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Myogenesis and muscle metabolism in juvenile Atlantic salmon (*Salmo salar*) made transgenic for growth hormone

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SUMMARY

Atlantic salmon (*Salmo salar*) made transgenic for growth hormone (GH) and non-transgenic salmon were sampled at 4 and 7 months of age to estimate myogenic factors, satellite cell proliferation and metabolic enzyme activities. The growth rate of 4 month old transgenic salmon was higher than that of non-transgenic salmon. Myosatellite cell (MC) proliferation rates were higher in cells isolated from GH-transgenic salmon compared with cells from non-transgenic salmon of the same mass. Moreover, MCs extracted from non-transgenic salmon demonstrated a higher proliferation capacity when exposed *in vitro* to salmon GH. White muscle MyoD I mRNA content was higher in transgenic and non-transgenic salmon at 7 months compared with that at 4 months, indicating an effect of age on MyoD I mRNA expression. White muscle myogenin mRNA content varied with fish age and presence of the transgene, and was higher in transgenic fish at 7 months, suggesting a higher differentiation capacity. MyoD I, MyoD II and myogenin mRNA content was higher in red muscle of GH-transgenic fish at 7 months compared with non-transgenic salmon at 7 months. However, red muscle myogenic factor expression was not different between transgenic and non-transgenic fish of the same weight. Enzyme activities in white muscle and liver were highly affected by the presence of the transgene, although this effect was generally dependent on the age of the fish. Glycolytic and oxidative enzyme activities were increased in transgenic salmon liver, indicating a higher metabolic rate in transgenics. This study demonstrates that (1) the higher growth rate of transgenic salmon particularly at 4 months of age could be explained at least in part by higher numbers and proliferation rates of MCs, (2) GH can directly stimulate the proliferation of myosatellite cells extracted from salmon, indicating that GH is one possible factor involved in the higher myosatellite cell proliferation rates in transgenic salmon, (3) MyoD and myogenin mRNA expression are affected by fish age, and (4) metabolic enzyme activities are affected by the age of the fish at least in liver and white muscle, and any transgene effect is dependent upon the age of the fish.

Key words: myosatellite cells, metabolism, enzymes, myogenin, MyoD I, MyoD II, RT-PCR.

INTRODUCTION

Atlantic salmon (*Salmo salar*) made transgenic for growth hormone (GH) represent a potential model to study and to better understand the role GH plays in growth processes in fish. The line of salmon used in this study grow to be 5–10 times larger than non-transgenic controls within the first 12 months of feeding (Fletcher et al., 2004) and are capable of reaching market size 1 year earlier than standard salmon currently cultured in Atlantic Canada (Fletcher et al., 2004; Deitch et al., 2006). The mechanism(s) responsible for such rapid growth in transgenic salmon especially at the very early stages (0–4 months) has yet to be resolved (Hill et al., 2000). A recent study using transgenic coho salmon (*Oncorhynchus kisutch*) points to their enhanced appetite as one possible explanation for the accelerated growth (Stevens and Devlin, 2005). However, it may be that the enhanced appetite is driven by the increased anabolic effects of GH on protein synthesis and growth of skeletal muscle and other tissues (Fauconneau et al., 1996). A number of studies have demonstrated that both juvenile and adult GH transgenic salmon have higher metabolic rates than non-transgenics (Stevens et al., 1998; Cook et al., 2000; Lee et al., 2003; Deitch et al., 2006).

Fish growth is generally indeterminate, with most species continuing to grow in mass and length throughout their life. Since skeletal muscle comprises a large proportion of body weight, growth in body mass is to a large extent dependent upon increased muscle mass (Weatherly and Gill, 1987; Houlihan et al., 1988). Post-larval growth of skeletal muscle is attributed to the proliferation and differentiation of muscle satellite stem cells or myosatellite cells (MCs) (Fauconneau and Paboeuf, 2001). These small, spindle-shaped cells have heterochromatic nuclei and are located between the sarcolemma and the basal lamina of differentiated muscle fibres (Koumans et al., 1990). During muscle growth, MCs either fuse with pre-existing muscle fibres resulting in hypertrophic growth or fuse together to form myotubes that differentiate into new fibres in hyperplastic growth (Allen et al., 1979; Veggetti et al., 1990; Koumans et al., 1993; Johnston et al., 1995).

Proliferation and differentiation of MCs are regulated by transcription factors called myogenic regulatory factors (MRFs) (Hawke and Garry, 2001). MyoD and myogenin are helix–loop–helix transcription factors that play an essential role in muscle cell determination, proliferation and differentiation (Feldman and Stockdale, 1991) during development and growth of vertebrates including fish (Xie et al., 2001). These two factors together with Myf5 and MRF4 comprise the MRF family (Watabe,

2001). In addition to transcription factors, hormones including but not restricted to GH, insulin and insulin-like growth factor-I (IGF-I) are implicated in vertebrate muscle growth (Mommsen and Moon, 2001). Circulating levels of these hormones are affected by ration size, and the composition and energy status of the fish (Mommsen and Moon, 2001).

Skeletal muscle consists of a mixture of small and large diameter fibres, and it is generally believed that the smaller fibres are earlier stages of the larger ones, and as such are diagnostic of hyperplastic growth (Weatherly and Gill, 1987). This is supported by the observation that a reduction occurs in the proportion of small muscle fibres with increasing length/weight of the fish (Stickland, 1983; Koumans and Akster, 1995).

Several studies have demonstrated that GH administration increases the proportion of small diameter fibres in rainbow trout (*Oncorhynchus mykiss*), suggesting increased hyperplasia (Weatherly and Gill, 1982; Fauconneau et al., 1997). A study of the white muscle composition revealed that GH transgenic coho salmon fry had a greater proportion of small muscle fibres relative to non-transgenics, thus supporting the hypothesis that GH promotes muscle hyperplasia (Hill et al., 2000). A more recent study of GH transgenic coho salmon suggests that the accelerated muscle growth is the result of decreased myostatin expression, a negative regulator of muscle growth (Roberts et al., 2004).

The goal of this study was to determine whether the ectopic expression of GH in transgenic Atlantic salmon influences the *in vivo* and *in vitro* proliferation and differentiation of skeletal muscle MCs. Proliferation was assessed by bromodeoxyuridine (BrdU) incorporation and differentiation was assessed by quantifying the expression of the MRFs MyoD I, MyoD II and myogenin. In view of the elevated metabolic rate reported for GH-transgenic salmon (Stevens et al., 1998; Cook et al., 2000; Deitch et al., 2006), the activities of a number of metabolic enzymes were also investigated to determine whether enzyme activities correlated with growth rate.

MATERIALS AND METHODS

Experimental design

This experiment examined various aspects of muscle growth in 4–7 month old Atlantic salmon, a time of very rapid growth when transgenic fish can grow to be 5–10 times larger than non-transgenics. These differences in growth rate make it impossible to compare weight-matched fish at the same age or time period. To overcome this difficulty the transgenic and non-transgenic salmon were first sampled at the same age (4 months after first feeding), a time when the non-transgenic fish had sufficient muscle mass for sampling. Both groups were also sampled at 7 months, a time when the non-transgenic salmon had achieved the same mass that the GH-transgenic salmon had at 4 months (i.e. the first sampling). This experimental design allowed comparisons between age-matched and weight-matched transgenic and non-transgenic salmon.

