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Continuous light affects mineralization and delays osteoid incorporation in vertebral bone of Atlantic salmon (*Salmo salar* L.)

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SUMMARY

In order to study the effects of photoperiod on fish bone, Atlantic salmon (*Salmo salar* L.) were exposed to two light regimes (natural and continuous light) from January until June. During the experimental period, several parameters related to the inorganic (minerals) and organic (osteoid) phases were measured. Changes in the organic phase were related to mechanical strength (yield-load) and the expression of the genes *sonic hedgehog* (*shh*) and *collagen type I alpha 2* (*col I*). Co-variation between yield-load and the expression of both *shh* and *col I* were detected in both groups. It was also shown that fish on the continuous light regime had delayed activation of osteoid incorporation. Mineralization properties were measured with stiffness, mineral incorporation per day and expression of *alkaline phosphatase* (*alp*) and *matrix Gla protein* (*mgp*). Stiffness, mineral incorporation and gene expression followed the same trend in both light groups in late spring, whereas an increase in the expression of *mgp* and *alp* was detected in April, followed by significantly higher stiffness at last sampling in both light groups. These results indicate that constant light affects mineralization and delays osteoid incorporation in Atlantic salmon during the spring. However, in this experiment light treatment did not promote the development of vertebral deformities. Our results also suggest that *shh* can be used as a marker of osteoblast proliferation and *col I* a marker of osteoid incorporation, and that both *alp* and *mgp* expression could be associated with a rapid increase in mineralization in Atlantic salmon vertebrae.

Key words: sonic hedgehog, collagen type I, alkaline phosphatase, matrix Gla protein, photoperiod, circadian rhythm.

INTRODUCTION

In Atlantic salmon (*Salmo salar* L.), continuous light has been shown to suppress nocturnal melatonin production and flatten out the diurnal pattern in melatonin secretion observed under natural light conditions (Porter et al., 1999). The pineal gland is the main source of melatonin production, and is the primary endocrine mediator of photoperiod changes. Pinealectomy has serious effects on the vertebral column in mammalians, birds and fish (reviewed by Pandi-Perumal et al., 2006). Studies of Atlantic salmon after 6 months under continuous light (Fjelldal et al., 2005) and 1 year after pinealectomy (Fjelldal et al., 2004) revealed a lower mineral content and mechanical strength in the bone of the vertebrae than under natural light, and after sham operation and under natural light. The primary mechanism of pineal mediation of the effect on the vertebral column is unknown. However, the action of melatonin on bone can be mediated directly by action on membrane-bound receptors on osteoblast cells or through the function of melatonin in setting the suprachiasmatic circadian rhythm in the brain. Recent reports support the hypothesis that osteoblasts can receive a direct innervation by sympathetic neurons controlled by the circadian rhythm in the suprachiasmatic nucleus (reviewed by Patel and Elefteriou, 2007). Studies in mammals have reported that light manipulation changes the pattern of osteoblast proliferation and bone mass in response to changes in circadian pattern. Seasonality in bone mass has also been reported in the teleost Atlantic salmon (Wargelius et al., 2005a). The molecular signals that bring about change in bone mass are unknown, but it is likely that light treatment stimulates a change in the formation rate of osteoid tissue and/or the mineralization ability of the tissue.

In this study the gene expression of the gene *sonic hedgehog* (*shh*) was selected as a marker of osteoblast cell proliferation since the protein product is crucial for development and growth of bone in mouse (Chiang et al., 1996; St-Jacques et al., 1999) and ectopic expression of *shh* results in excess dermal bone formation in regenerating zebrafish (*Danio rerio*) caudal fins (Quint et al., 2002). Alkaline phosphatase (Alp) was selected because of its involvement in the mineralizing function of the osteoblasts, and the expression of its gene is commonly used in mammalian species to measure the level of extracellular matrix mineralization (ECM) (Rawadi et al., 2003). Matrix Gla protein (Mgp) was selected based on its involvement in the calcification of ECM, and *mgp* deficiency in mice results in overcalcification of bone and cartilage (Luo et al., 1997), suggesting that the protein functions, to some extent, as an inhibitor of calcification. The expression of this gene has also been found to be induced during mineralization of a bone-derived cell line from sea bream (*Sparus aurata* L.) (Pombinho et al., 2004). Another marker, *collagen type I alpha 2* (*col I*; also known as *col1a2*) was selected, because collagen I is the major structural protein that is incorporated into bone. Two of the genes used in this study, *shh* (Wargelius et al., 2005b) and *mgp* (Laize et al., 2005), have been cloned and/or identified previously in Atlantic salmon, and the other genes have been identified by sequence homology; *alp*, has been identified in fugu (*Takifugu rubripes*, GenBank acc. no. NM_001032651) and Atlantic salmon (acc. no. CO472235). Collagen type I precursors have been identified in a number of fish species, including rainbow trout (*Oncorhynchus mykiss* Walbaum) (Saito et al., 2001). In rainbow trout, type I procollagen has a trimer

