μm diameter, the mean myonuclear content was 27% higher in the photoperiod-manipulated (3118) than ambient (2448) groups.

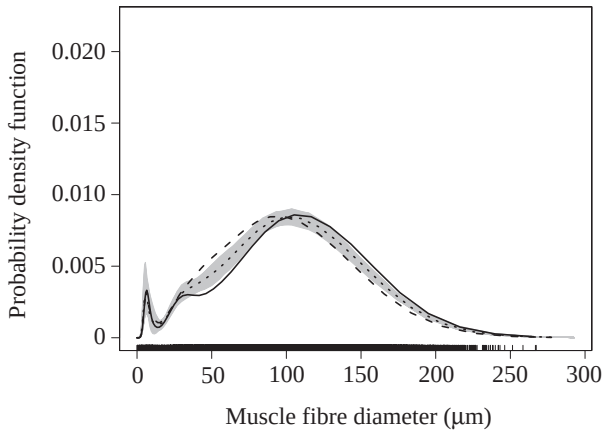

Fig. 4. The mean probability density function (pdf) of fibre diameter in the fast muscle of Atlantic salmon in June 2001 at the end of the period of photoperiod manipulation. Ambient photoperiod (solid line; N=10) and 24 h continuous lighting regime (dashed line; N=10). The dotted line represents the average probability of the combined population, and the grey shaded area represents 100 bootstrap estimates of the probability density. Areas where the mean pdf of the ambient and photoperiod-manipulated treatments fall outside the shaded area provide a graphical representation of the parts of the distribution that are significantly different. The position of data points is shown on the abscissa.

Myogenic cell density

The effect of photoperiod manipulation on the density of c-met immuno-positive cells is shown in Fig. 7. An ANOVA with fixed factors of treatment and treatment × *GP*, and *GP* as covariate, revealed a significant effect of photoperiod on the density of c-met^{+ve} cells ($F_{1,75}$=50.63; P<0.001). In fish sampled 24–30 h after the lights were switched on, the density of myogenic cells was 12% higher in fish exposed to continuous light than ambient photoperiod (P<0.01), rising to a peak of 72% higher after 40 days (P<0.0001; Tukey's tests; Fig. 7).

Discussion

The results supported the hypothesis, namely that 1-SW salmon exposed to continuous light treatment in the winter and spring would show an increase in muscle fibre recruitment relative to fish under natural light (Fig. 2). The extent of phenotypic plasticity of fibre recruitment was equivalent to 23% of FN_{max}. The intensity of fibre recruitment varies markedly during ontogeny and is greatest between smoltification and the end of the first summer spent in seawater, with fibre number increasing around 10-fold over

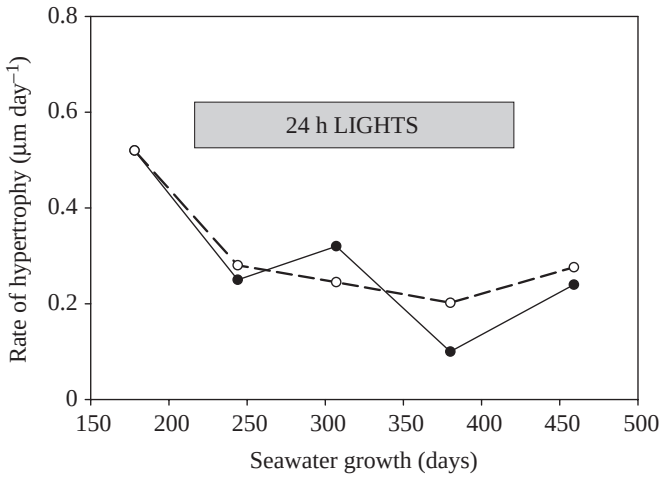

Fig. 5. The mean rate of hypertrophy of fast muscle fibres between successive sample points plotted against seawater growth for the ambient (solid circles) and photoperiod-manipulated (open circles) fish. The period of continuous lighting in the photoperiod manipulated treatment is illustrated by the grey box. The rate of hypertrophy has been plotted at the midpoint of the time period over which it was calculated. Hypertrophy was calculated as the mean of the difference between the observed fibre diameter and the mean of the fibre diameter in the preceding sample.

this period (Higgins and Thorpe, 1990; Johnston et al., 2000b). Smoltification of the freshwater parr is known to be under complex genetic and environmental control, occurring after 1–5 years in the wild depending on latitude (Saunders et al., 1989). Fibre number increased markedly during the two months prior to seawater adaptation, and the timing of this burst of fibre recruitment was synchronised with the numerous physiological changes associated with smoltification (Higgins and Thorpe, 1990). Fibre recruitment in post-smolts decreased significantly as water temperature fell and the day length decreased during the winter (Johnston et al., 2000b, 2002). Winter signalled the end of fibre recruitment in some populations whilst in others myotube formation increased in intensity again as water temperature and day length increased in early summer (Johnston et al., 2000b). It appears that there is a seasonal cycle of myotube formation, which is superimposed on an endogenous rhythm related to age and/or life history stage.