Experimental animals

The transgenic Atlantic salmon strain (termed EO-1α) used in this study is hemizygous for a single functional ectopic copy of a GH transgene (Fletcher et al., 2004; Yaskowiak et al., 2006). This strain was created in 1989 by injecting fertilized eggs with a chimeric GH gene construct (opAFP-GHc2) consisting of a Chinook salmon (*Oncorhynchus tshawytscha*) GH cDNA regulated by an ocean pout (*Macrozoarces americanus*) antifreeze protein gene promoter.

The salmon selected for this study were from the 5th generation (F5) of this lineage. The transgenic parents had been cultured in fresh water under ambient temperatures and photoperiod. Control, wild-type salmon were collected from the North East River, Placentia, NF (Canada), in November 2002. The F5 transgenic offspring were obtained by fertilizing a pool of eggs from seven control (wild-type) females with milt from one GH transgenic male that was hemizygous for the transgene. Fifty per cent of the offspring from this cross inherited the GH transgene in accordance with simple Mendelian inheritance rules. The non-transgenic salmon used as comparators in this study were obtained by crossing the same pool of eggs from non-transgenic females with sperm from a single wild-type non-transgenic male. Eggs were incubated at 6–7°C in a re-circulating vertical stacked tray system at pH 6.8–7. Approximately 1000 fry were transferred to re-circulating rearing tanks (~1 m^3) on March 3, 2003. The water pH was maintained at 6.8–7 and the temperature held at 13.0–13.8°C. All offspring were raised in the same tank under natural photoperiod. The sample of fish (transgenics and non-transgenics) selected for analyses at 4 and 7 months after first feeding were representative of the modal size range of the total population under culture. Fin clips were removed from all fish sampled, frozen in liquid nitrogen and stored in a –80°C freezer until analysis by PCR for the presence or absence of the GH transgene (see Deitch et al., 2006) (Fig. 1).

Specific growth rate (SGR as %/day) was calculated using the formula $SGR=100(\ln W2 - \ln W1)/t$, where W1 and W2 were the mean body masses (g) of the fish at the beginning and end of the observation period, and t the time (days) between observations (Ricker, 1979). The mean fish mass at the beginning of the experiment was estimated at 0.5 g for both controls (non-transgenics) and GH-transgenics.

All experiments were conducted in accordance with the guidelines of the Canadian Council on Animal Care for the use of animals in teaching and research.

Myosatellite cell isolation and culture

MCs were isolated from salmon white muscle using the standard procedures developed by Fauconneau and Paboeuf (Fauconneau and Paboeuf, 2000) with minor modifications. Briefly, Atlantic salmon were killed by a blow to the head, rinsed with cold water to remove excess mucus and disinfected by washing with a bleach solution (1/2000) followed by phosphate-buffered saline (PBS) and 70% ethanol. White muscle was filleted from both sides of the fish inside a laminar flow hood at room temperature and collected in basal medium (DMEM, Dulbecco's modified Eagle's medium with 9 mmol l^{-1} $NaHCO_3$, 20 mmol l^{-1} Hepes and an antibiotic cocktail – A5955, Sigma-Aldrich, St Louis, MO, USA). Muscle pieces were then incubated for 1 h at 4°C in basal medium with 15% horse serum and a 4-fold antibiotic concentration. Muscle pieces were then chopped into small cubes and transferred into fresh basal medium containing 1-fold antibiotic concentration and cell extraction proceeded at room temperature. MCs were cultured at 18°C in DMEM supplemented with 10% fetal calf serum and the cocktail of antibiotics at 3×10^5 cells cm^{-2}, in either sterile 24 well plates or 75 cm^2 flasks.

MC proliferation was assessed by BrdU incorporation (Valente et al., 2002). BrdU was added to the cell culture medium at a concentration of 10 μmol l^{-1}. Incorporation of BrdU into the MCs was measured after 24 h using an immunofluorescence kit (Roche Diagnostic, QC, Canada). Total nuclei were stained using Hoechst 33258 dye solution (355 nm/450 nm). Total nuclei and nuclei that incorporated BrdU were counted using the Image J 1.33 program (National Institutes of Health, Rockville, MD, USA) from computer-generated pictures taken with a fluorescent microscope

Table 1. Sequences of the forward and reverse primers used in this study and the length of the expected PCR amplicon

Gene amplified	GenBank accession no.	Forward primer	Reverse primer	Product (bp)
MyoD I	AJ557148	5′GTGCTTGGATAAAAACGTGCGC3′	5′CAAAGAAATGCATGTCGC3′	195
MyoD II	AJ557149	5′GAGCCAGGATTACACTCGTTAC3′	5′CGAAGAAATGCATGTCGC3′	141
Myogenin	AJ534875	5′GTGGAGATCCTGAGGAGTGC3′	5′GGTCCTCGTTGCTGTAGCTC3′	232
β-Actin	AF012125	5′CGCCGCACTGGTTGTTGACA3′	5′GCGGTGCCCATCTCCTGCT3′	674
18S	AJ427629	5′GGAGGTTCGAAGACGATCAG3′	5′CTCAATCTCGTGTGGCTGAA3′	438
GH transgene	(Dietch et al., 2006)	5′GCTCTTCAACATCGCGGTCA3′	5′ATATGGAGCAGCTTCAGGGAC3′	207

GH, growth hormone.

(Axiophot, Carl Zeiss, Oberkochen, Germany). Three random fields on each slide were counted and each field contained at least 100 cells. Duplicate slides were made for each time point and experiments were repeated 3 times giving a sample size of 3 using different pools of white muscle.

In addition to comparing MC proliferation rates in white muscle isolated from GH-transgenic and non-transgenic salmon, recombinant trout GH actions were assessed at concentrations of 10 and 50 ng ml^{-1} (GroPep, Adelaide, Australia, GHBU020 lot: IJH-GHB1) on MCs isolated from 7 month old non-transgenic salmon white muscle. Cells were cultured for 24 h in serum replacement medium (Sigma, S0638) in 24 well plates at an initial density of 3×10^5 cells cm^{-2}. Proliferation was assessed using BrdU as described above.

Myogenesis

Myogenesis was assessed by using RT-PCR to quantify mRNA levels of the MRFs MyoD I, MyoD II and myogenin in freshly isolated muscle tissue. Since salmon have two homologous, non-allelic MyoD genes, MyoD I and MyoD II (Watabe, 2001), the expression levels of both were assessed.

Total RNA was isolated from red and white muscle tissues using TRIzol® reagent (Invitrogen, Burlington, ON, Canada) in accordance with the manufacturer's instructions. Red muscle could not be isolated from the 4 month non-transgenic salmon as red muscle and white muscle are indistinguishable at this age. Muscle samples were removed immediately after the salmon had been killed, frozen on dry ice and stored at –80°C. Muscle tissue was powdered with a mortar and pestle in liquid nitrogen and RNA extracted using 1 ml TRIzol per 65–85 mg tissue. All RNA samples were run on a gel to ensure integrity. Following isolation, total RNA was treated with DNase I (Invitrogen, 18068-015), transformed into cDNA using a reverse transcriptase (Invitrogen, MMLV reverse transcriptase 28025-013) and amplified using Taq polymerase (Invitrogen, 1803842) according to the manufacturer's instructions. A control using water rather than reverse transcriptase was incorporated into the PCR reaction to ensure the absence of DNA contamination.