structure α1(I) α2(I) α3(I) (Saito et al., 2001) and in this study it was used as a homologous sequence to the α2 chain (acc. no. CA064459).

To summarize, the effects of photoperiod and pinealectomy on the gene expression of proteins involved in bone cell proliferation, and in the production and mineralization of osteoids, remain to be elucidated. To study the photoperiodic effect on vertebral bone, we reared Atlantic salmon under continuous light or natural light [following the protocol by Porter et al. (Porter et al., 1999)], and the gene expression levels of proteins involved in cell proliferation, mineralization and osteoid formation were measured in vertebral bone once a month from January until the summer solstice. In addition, yield-load and stiffness were used as markers of osteoid incorporation and mineralization respectively, as it is known that stiffness reflects the mineral content of bone, whereas yield-load is a measure of the collagen content and structure of the bone (reviewed by Currey, 2003).

MATERIALS AND METHODS

Fish stock and rearing conditions

Atlantic salmon (*Salmo salar* L.) under-yearling post-smolts (*N*=2264) with an average mass of 75 g were transferred to a seawater cage at the Institute of Marine Research, Matre, Norway (61°N) 2 months before the start of the experiment. One week after transfer to seawater, 924 fish were tagged with Trovan® transponders ID 101 (BTS Scandinavia AB, Åhus, Sweden), and their adipose fins removed.

The experiment was designed with one experimental and one control group, each with three replicates in separate cages, and the experiment lasted from mid January until the summer solstice (19 June). At the start of the experiment, the fish were randomly allocated to one of six cages (5 m×5 m×7 m), so that each cage contained 154 tagged and 220 untagged fish. Three cages of the experimental group were reared under a natural photoperiod with additional 24 h continuous light, while the fish in the three cages of the control group were reared under a natural photoperiod. For continuous illumination, one asymmetric metal halide lamp per cage was employed (Euroflood, Siemens, Trondheim, Norway; Osram Powerstar HQI-TS 150W/NDL UVS). The lamps were mounted on the side of the cages, 2 m above the water surface, yielding an illuminance of 105±7 lux at a depth of 1 m. A light-tight barrier separated the illuminated cages from the others. The fish were reared at ambient temperature, which decreased from 7.9°C in January to 5.6 in March and then increased to 10.5°C in June (measured at a depth of 5 m). The salinity at 5 m was stable at approximately 31‰.

The fish were fed Bio-optimal® dry feed (BioMar Ltd, Trondheim, Norway), to excess, using a computer-operated feeding system (ARE, Storebø, Norway). Three pellet sizes (3 mm, 4 mm and 6 mm) were used as the fish grew throughout the experiment. To control sea lice infestation, the fish were given SLICE® (Schering-Plough AS, Farum, Denmark) at a dose of 0.5% of biomass per day for 1 week in the middle of the experimental period. The growth performance of the tagged fish during the experimental period is published in Fjelldal et al. (Fjelldal et al., 2005) and Nordgarden et al. (Nordgarden et al., 2006). The mean mass at the start of the experiment was 135 g, and at the end of the experiment was 457 g and 372 g in the continuous and natural light groups, respectively. The mortality rate during the experimental period was 0.7% in the continuous light group and 0.6% in the natural light group. Throughout the experiment 16 fish from each tank were sampled for gene expressional studies (*N*=3/tank) and biomechanical analysis (*N*=3/tank: stiffness, yield-load and mineral content) and X-ray (*N*=10/tank). Owing to the monthly sampling and the general mortality in the fish groups 264 and 276 fish remained in the two light treatment groups and the end of the experiment.