In the present study, continuous light treatment was shown to produce a marked increase in fibre number within 40 days of the lights being switched on, equivalent to an average recruitment of 4400 fibres day^{-1} per myotomal cross-section. The corresponding rate of fibre recruitment was less than 200 fibres day^{-1} per myotomal cross-section in fish exposed to natural daylight. By contrast, over the next 53 days, 900–1000 fibres day^{-1} per myotomal cross-section were added in both treatments, and recruitment had ceased entirely by June in both cases (Fig. 2). Thus, photoperiod manipulation of fibre number only occurred in a relatively narrow window, which corresponded to the period of decreasing day length (Fig. 2). It is therefore possible that an earlier onset of continuous light would produce a greater effect on fibre recruitment and *vice versa*. The results are consistent with the view that distinct genetic mechanisms control the duration of fibre recruitment on one hand and its intensity on the other. Once fibre recruitment had ceased, growth occurred entirely *via* the hypertrophy of fibres formed at earlier stages of ontogeny.

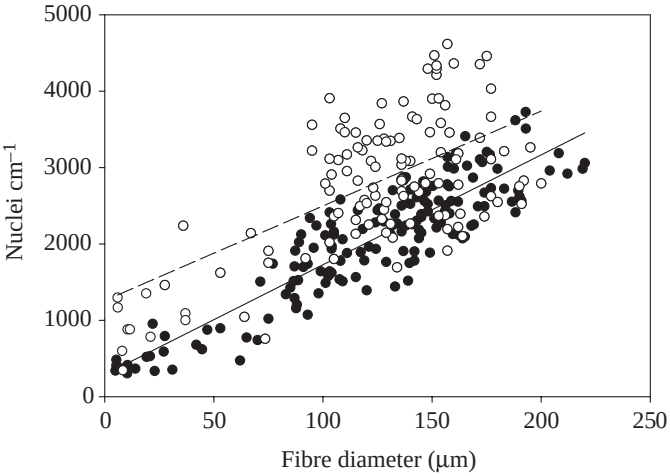

Fig. 6. The number of myonuclei in single muscle fibre segments of 1 cm length in relation to muscle fibre diameter for the 4 June sample (see Table 1) for the ambient (closed circles) and photoperiod-manipulated treatment (open circles). First-order linear regressions were fitted to the data with the following equations. For the ambient photoperiod: myonuclei number=288.6+14.4(fibre diameter) (r^2=0.80; ANOVA: $F_{1,159}$=627.2, P<0.001). For the manipulated photoperiod: myonuclei number=1258.1+12.4(fibre diameter) (r^2=0.34; ANOVA: $F_{1,230}$=118.4, P<0.001).

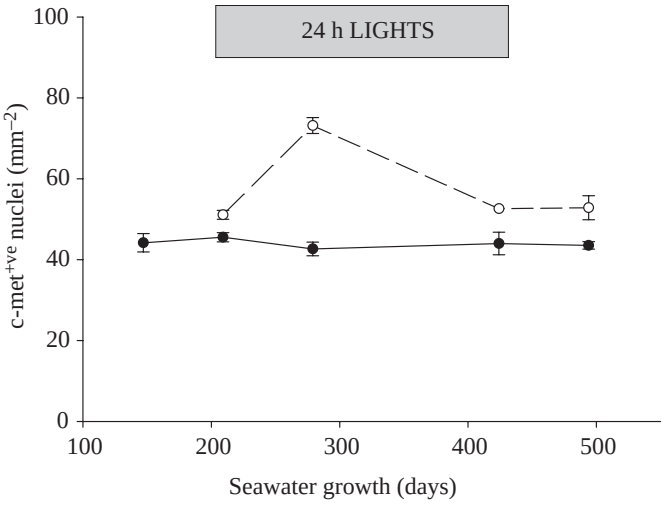

Fig. 7. The density of c-met immuno-positive cells per mm^2 fast muscle cross-sectional area for the ambient (closed circles) and photoperiod-manipulated treatment (open circles). Values represent means ± S.E.M. for six fish per treatment group.