Specific primers were designed for MyoD I, MyoD II, myogenin and 18S using sequences available on GenBank (accession nos AJ557148, AJ557149, AJ534875 and AJ427629, respectively). The primer sets used are presented in Table 1. A cycle gradient curve was performed for each gene in each tissue to select the number of cycles necessary to amplify the myogenic factors and the control gene according to Mimeault et al. (Mimeault et al., 2005; Mimeault et al., 2006) (based on the linear phase of amplification for which the R^2 varied between 0.96 and 0.99). MyoD I, myogenin and 18S were amplified for 25, 26 and 11 cycles, respectively, for white muscle tissue RNA. MyoD I, MyoD II, myogenin and 18S were amplified for 26, 28, 24 and 11 cycles, respectively, for red muscle tissue RNA. The PCR conditions used were 94°C for 45 s, 59°C for 30 s and 72°C for 45 s, except for myogenin, where the annealing temperature was 62°C rather than 59°C. For each gene, increasing amounts of cDNA were amplified at the selected cycle number in order to verify the linearity of the analysis; amplification was shown to be linear for each primer (R^2 varied between 0.97 and 0.99). The cycle gradient and cDNA amount studies used a pool of control and transgenic cDNA for each muscle type run separately.

The PCR amplification products were electrophoresed on a 1.5% agarose gel containing ethidium bromide. PCR products from each gene of interest were mixed with the PCR product of the control gene in equal amounts and run on the same gel. The gel was scanned under UV light and the density of the bands quantified using the Quantity one computer program (BioRad, Hercules, CA, USA). Data are expressed as a ratio of the band density for the gene of interest to the band density for 18S from the same tissue. In order to ensure that the PCR products corresponded to the mRNA of interest, each amplification product was cloned using a Topo cloning kit and One Shot Topo II vector (Invitrogen, K4550-01). Plasmid DNA containing the insert of interest was then purified using the QIAprep spin miniprep kit protocol (Qiagen, 27104) and sequenced by the Core DNA Facility Centre (Ottawa, ON, Canada).

Enzyme activities

Enzyme activities were determined in liver, intestine, red muscle and white muscle tissues from transgenic and non-transgenic salmon at 4 and 7 months of age; red muscle could not be recovered from 4 month non-transgenics. Red muscle and white muscle were always taken from the same side and in the same region just below the dorsal fin for all fish. Tissues were immediately frozen on dry ice and held at –80°C until analyses were performed. Tissues were powdered by grinding with a mortar and pestle in liquid nitrogen. The powder was homogenized (1:5 w/v) in a glycerol-containing buffer (see Moon and Mommsen, 1987) and centrifuged for 10 min at 4°C and 10 000 ***g*** (Micro Centaur, Sanyo, VWR Scientific); the resulting supernatant was used immediately for enzyme analyses. Enzyme activities were measured under saturating substrate conditions using standard procedures (Moon and Mommsen, 1987). Reaction rates were assayed spectrophotometrically at 340 nm following the appearance or disappearance of NAD(P)H for all enzymes except citrate synthase (CS) where the oxidation of DTNB was monitored at 412 nm. The extinction coefficients used for NAD(P)H and DTNB were 6.22 and 13.61 $(mmol\ l^{-1})^{-1}\ cm^{-1}$, respectively. Enzyme activities were measured at room temperature (22°C) using a plate reader (Spectra Max Plus 384; Molecular Devices, Sunnyvale, CA, USA) and SOFTmax Pro software to calculate activities. Tissue protein content was

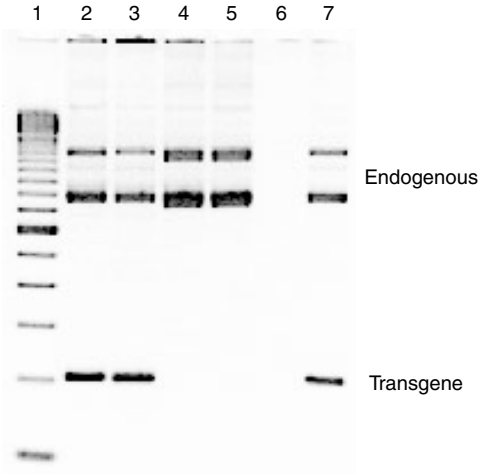

Fig. 1. PCR analysis to detect the presence of the growth hormone (GH) transgene from fin clips of GH-transgenic and non-transgenic Atlantic salmon (*Salmo salar*). The PCR conditions were 92°C for 30 s, 60°C for 30 s and 72°C for 30 s, for 32 cycles. The primers anneal to the Chinook salmon GH cDNA within the EO-1α transgene producing a 207 bp amplicon. In addition, the primers also anneal within the endogenous Atlantic salmon GH DNA sequence, producing amplicons of 798 bp and 1150 bp that serve as internal positive controls for the PCR reaction. Lane 1, DNA ladder; lanes 2 and 3, fin clip from GH-transgenic salmon; lanes 4 and 5, fin clip from non-transgenic salmon; lane 6, negative control (H_2O); lane 7, positive control.

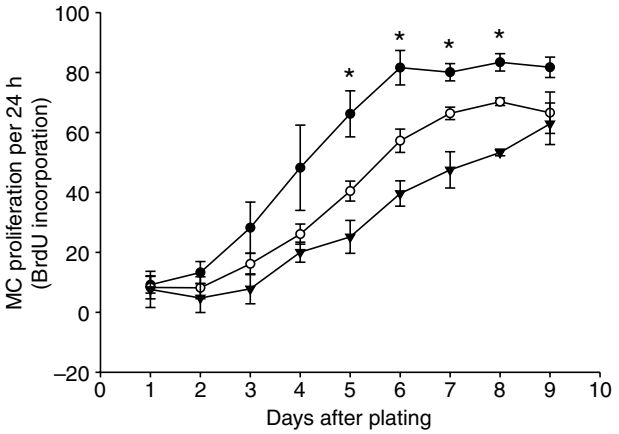

Fig. 2. White muscle myosatellite cell (MC) proliferation *in vitro*. ●, MCs extracted from 4 month old GH-transgenic salmon; ○, MCs extracted from 4 month old non-transgenic salmon; ▼, MCs extracted from 7 month old non-transgenic salmon. Sample size equals 3 for transgenic and non-transgenic salmon at 4 months (control age) and 5 for non-transgenic salmon at 7 months (control size). Results are means ± s.d.; a two way ANOVA was performed using the effects of days after plating (P<0.001), presence of transgene (P<0.001) and the interaction (P=0.0029); asterisk indicates significant differences between transgenic and non-transgenic fish at 7 months (Bonferroni, P<0.05).

measured using the bicinchononic acid (BCA) method (Sigma-Aldrich) with bovine serum albumin as a standard. However, protein content between groups changed significantly, so enzyme activities were expressed as units (μmol min^{-1}) of activity per g tissue.