Tissue sampling

First sampling was carried out on 13 January, followed by samplings after 3, 6, 10, 15 and 22 weeks. At each sampling, 16 fish from each cage were anaesthetized with metomidate hydrochloride (Wildlife Pharmaceuticals, Fort Collins, CO, USA) according to Olsen et al. (Olsen et al., 1995), and killed by a blow to the head, before sampling of vertebral tissue (six fish), and dissection of vertebral columns for lateral radiographs (10 fish). The fortieth vertebra (V40), which is located in the caudal region of the vertebral column, was carefully dissected out and immediately frozen in liquid nitrogen for later analysis of gene expression, and whole fish were frozen (–20°C) for measurement of vertebral mineral content and mechanical strength.

Radiology

Radiographs were taken using a portable X-ray apparatus (HI-Ray 100, Eickenmeyer Medizintechnik für Tierärzte e.K., Tuttlingen, Germany) and 30×40 cm film (AGFA Structurix D4 DW ETE, Agfa-Gevaert N.V., Mortsel, Belgium). The film was exposed twice for 50 mA and 72 kV, and developed using a manual developer [Cofar Cemat C56D, Arcore (MI), Italy] with Kodak Professional manual fixer and developer (Kodak S.A., Paris, France). The images were digitized using an A3 positive scanner (Epson Expression 10000 XL, Seiko Epson, Kagano-Ken, Japan). Each fish was evaluated for vertebral deformities, and the number of affected vertebrae was recorded.

Bone properties

The vertebrae were compressed in the cranial–caudal direction using a texture analyzer (TA-XT2 Texture Analyzer, Stable Micro Systems, Haslemere, UK) with a steadily advancing piston (6 mm min^{-1}). Load-deformation data were continuously recorded, and the stiffness (g mm^{-1}) and yield-load (g) were calculated for each vertebra according to the method of Fjelldal et al. (Fjelldal et al., 2004). After mechanical testing, the vertebrae were defatted in hexane baths, dried overnight at 90°C, and then incinerated for 13.5 h in a muffle furnace (115°C for 0.5 h, 540°C for 5 h, and 750°C for 8 h). The ash (mineral) weight of each vertebra was weighed to within 1×10^{-2} mg. Mineralization per day was calculated as increase in mineral mass per day (mg day^{-1}). This was done based on the ash mass of the sampled vertebrae, using the following formula; {[average ash mass in tank X_n at time (t) 2]–(average ash mass in tank X_n at t1)}/(number of days between samplings). As stated above, we used three fish (*N*=3) in each replicate tank and three replicate tanks (X_1, X_2 and X_3) were used per treatment.

Real-time PCR

Total RNA was extracted from vertebral tissue using FastRNA Pro Green Kit (Qbiogene, Cambridge, UK). RNA was DNAse treated (37°C for 30 min, Promega, Madison, WI, USA) and then extracted once more with phenol pH 4.5. First-strand cDNA was reverse transcribed using a Reverse Transcription Core Kit (RT-RTCK-05; Eurogenetec, Seraing, Belgium) using 500 ng of RNA. For amplification of *shh* (GenBank acc. no. AY370830), *col I* (acc. no. CA064459), *alp* (acc. no. CO472235), *mgp* (acc. no. AY182239) and the normalization gene, elongation factor 1α [*e1α*; Acc no