In the present study, the density of c-met-expressing cells was significantly higher in fish under continuous light than natural day length, and this was associated with an increase in both myotube formation and the myonuclei content of muscle fibres (Fig. 7). The myogenic precursors expressing c-met are thought to represent a relatively rare muscle stem cell population and their progeny at various stages towards terminal differentiation (Hawke and Garry, 2001; Zammit and Beauchamp, 2001). Wada et al. (2002) reported that, in the mouse, single undifferentiated muscle progenitor cells derived from a single satellite cell were multipotent and able to differentiate into myotubes, adipocytes or osteoblasts depending on the culture conditions. Perhaps the simplest model for muscle growth in fish would be a single population of myogenic precursors in each muscle type with the fate of the cells destined to form myotubes determined by local signalling, thereby ensuring that fibres were added in the correct places as the myotomal cones expand in volume (Johnston et al., 2003b).

The major increase in myogenic progenitor cells was transient and coincident with the increase in myotube formation that accompanied the onset of continuous light treatment (Fig. 7). The relatively low correlation coefficient (0.34) for the relationship between fibre myonuclei content and fibre diameter in the continuous light treatment suggests that not all the fibres absorbed additional nuclei (Fig. 7). Our working hypothesis to explain these results is that the continuous light treatment affected cell cycle duration and/or the number of times the myogenic progenitor cells divided prior to exiting the cell cycle and differentiating (illustrated diagrammatically in Fig. 8).

The cellular mechanisms underlying developmental plasticity of fibre recruitment (Johnston et al., 2000b, 2003b) and the phenotypic plasticity observed with continuous light treatment (Fig. 2) are almost certainly different. Salmon reared at different temperatures during the freshwater stages showed differences in FN_{max} of up to 22% (Johnston et al., 2003b). In this case, temperature probably influenced the number of myogenic stem cells but not their subsequent behaviour, since the different freshwater-temperature-treated fish were reared under identical conditions during the seawater stages.

The minimum time required for myogenic progenitors to respond to the onset of continuous light will be limited by their cell cycle duration, which is known to vary with temperature, growth rate and feeding status (Brodeur et al., 2003a). The cell cycle time of myogenic progenitors was estimated at 32 h in rat skeletal