Statistics

Statistical analyses were performed using InStat 3 (for normality testing) and SYSTAT (version 10). Two-way analyses of variance (ANOVA) were performed on enzyme activities and myogenic factor expression, except for red muscle where a one-way ANOVA was used as the 4 month non-transgenic group was absent. When the one-way ANOVA showed significant effects, multiple mean comparisons were made using the Tukey–Kramer comparison. Data were transformed to obtain normality (Kolmogorov–Smirnov test) when necessary. When it was not possible to obtain normality, non-parametric analyses (Kruskall–Wallis) were employed. Simple correlations using SYSTAT were performed between enzymes and fish mass.

RESULTS

Salmon growth rate and white muscle MC yield

PCR analysis revealed the presence of the GH transgene (207 bp amplicon) and the endogenous GH genes (798 and 1150 bp amplicons; Fig. 1) confirming the transgenic and non-transgenic status of the salmon used in this investigation.

The specific growth rates of the transgenic salmon were approximately double those of the non-transgenics over the first 4 months of growth (Table 2). This resulted in the transgenics being approximately 5 times the weight of the non-transgenics at 4 months after first feeding. Over the 4 to 7 month period, growth rates of the transgenic fish declined significantly while those of the

Table 2. Mass, length, condition factor (CF), specific growth rate (SGR) and myosatellite cell (MC) yield of GH-transgenic and non-transgenic Atlantic salmon used in the experiment

	N_1	Fish age (months)	Mass (g)	Length (cm)	CF	Mean SGR (%/day)	N_2	MC yield
Non-transgenic salmon	12	4	2.83±0.75	6.57±0.49	0.98±0.07	1.44	5	2.95±0.24
	12	7	16.15±2.18	11.13±0.58	1.17±0.11	1.8	5	1.92±0.10
Transgenic salmon	10	4	14.33±3.32	11.75±0.81	0.87±0.10	2.89	5	2.71±0.21
	10	7	93.71±21.03	21.38±1.80	0.94±0.06	1.96	5	2.17±0.11
Effect of age			P<0.001	P<0.001	P<0.001			P<0.001
Effect of transgene			P<0.001	P<0.001	P<0.001			P=0.968
Interaction			P<0.001	P<0.001	P<0.001			P=0.196

Data are presented as means ± s.e.m. MC yield is expressed as 10^6 cells g^{-1} tissue. Two-way ANOVA was used, assessing effect of age, presence of the transgene and the interaction of age and the transgene. Specific growth rate, SGR (%/day), was calculated as SGR=100(ln W2 – ln W1)/t, where W1 and W2 are the body masses at time 1 and 2, respectively and t is time (days) between observations; N_1, sample size for mass, length, CF and mean SGR; N_2, sample size for MC yield.

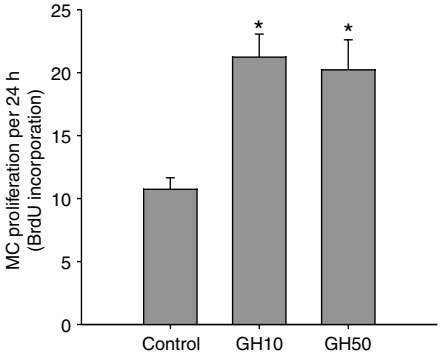

Fig. 3. Non-transgenic salmon white muscle MC proliferation *in vitro* exposed to GH at 10 ng l^{-1} (GH10) and 50 ng l^{-1} (GH50). Sample size equals 3 for each group. Results are means ± s.d.; asterisk indicates significant difference from control (one-way ANOVA, P=0.00072, Tukey–Kramer, α<0.05).

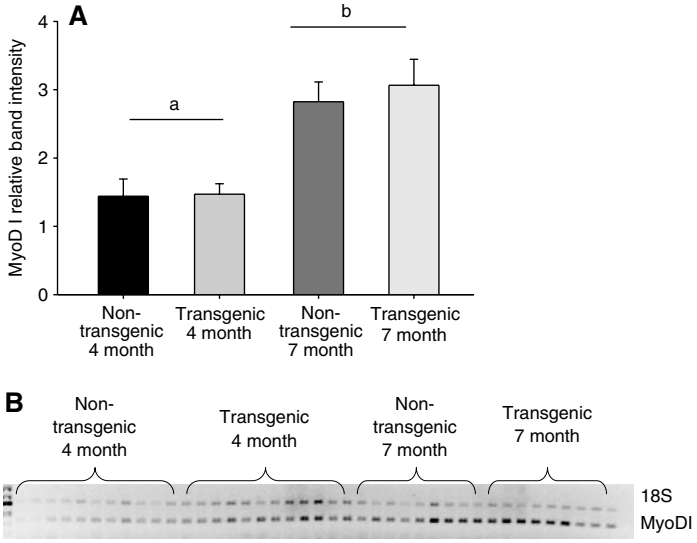

Fig. 4. (A) Relative expression of MyoD I mRNA in white muscle of GH-transgenic and non-transgenic Atlantic salmon at 4 and 7 months of age. Sample size equals 11 for non-transgenic salmon at 4 months, 12 for transgenic salmon at 4 months, 9 for non-transgenic salmon at 7 months and 9 for transgenic salmon at 7 months. Results are means ± s.e.m. Data were log transformed to obtain normality. Two-way ANOVA was used, assessing the effect of age (P<0.001), presence of the transgene (P=0.452) and the interaction of age and the transgene (P=0.790). Different letters and horizontal bars indicate significant differences between ages of the fish. Relative expression is the ratio of the band intensity of the gene of interest to that of the comparator gene, 18S. (B) Picture of the gel used to analyse band intensities.

controls increased slightly (Table 2). Body mass of the 7 month non-transgenic salmon did not differ significantly from that of the 4 month transgenics. Thus the 7 month non-transgenic salmon meet the criteria of being appropriate weight-matched controls for the 4 month GH-transgenics (Pitkänen et al., 2001).

Condition factor (CF) was affected by the age of the fish, with the CF of the 7 month GH-transgenics and non-transgenics being significantly greater than that of the same group at 4 months (P<0.001; Table 2). In addition, the CF was affected by the presence of the transgene with the 4 and 7 month old GH-transgenics having a significantly lower CF than the 4 and 7 month weight-matched controls (P<0.001; Table 2).

MC yield was significantly affected by the age of the fish, with a significantly higher yield from both transgenic and non-transgenic salmon at 4 than at 7 months (P<0.001; Table 2). In addition, despite the fact that the 4 month GH-transgenics were similar in weight to the 7 month non-transgenic salmon, the yield of MCs was generally greater (Table 2).