Table 1. The primers and probes used

Gene	Sequence
shh	Forward: 5′-GGCGTTTGCACCTGTCAGA-3′
	Reverse: 5′-TGGGCATGGAGTATTCTTTCG-3′
	Probe: 6-FAM-TACTGGCTGTCCTCGTTGCGA-TAMRA
col I	Forward: 5′-GAGGGTGGATGCAGGTGTGT-3′
	Reverse: 5′-TACTGGATCGACCCCAACCA-3′
	Probe: 6-FAM-TGGAGAAGTCGCAGTGGGCCTTGAT-TAMRA
alp	Forward: 5′-CTACACGCCAAGAGGGAACAC-3′
	Reverse: 5′-GGTAAAGGGTTTCTGGTCCACAT-3′
	Probe: 6-FAM-ATTTTTGGACTGGCTCCCATGTTGAGT-TAMRA
mgp	Forward: 5′-GAAAGCACAGAATCCTTTGAAGATGT-3′
	Reverse: 5′-GTGGACTCTGTGGGTTGATGAA-3′
	Probe: 6-FAM-TTTGTCAGTCCATACCGAGCCAAC-TAMRA
e1α	Forward: 5′-CCCCTCCAGGACGTTTACAAA-3′
	Reverse: 5′-CACACGGCCCACAGGTACA-3′
	Probe: 6-FAM-ATCGGTGGTATTGGA-MGB

AF321836 (Olsvik et al., 2005)], the primers and probes listed are listed in Table 1. Primers were tested using conventional PCR and shown to amplify a single band of approximately 80 bp. Real-time PCR was carried out on an ABI 7700 system (Applied Biosystems, Foster City, CA, USA). Thermal cycling conditions were 50°C for 2 min followed by 98°C for 10 min. Subsequently, the reactions proceeded through 40 cycles of 95°C for 15 s followed by 60°C for 1 min. Each reaction (25 µl) contained 5 µl of cDNA diluted 1:5 in ddH_2O, 12.5 µl of Taqman Universal PCR master mix (Applied Biosystems) and 0.9 µmol l^{-1} of forward and reverse primers. Each sample was run in triplicate and each stage contained six RNA replicates (samples from six different fish). The efficiency of the targets (*shh*, *mgp*, *col I* and *alp*) in relation to the reference (*elα*) was determined using a standard curve method together with a validation experiment (Applied Biosystems, User Bulletin #2 for ABI 7700 sequence detections system). In the validation experiment, 500, 250 and 125 ng of RNA were used for cDNA synthesis and the slope of log input amount of RNA *versus* delta Ct for *shh*/*e1α* was 0.028, for *mgp*/*e1α* was 0.061, *col I*/*e1α* was 0.025 and *alp*/*e1α* was 0.042, which is <0.1, which demonstrates that the efficiency of target and reference were approximately equal. The relative expression level was calculated using the Comparative Ct method (Applied Biosystems, User Bulletin #2 for ABI 7700 sequence detection system). In all experiments no-template controls were run together with the samples.

Statistics

All data was subjected to Kolmogorov–Smirnov test for Gaussian distribution. Stiffness, Yield-load and gene expression of *col I*, *shh* and *alp* were confirmed to have Gaussian distribution and were subjected to two-way ANOVA with photoperiod and time as the dependent factors when analyzing for differences among photoperiod groups or variation over time, respectively. The data were further subjected to Bonferroni *post-hoc* tests. Expression of *mgp* and mineral incorporation per day did not meet the assumption of Gaussian distribution; these data were subjected to an unpaired *t*-test with Welch's correction. Data analyses were performed using GraphPad Prism 5.0 (La Jolla, CA 92037, USA). A P value <0.05 was taken to indicate statistical significance.

RESULTS

Yield load

In the natural light group, the yield-load (g) of the vertebrae rose from 13 January until 24 March (P<0.05) and then again from 28 April to 19 June (P<0.001; Fig. 1A, white bars). In the continuous light group, the yield-load increased from 13 January until 28 April (P<0.05; Fig. 1A, black bars). Yield-load increased significantly in both light groups between 28 April and 19 June (P<0.001 in both light groups). When comparing light groups at each time point no differences were detected between light groups.