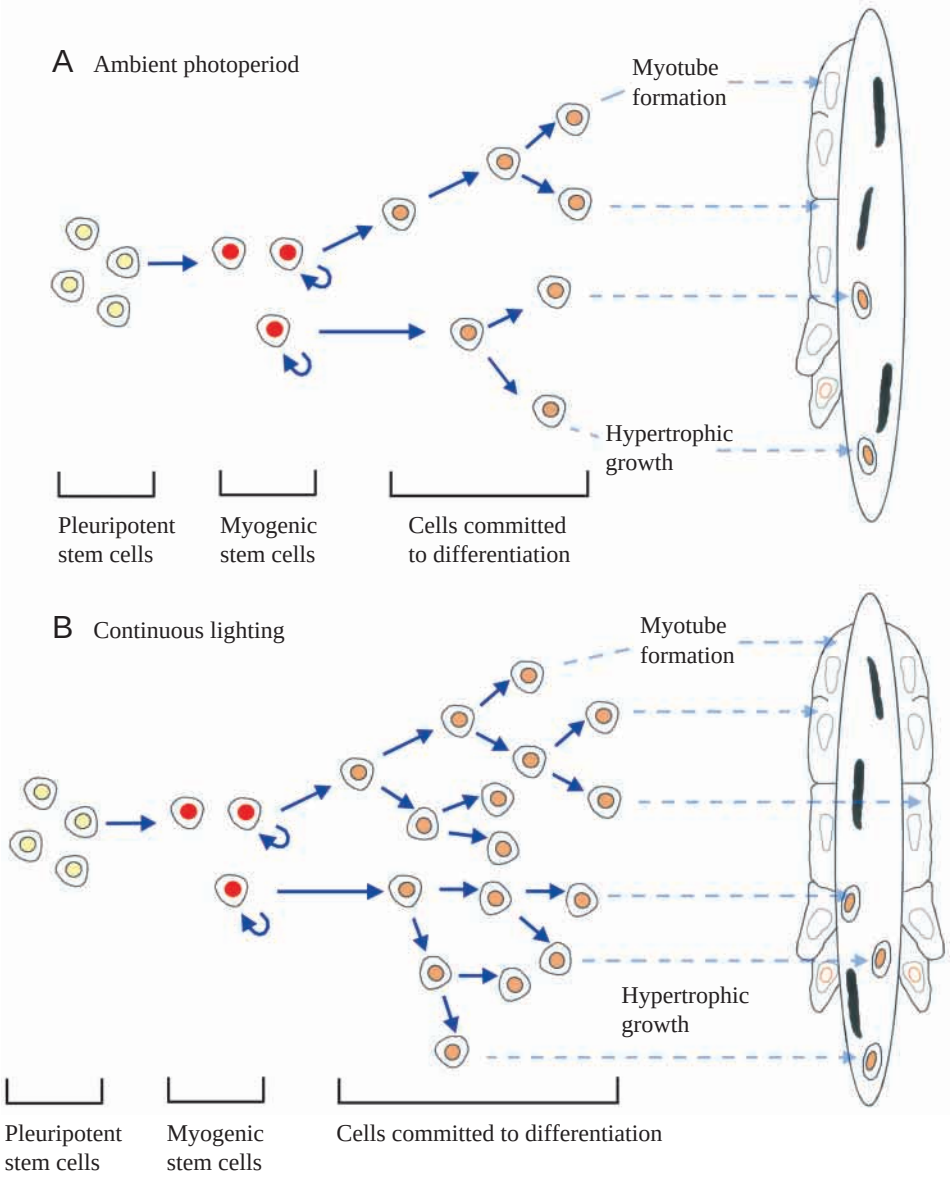

Fig. 8. Working hypothesis to explain the cellular basis of the results obtained. Myogenic stem cells (red nuclei) are derived from a pleuripotent stem cell (yellow nuclei) population at an earlier stage in ontogeny. The myogenic stem cells are assumed to undergo an asymmetric division to regenerate the stem cell and produce a daughter cell (orange) capable of a limited number of further divisions before terminal differentiation. Relative to ambient winter photoperiod (illustrated in A), the cells committed to differentiation (orange) undergo more divisions and/or have a shorter cell cycle time than similar cells in the continuous light treatment (illustrated in B), resulting in a higher standing population of c-met immuno-positive cells (see Fig. 7), a higher content of myonuclei (see Fig. 6) and a higher fibre number (Fig. 2).

muscle (Schultz, 1996) and at 81 h in adult stages of the sub-Antarctic fish *H. bispinis* at 10°C under conditions of zero growth (Brodeur et al., 2003a). Interestingly, in the present study, a significant increase in c-met^{+ve} cells was observed 24–30 h after the lights were switched on. A key factor regulating myocycte cell cycle exit and viability is the cyclin-dependent kinase inhibitor p21. Gene targeting experiments in mice have shown that myostatin-I (MSTN-I), a member of the TGF-B superfamily of secreted growth and differentiation factors, is a powerful negative regulator of muscle fibre number and size (McPherron et al., 1997). The overexpression of MSTN-I in C2C12 myoblasts resulted in a decrease in their proliferation and an increase in their resistance to apoptosis (Ríos et al., 2001). Based on the analysis of cell cycle control proteins, it has been suggested that MSTN signalling upregulates p21, inhibiting cyclin-E–Cdk2 activity, causing the hypophosphorylation of retinoblastoma protein and arrest at the G1 gap phase of the cell cycle (Thomas et al., 2000). Thus, MSTN is a potential candidate for mediating the control of muscle fibre recruitment by photoperiod, possibly in conjunction with growth hormone and IGF-I (Björnsson, 1997).

There was no evidence that the rate of fibre hypertrophy was affected by light treatment, suggesting that the genetic mechanisms controlling this process are distinct from those regulating myotube formation and myonuclei production. Short days appear to inhibit the proliferation of myogenic progenitors and hence muscle growth. The delayed increase in body mass observed in the continuous light treatment probably reflects the time required for hypertrophy of the muscle fibres produced once the inhibitory effects of short day length are removed. A similar delay in the growth-stimulating effects of continuous light treatment has been observed previously (Oppedal et al., 1997). It has been suggested that the ratio between day/night light intensity is important with respect to the timing and increase in growth rate observed (Steffansson et al., 1991).

While there were effects of the extended day length in winter on growth and muscle fibre recruitment, the expected reduction in the percentage of fish sexually maturing (Hansen et al., 1992; Porter et al., 1999) was not found. Endal et al. (2000) also reported that holding fish on long days from November to July failed to reduce maturity; indeed, in their study maturity was significantly enhanced. The relationships between the light intensity of the extended photoperiod, growth rate and maturity may be more complex than previously considered (Oppedal et al., 1997).

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