White muscle MC proliferation *in vitro*

Effects of GH transgene on cell proliferation

The two-way ANOVA performed on MC proliferation rates showed an effect of days after plating (P<0.001), an effect of presence of the transgene (P<0.001) and an effect of the interaction (P=0.003). The comparison using Bonferroni indicated that *in vitro* MC proliferation rates differed between the 4 month transgenic and the 7 month non-transgenic salmon (Fig. 2). No significant differences existed between proliferation rates of the 4 month transgenic and non-transgenic or between the 4 and 7 month non-transgenic salmon. Initial proliferation rates were approximately 10% per day for all groups but rates increased more rapidly for the transgenic than the non-transgenic salmon MCs before reaching a plateau of 80% per day after 6 days. Proliferation rates for the non-transgenic salmon MCs were lower, with the 4 month group achieving a plateau by 8 days at 65% and the 7 month group also reaching 65% by 9 days but never achieving a plateau.

Effects of growth hormone on cell proliferation

Proliferation rates of MCs isolated from 7 month non-transgenic salmon after 24 h of culture was 10% per day (Fig. 3), a value noted in the previous experiment for this group (Fig. 2). The addition of recombinant trout GH to the culture media doubled cell

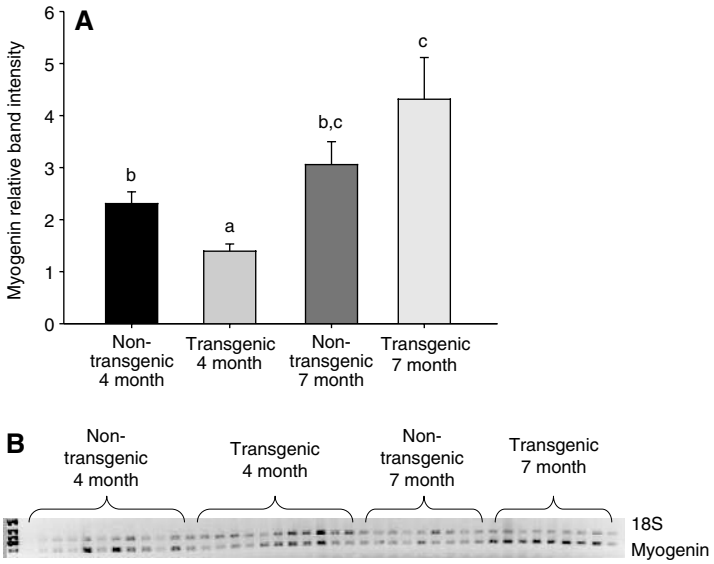

Fig. 5. Relative expression of myogenin mRNA in white muscle of GH-transgenic and non-transgenic Atlantic salmon at 4 and 7 months of age. Sample size equals 11 for non-transgenic salmon at 4 months, 12 for transgenic salmon at 4 months, 9 for non-transgenic salmon at 7 months and 9 for transgenic salmon at 7 months. Results are means ± s.e.m. Different letters indicate significant differences. Myogenin data were log transformed to obtain normality. Two-way ANOVA was used, assessing the effect of age (P<0.001), presence of the transgene (P=0.528) and the interaction of age and the transgene (P=0.002). See Fig. 4 legend for the definition of relative expression. (B) Picture of the gel used to analyse band intensities.

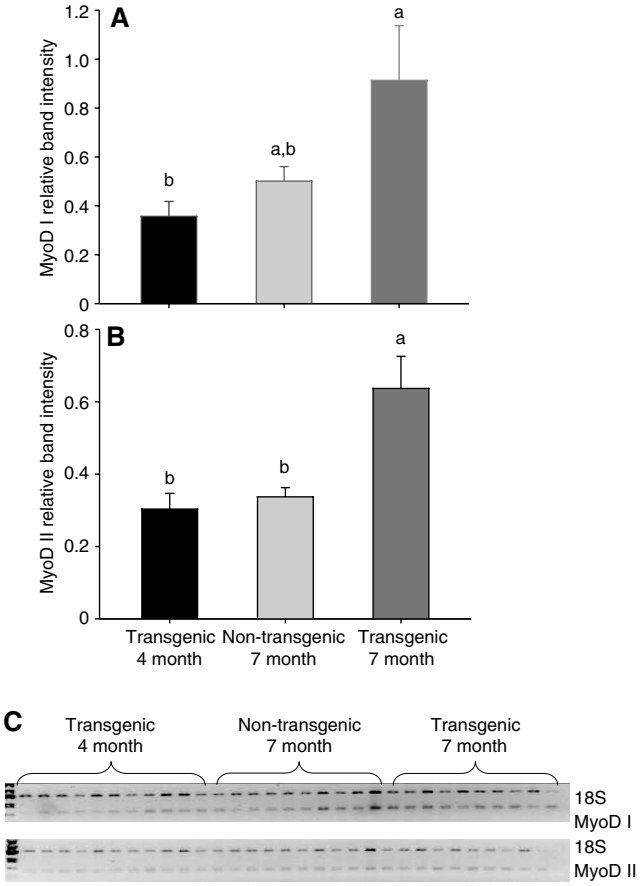

Fig. 6. Relative expression of (A) MyoD I and (B) MyoD II mRNA in red muscle of GH-transgenic and non-transgenic Atlantic salmon at 4 and 7 months of age. Sample size equals 11 for transgenic salmon at 4 months, 10 for non-transgenic salmon at 7 months and 10 for transgenic salmon at 7 months. Results are means ± s.e.m. MyoD I data were log transformed to obtain normality. Data were analysed using one-way ANOVA, P=0.006 for MyoD I and P<0.001 for MyoD II (Tukey–Kramer, α<0.05). Red muscle could not be sampled from control salmon at 4 months due to the small size of the fish. Different letters indicate significant differences. See Fig. 4 legend for the definition of relative expression. (C) Picture of the gel used to analyse band intensities.

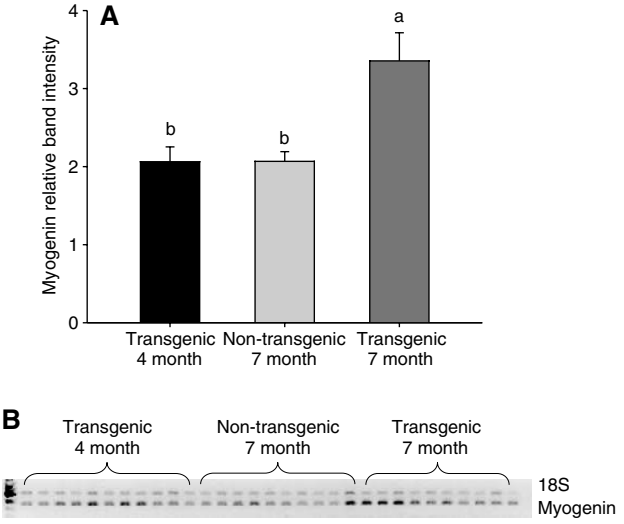

Fig. 7. Relative expression of myogenin RNA expression in red muscle of GH-transgenic and non-transgenic Atlantic salmon at 4 and 7 months of age. Sample size equals 11 for transgenic salmon at 4 months, 10 for non-transgenic salmon at 7 months and 10 for transgenic salmon at 7 months. Results are means ± s.e.m. Myogenin data were log transformed to obtain normality. Data were analysed using one-way ANOVA, P=0.001 (Tukey–Kramer, α<0.05). Red muscle could not be sampled from control salmon at 4 months due to the small size of the fish. Different letters indicate significant differences. See Fig. 4 legend for the definition of relative expression. (B) Picture of the gel used to analyse band intensities.

proliferation rates over the 24 h culture period at both GH concentrations tested (Fig. 3).