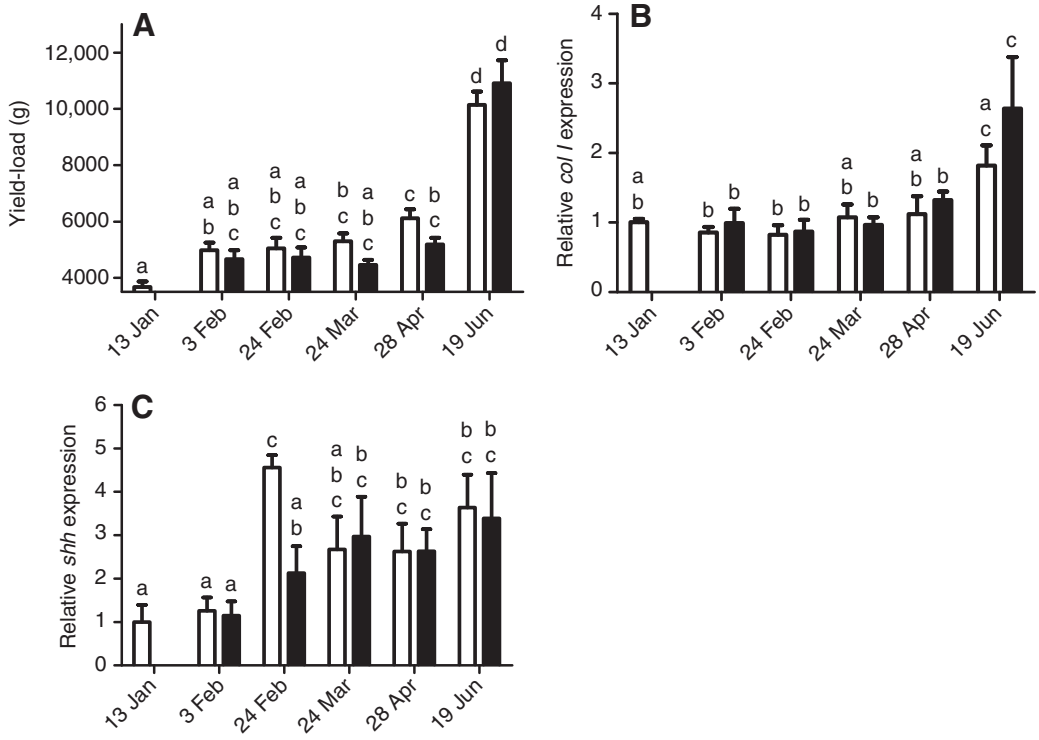

Fig. 1. The effect of different light regimes on the yield-load (A; N=6–13), *col I* expression (B; N=6) and *shh* expression (C; N=6). White bars, natural light; black bars, continuous light. Values are means ± standard error of mean (± s.e.m.). Significant differences are indicated by different lower case letters above the bars.