Tissue myogenesis *in vivo*

White muscle MyoD I mRNA expression in 7 month transgenic and non-transgenic salmon was higher than that observed in 4 month equivalent groups (Fig. 4). Statistically the age of the fish was significant (P<0.001) but not the presence of the transgene (P=0.452) or the interaction (P=0.790; Fig. 4). There was no evidence for MyoD II mRNA expression in white muscle from any salmon group.

White muscle myogenin mRNA expression was significantly affected by the age of the fish (P<0.001) and the interaction between age and presence of the transgene (P=0.002) but not by the presence of the transgene (P=0.528). Relative myogenin mRNA was lower in 4 month transgenics compared with non-transgenic salmon of the same age (4 months) and mass (7 months; Fig. 5).

Red muscle MyoD I, MyoD II and myogenin mRNA expression were significantly higher in transgenic salmon at 7 months (P<0.001) compared with non-transgenic salmon of the same age and transgenic salmon at 4 months (Figs 6 and 7).

Tissue enzyme activities

White muscle protein content, pyruvate kinase (PK), aspartate aminotransferase (AST) and malate dehydrogenase (MDH) were positively correlated with body mass (Table 3, Fig. 8). White muscle protein content and activities of alanine aminotransferase (ALT), MDH and citrate synthase (CS) were significantly affected by the age of the fish. White muscle protein content, lactate dehydrogenase (LDH) and CS were significantly affected by the presence of the transgene. For all the enzymes tested in white muscle, except AST and isocitrate dehydrogenase (IDH), there was an interaction between age and the presence of the transgene (Table 3), with enzyme activities being higher in transgenic salmon at 4 months and lower in transgenic salmon at 7 months compared with non-transgenic salmon. Intestinal CS was positively correlated with fish body mass (P<0.001) and affected by age (P=0.001) and the presence of the transgene (P=0.012); CS activity was significantly higher in transgenic salmon compared with non-transgenic salmon (Fig. 9).

Red muscle PK, MDH, IDH and CS were higher in 7 month non-transgenics than in the 4 or 7 month old transgenic salmon (Table 4). There was insufficient red muscle in the 4 month non-transgenics to sample. Red muscle MDH, IDH and CS were significantly negatively correlated with fish body mass.

Liver phosphoenolpyruvate carboxykinase (PEPCK), PK, AST, glutamate dehydrogenase (GDH), LDH and CS were all significantly affected by the age of the fish, being higher in 7 month than in 4 month old salmon. Liver protein, LDH, MDH, IDH and CS were significantly affected by the presence of the transgene, with the activity of the enzyme being lower in the transgenic

Table 3. Enzyme activities in the white muscle and intestine of GH-transgenic and non-transgenic Atlantic salmon

	Non-transgenic 4 month	Transgenic 4 month	Non-transgenic 7 month	Transgenic 7 month	Effect of age	Effect of transgene	Interaction	Mass correlation	Probability
White muscle									
Protein	30.6±1.9	44.7±1.9	62.8±2.2	61.0±2.2	<0.001	0.004	<0.001	0.569	<0.001
AST	13.5±0.6	15.4±1.2	35.3±2.3	24.9±4.1	0.387	0.974	0.621	0.57	0.003
ALT	1.1±0.1	1.6±0.1	2.3±0.1	1.2±0.1	<0.001	0.055	<0.001	–0.17	1.000
GDH	1.0±0.1	1.5±1.4	1.4±0.1	1.0±0.1	0.246	0.82	<0.001	–0.28	1.000
LDH	248.9±17.9	459.8±35.3	1227.1±78.5	147.2±51.5	0.056	<0.001	<0.001	–0.41	0.226
MDH	70.5±4.8	55.3±7.6	108.7±6.0	88.4±17.5	<0.001	0.553	0.009	0.64	<0.001
IDH	5.6±0.2	6.0±0.7	7.0±0.4	4.6±0.5	1.637	0.303	0.089	–0.13	1.000
CS	20.5±1.6	27.5±1.0	38.1±1.4	22.3±2.8	<0.001	0.009	<0.001	–0.15	1.000
Intestine									
Protein	13.7±0.8	15.6±0.6	16.1±0.7	15.3±0.66	0.153	0.443	0.059	0.023	0.894
CS	6.1±0.2	7.2±0.3	7.9±0.2	8.5±0.3	<0.001	0.012	0.289	0.51	0.001

All data are means ± s.e.m. and enzyme activities are expressed as μmol min^{-1} g^{-1} tissue and protein as mg g^{-1} tissue; sample size equals 8 or more per group. Enzyme activities were assessed at 22°C. Data were transformed when necessary to obtain normality (log for ALT, sine for AST and i^2 for PK and LDH). Two-way ANOVA was used, comparing the effect of age, presence of the transgene and the interaction of age and transgene. Pearson correlations with Bonferroni probability were used to assess correlation between mass and transformed enzyme activities. AST, aspartate aminotransferase; ALT, alanine aminotransferase; GDH, glutamate dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; IDH, isocitrate dehydrogenase; and CS, citrate synthase.

compared with the non-transgenic salmon. Liver protein, PK, MDH, IDH and CS were significantly affected by the interaction between the transgene and the age of the fish (Table 5).

DISCUSSION

The line of GH-transgenic Atlantic salmon used in this study show accelerated growth rates compared with non-transgenic salmon within a few weeks of first feeding, with the greatest difference between the two groups occurring prior to reaching a size equivalent to that of smolts (50–100 g) (G.L.F., unpublished data). In this study, growth rates during this critical period doubled in the GH-transgenics over that of the non-transgenics within the first 4 months of life (Table 1).

During this rapid growth period the GH-transgenic salmon had higher numbers of white muscle MCs than did the weight-matched non-transgenics. In addition, although the 4 month transgenics had the same number of MCs per gram of white muscle as the age-matched controls, their 5-fold greater body mass indicates that they had a greater total number of MCs. These results suggest that MC number in addition to other factors (e.g. humoral factors, MC sensitivities) may contribute to the more rapid growth rates observed in the transgenic salmon. This result contrasts with the study of Valente et al. (Valente et al., 2002), which reported no difference in white muscle MC yield between two strains of rainbow trout (*Oncorhynchus mykiss*) that differed considerably in growth rates.

The decline in MC number with body mass in both transgenic and non-transgenic salmon is consistent with observations made on rainbow trout (Greenlee et al., 1995; Fauconneau and Paboeuf, 2001) and common carp (*Cyprinus carpio*) (Koumans et al., 1991; Alfei et al., 1994; Koumans and Akster, 1995). This decrease in MC number suggests that the recruitment of new muscle fibres declines as the salmon age and as body mass increases. However, despite the loss in this capacity with age the 7 month transgenic salmon are potentially as capable of recruiting new muscle fibres as the smaller non-transgenic age-matched comparators (Table 1).