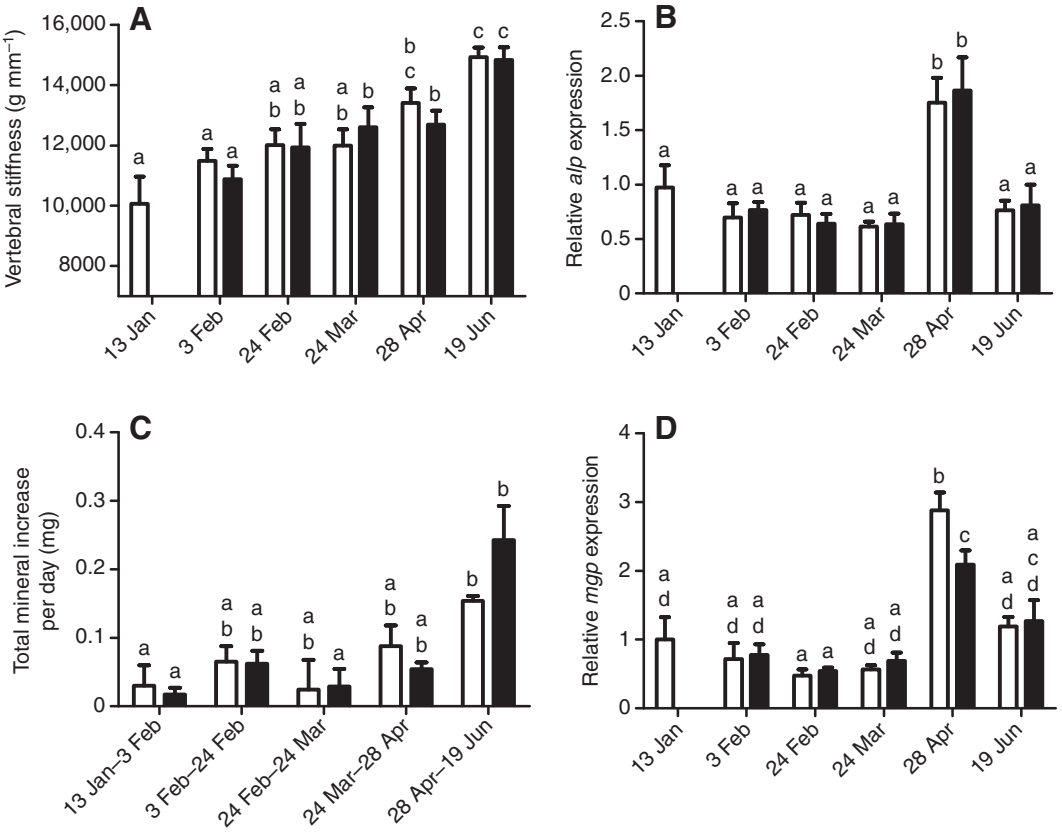

Fig. 2. The effect of different light regimes on the vertebral stiffness (g mm^{-1}; A; N=6–12), *alp* expression (B; N=6), total mineral increase per day (mg; C; N=3) and *mgp* expression (D; N=6). White bars, natural light; black bars, continuous light. Values are means ± standard error of mean (± s.e.m.). Significant differences are indicated by different lower case letters above the bars.

col I expression

In the natural light group, *col I* expression increased from 24 February until 19 June (P<0.05; Fig. 1B, white bars). In the continuous light group, the expression of *col I* increased from 28 April to 19 June (P<0.01; Fig. 1B, black bars). When light groups at each time point were compared, no differences were detected.

shh expression

In the natural light group, *shh* expression increased from 3 February until 24 February and remained high throughout the study (P<0.001; Fig. 1C, white bars). In the continuous light group *shh* expression increased on 28 April in comparison with 13 January (P<0.05; Fig. 1C, black bars). When light groups at each time point were compared, *shh* showed higher expression in the natural light group on 24 February (P<0.05).

Stiffness

In the natural light group, vertebral stiffness (g mm^{-1}) increased from 3 February to 28 April (P<0.05; Fig. 2A, white bars). In the continuous light group, stiffness increased from 13 January to 24 March (P<0.05) and then again from 28 April to 19 June (P<0.05; Fig. 2A, black bars).

alp expression

In both light groups the expression of *alp* (Fig. 2B, white and black bars) was low and stable in the period between 13 January and 24 March, and increased from 24 March until 28 April (P<0.001 in both light groups), and then decreased significantly from 28 April until 19 June (P<0.001 in both light groups). When light groups at were compared, no differences were detected between light groups.

Mineral incorporation per day

In the natural light group the *per diem* mineral incorporation was higher between 28 April and 19 June than between 13 January and 3 February (P<0.05). In the continuous light group, mineral incorporation per day was higher between 28 April and 19 June than between 13 January and 3 February (P<0.05) or 24 February and 28 April (P<0.05; Fig. 2C). When light groups at each time point were compared, no differences were detected.

mgp expression

In both light groups the expression of *mgp* (Fig. 2D, white and black bars) was at a low steady state between 13 January and 24 March, increasing on 28 April (P<0.001 in both light groups), and then decreasing between 28 April and 19 June in the natural light group (P<0.005). When light groups at were compared, lower *mgp* expression was detected in the continuous light group on 28 April (P<0.05).

Vertebral deformities

To assay if continuous light treatment affected the occurrence of vertebral deformities, fish were X-rayed at the end of the experiment (N=30/light group). The prevalence of individuals with one or more deformations of vertebral bodies was 6.7% at the start of the experiment, and 3.3% and 10% in the natural and continuous light groups, respectively, at the end of the experiment on 19 June. The deformities were located in all regions of the vertebral column, and there were no severe cases. The number of affected vertebrae per deformed fish ranged between 1 and 3.