If the enhanced growth rates of the GH-transgenics are at least in part attributable to their greater MC number, then it is likely that the time at which this acceleration in growth is initiated following first feeding is facilitated by the presence of greater numbers of

Table 4. Enzyme activities in the red muscle of GH-transgenic and non-transgenic Atlantic salmon

	Non-transgenic 4 month	Transgenic 4 month	Non-transgenic 7 month	Transgenic 7 month	Mass correlation	Probability
Protein	n.a.	10.4±0.4	11.7±0.4	9.9±0.4	–0.32	1.000
PK	n.a.	98.5±5.1^{b}	149.6±8.2^{a}	101.1±4.2^{b}	–0.31	1.000
AST	n.a.	41.6±2.3	72.3±1.4	41.8±2.4	–0.43	1.000
ALT	n.a.	3.6±0.1	5.9±0.2	3.6±0.1	–0.44	0.992
GDH	n.a.	0.66±0.04	0.71±0.04	0.57±0.04	–0.35	1.000
LDH	n.a.	406.7±20.1	804.8±36.1	398.9±15.8	–0.43	1.000
MDH	n.a.	140.4±6.3^{b}	276.2±7.4^{a}	79.9±2.3^{c}	–0.69	0.002
IDH	n.a.	19.9±0.9^{b}	26.6±1.4^{a}	15.2±0.8^{c}	–0.65	0.012
CS	n.a.	16.7±0.7^{b}	20.2±0.9^{a}	10.2±0.4^{c}	–0.83	<0.001

All data are means ± s.e.m. and enzyme activities are expressed as μmol min^{-1} g^{-1} tissue and protein as mg g^{-1} tissue; sample size equals 8 or more for each group. Data were transformed when necessary to obtain normality (sine for AST, ALT and LDH, and log for MDH). Pearson correlations with Bonferroni probability were used to assess the correlation between mass and transformed enzyme activities. Enzymes were assessed at 22°C. PK, pyruvate kinase; see Table 3 for the list of other enzyme abbreviations. n.a.: red muscle could not be sampled from non-transgenic salmon at 4 months due to the small size of the fish. Different superscript letters indicate significant differences (one-way ANOVA, P<0.05, Tukey–Kramer, α<0.05) between non-transgenic and transgenic salmon.

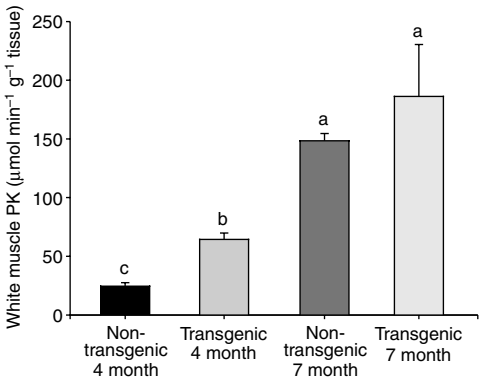

Fig. 8. Pyruvate kinase (PK) activity in GH-transgenic and non-transgenic salmon white muscle. Sample size equals 8 or more for each group. Results are means ± s.e.m. PK data were l^2 transformed to obtain normality. Two-way ANOVA was used, assessing the effect of age (P<0.001), presence of the transgene (P<0.001) and the interaction of age and the transgene (P=0.019). Pearson correlations between mass and white muscle PK equal 0.906, P<0.001 (Bonferroni). Different letters indicate significant differences.

these stem cells. MCs are thought to originate during late embryogenesis (Stoiber and Sänger, 1996); therefore, the increase in MC number in the transgenic salmon should be established during this stage in development.

This study clearly demonstrates that the *in vitro* proliferation rate profile of the MCs from GH-transgenic salmon differed from that of the non-transgenics in four ways: (1) the daily proliferation rate began to increase earlier; (2) the daily rate of increase in proliferation was accelerated; (3) the proliferation rate reached a plateau value more quickly; and (4) the plateau value for the proliferation rate was greater. Thus, not only did the white muscle from the transgenic salmon have a greater concentration of MCs than the size-matched controls, but also the MCs themselves had a greater capacity to proliferate than did the cells from non-transgenics.

The precise mechanism(s) to explain how the ectopic expression of GH influences MC number and/or their proliferation and differentiation is unknown. The GH-enhanced proliferation of white muscle MCs *in vitro* certainly indicates that GH can have a direct effect on these MCs as reported by Halevy et al. (Halevy et al., 1996) and Hodik et al. (Hodik et al., 1997) in avian muscle and Michal et al. (Michal et al., 2002) in canine satellite cells. Although the levels of GH in the plasma of the transgenic salmon have not been quantified, GH transgene mRNA is expressed in most tissues including muscle of these transgenic Atlantic salmon (Hew et al., 1995; Hobbs and Fletcher, 2007). Therefore, it is possible that GH production by the muscle tissue and/or higher levels of circulating GH is responsible for the increased proliferation directly or *via* IGF-I production in muscle as reported for mice (Kim et al., 2005) and the C2C12 cell line (Sadowski et al., 2001). The absence of a dose-dependent response in MC proliferation rate when cells were exposed to GH *in vitro* in our experiment suggests that the two doses tested in our study were too high. Similarly, there was no dose–response effect in avian satellite cells at a GH concentration between 2 and 50 ng ml^{-1} (Halevy at al., 1996), and between 10 and 75 ng ml^{-1} in canine satellite cells (Michal et al., 2002). The use of lower GH doses would be necessary to observe a dose–response effect.

GH is reported to activate myogenesis by increasing MC fusion to existing myotubes *in vitro* in mice (Sotiropoulos et al., 2006), and MC proliferation in mice (Kim et al., 2005), mammalian cell lines (Sadowski et al., 2001) and birds (Halevy et al., 1996). It is generally believed that GH acts on white muscle through circulating or local production of IGF-I (Kim et al., 2005), and it was shown in rodents that IGF-I increased both MC proliferation and MC differentiation (Allen and Rankin, 1990; Hawke and Garry, 2001). However, in our study the age of the fish seems to have a greater impact than GH level, at least for MC proliferation. The fact that differentiation is decreased in salmon white muscle at 4 months in our experiment implicates different factors in the regulation of the expression of myogenin mRNA.

Red muscle MyoD I, MyoD II and myogenin mRNA expression did not differ between 4 month transgenics and their 7 month weight-matched controls, suggesting that in red muscle the MC proliferation and differentiation rates did not differ between transgenics and controls at this growth stage. However, the increased level of MyoD II and myogenin expression in the 7 month old transgenics suggests that MC proliferation and differentiation increases as the fish increase in size. To date, nothing is known regarding the different regulation of MyoD I and MyoD II in red muscle in adult fish; however, our study confirms the suggestion by Delalande and Rescan (Delalande and Rescan, 1999) that MyoD I and II have a distinct role in red muscle.

Table 5. Enzyme activities in the liver of GH-transgenic and non-transgenic Atlantic salmon

	Non-transgenic 4 month	Transgenic 4 month	Non-transgenic 7 month	Transgenic 7 month	Effect of age	Effect of transgene	Interaction
Protein	100.1±4.1	83.2±2.9	96.9±3.4	97.9±3.2	0.103	0.027	0.014
PEPCK	1.3±0.1	1.2±0.1	1.5±0.1	1.4±0.1	<0.001	0.132	0.454
PK	39.9±3.9	48.7±3.3	65.2±1.9	51.7±2.5	<0.001	0.463	0.001
AST	114.1±8.3	128.8±12.5	158.1±10.2	137.5±8.3	0.027	0.798	0.132
ALT	71.3±8.8	67.5±6.4	54.8±3.3	63.6±4.3	0.097	0.681	0.302
GDH	36.6±5.2	25.3±1.6	23.6±1.7	25.2±3.1	0.027	0.118	0.063
LDH	479.7±53.7	406.9±32.2	568.8±40.7	487.6±25.9	0.03	0.048	0.911
MDH	878.1±111.5	932.5±67.4	1185.2±67.7	761.1±53.9	0.365	0.017	0.003
IDH	13.7±1.0	13.2±0.7	16.9±0.4	12.5±0.5	0.072	<0.001	0.006
G6PDH	6.8±0.7	6.9±0.4	6.7±0.3	7.5±0.5	0.461	0.452	0.576

All data are means ± s.e.m. and enzyme activities are expressed as μmol min^{-1} g^{-1} tissue and protein as mg g^{-1} tissue; sample size equals 8 or more for all groups. Enzymes were assessed at 22°C. Data were transformed when necessary to obtain normality (log for PEPCK and GDH). Two-way ANOVA was used, comparing the effect of age, presence of the transgene and interaction of age and the transgene. Pearson correlations between mass and transformed enzyme activities were not significant. PEPCK, phosphoenolpyruvate carboxykinase; G6PDH, glucose 6-phosphate dehydrogenase; see Table 3 for list of other enzyme abbreviations.

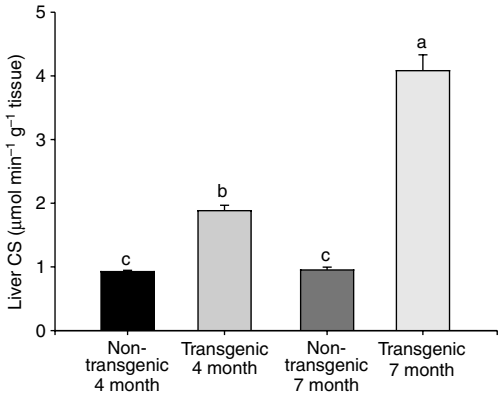

Fig. 9. Citrate synthase (CS) activity in GH-transgenic and non-transgenic salmon liver. Sample size equals 8 or more for each group. Results are means ± s.e.m. Two-way ANOVA was used, assessing the effect of age (P<0.001), presence of the transgene (P<0.001) and the interaction of age and the transgene (P<0.001). Pearson correlations between mass and liver CS equal 0.905, P<0.001 (Bonferroni). Different letters indicate significant differences.

It is apparent from the foregoing that the ectopic expression of the GH transgene in salmon altered the temporal patterns of expression of MyoD and myogenin in both white muscle and red muscle. Comparing red muscle and white muscle in transgenics, MyoD expression increased as the transgenic salmon increased in age and mass from 4 to 7 months after first feeding. This implies an increase in MC proliferation rates in both tissues over this time period. White muscle from non-transgenic salmon demonstrated the same changes in MyoD expression over the same period of time. In the case of myogenin expression, an indicator of cell differentiation, white muscle and red muscle showed increased mRNA levels as the transgenic fish aged and increased in size from 4 to 7 months. This result shows a direct link between myogenesis and fish growth rate, with MC proliferation occurring when growth rate increased.

The GH transgene also increased the activity of the glycolytic enzyme LDH in white muscle and liver and the oxidative enzyme CS in white muscle, liver and intestine, a result possibly reflecting a GH-mediated improvement in feed conversion efficiency as reported for both GH-treated (Campbell et al., 1989) and GH-transgenic (Pursel et al., 1990) domestic pig, and in GH-transgenic tilapia (De La Fuente et al., 1999), and an increase in basal metabolism of transgenic fish (Hill et al., 2000). An effect of the interaction between age and transgene was found in all the enzymes assessed in white muscle, except for AST and IDH, indicating that depending on the age of the fish the effect of the transgene was different, with higher activity in the transgenics at 4 months and lower activity in the transgenics at 7 months compared with the age-matched non-transgenics, except for MDH where the opposite happens, indicating that these enzymes could be affected by the growth rate of the fish.

The activities of all enzymes measured in the red muscle were higher in the non-transgenic salmon at 7 months compared with both GH-transgenic groups of salmon. This result may be explained by the higher quantity of red muscle found in transgenic salmon compared with non-transgenic salmon (Hill et al., 2000), resulting in no change in the total enzyme activities per tissue. However, we did not compare the proportion of red muscle and white muscle in our fish. Within the liver, most of the enzymes (except ALT, glucose 6-phosphate dehydrogenase and MDH) were affected by the age of the fish. However, PK, LDH, MDH, IDH and CS were affected by the presence of the transgene and/or the interaction of age and transgene. These results suggest that both oxidative and glycolytic metabolism increase in the liver in GH-transgenic salmon at 4 and 7 months. This statement supports previous studies reporting higher oxygen consumption in GH-transgenic tilapia (McKenzie et al., 2003) and in exercised GH-transgenic salmon (Stevens et al., 1998) compared with non-transgenic fish of the same mass. IDH provides NADPH for lipid synthesis, suggesting a lower lipid synthesis in the liver of 7 month old GH-transgenics. This result agrees with previous findings that GH-treatment inhibited lipogenesis, and a reduced fat content was reported in the carcasses of GH-treated (Sorensen et al., 1996) and GH-transgenic (Pursel et al., 1990) farm animals. Moreover, Fauconneau et al. (Fauconneau et al., 1997) also reported a decrease in the size of adipose cells after GH treatment in rainbow trout. The extra GH in these transgenics could also increase appetite and food conversion and ultimately act on fish metabolism to reduce fat accumulation (Fletcher et al., 2004).

CONCLUSIONS

We found that MC proliferation, differentiation (as inferred by changes in myogenic factor RNA content) and metabolic enzymes were significantly affected by the presence of the GH transgene in Atlantic salmon. The larger total number and potentially greater GH-induced proliferative capacity of MCs extracted from white muscle of GH-transgenic Atlantic salmon may at least in part contribute to the higher growth rates observed in this group compared with non-transgenic salmon. Other hormones, including IGF-1, may also be involved, but only GH was studied. We report similar patterns in the expression of myogenin and MyoD in red muscle and white muscle, which appear to be linked to changes in size over time in transgenic salmon. This finding will need to be investigated at other life stages and ages of transgenic and non-transgenic salmon in further studies. We also found an increased activity of liver glycolytic and oxidative enzymes in transgenic salmon liver and white muscle, implicating a higher metabolic rate compared with the non-transgenic salmon, at least at 4 months of age, when the growth rate of the transgenic salmon is higher than in non-transgenic salmon.

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