DISCUSSION

The similarity in the trends of *col I* expression and yield-load in the natural light group can be attributed to the link between the collagen

I content and the toughness of the vertebrae. Alterations in the amount of collagen I can then affect the yield-load of the bone and possibly change susceptibility to fracture. However, the first increase in yield-load in both light groups was not reflected in the expression of *col I*, and it is possible that the incorporation of collagen I into the matrix is controlled by modifying proteins instead of at the level of gene expression at this stage. The modification of the procollagen I protein is controlled by a set of factors that involve the bone morphogenetic protein 1 (BMP-1) and tolloid-related metalloprotein kinase (MMP) which then controls the assembly of the fibrillar collagen (Ge and Greenspan, 2006). These factors may also contribute to the actual level of collagen fibril incorporation into the bone. In bovine cortical bone, the extracellular post-translational modifications of collagen are the major determinants of its biomechanical properties (Garnero et al., 2006). This suggests that collagen I abundance in bone was controlled at the post-translational level during the first phase of the experiment, while its abundance was regulated at the level of gene expression during the last phase.

In the natural light group, the increase in *shh* expression on 24 February was followed by an increase in *col I* expression and yield-load between 24 February and 19 June. Shh has been shown to be involved in osteoblast proliferation in zebrafish bone (Quint et al., 2002), and this suggests an increase in osteoblast proliferation between 24 February and 19 June in the present experiment. Furthermore, increased osteoblast proliferation could explain why both *col I* and yield-load increased between 24 February and 19 June. Taken together, these results suggest that in Atlantic salmon the expression of both *col I* and *shh* can be used as markers of osteoid incorporation and osteoblast proliferation, respectively.

This experiment found a clear link between stiffness ($g\,mm^{-1}$) and expression of both the alkaline phosphatase and matrix Gla protein genes. Stiffness provides a measure of the mineral phase of the bone (reviewed by Currey, 2003) and this result suggests that both *mgp* and *alp* are involved in bone mineralization in salmon. If *mgp* and *alp* are involved in mineralization, our results imply that mineralization has a seasonal component. Similar results have been found previously, with a significant increase in expression of the genes for both IGF-1 and its receptor during the same time of year (Nordgarden et al., 2006; Wargelius et al., 2005a). However, mineral incorporation (mg mineral per day) was not directly related to expression of the *alp* and *mgp* genes. Mineral incorporation per day seems to be related to both stiffness and *alp* and *mgp* expression during the last phase of the experiment (28 April to 19 June), since stiffness increased from April to June and both *alp* and *mgp* showed a peak in expression on 28 April. Moreover, the gradual increase in stiffness between February and April was not reflected in mineral incorporation per day or the expression of *alp* or *mgp*, which suggests that there are other factors promoting the long-term increase in mineralization in Atlantic salmon vertebrae.

The increase in the relative expression of *col I* over the last three samplings in the natural light group compared to the increase in *col I* between the last two samplings in the continuous light group indicates that there was a delay in the induction of collagen production in the fish exposed to continuous light. Similarly, it is known in mammals and birds that the increase in collagen production takes place at night (Hassager et al., 1992; Simmons and Nichols, 1966). Our results suggest that the dark phase of the photoperiod plays a role in the regulation of *collagen I* expression in salmon. It has previously been reported that DNA synthesis in rat osteoblasts peaks during the night (Fu et al., 2005). However, this study is the first to demonstrate an effect of photoperiod at the level of expression of the collagen I gene.

The expression of *Shh* rose 1 month later in the continuous light group. If *shh* is involved in the recruitment of osteoblasts in salmon, our results suggest that there is a lower early recruitment of osteoblasts in fish exposed to continuous light. This presumption is based on results from zebrafish dermal bone, where ectopic expression of *shh* induces osteoblast proliferation (Quint et al., 2002). The delay in induction of both *shh* and *col I* expression in the continuous light group is then reflected in a 1 month delayed increase in yield-load, which is probably is a result of a delay in osteoblast recruitment (*shh* expression) and a subsequent reduction in organic matrix production (*col I* expression). These results leads us to suggest that constant light delays both osteoblast proliferation and thereafter collagen I production, which lead to temporal changes in the properties of the extracellular matrix.

If Mgp plays a similar role in salmon as in mammals, i.e. inhibition of mineralization (Luo et al., 1997), the lower *mgp* expression in the continuous light groups in late April would suggest that mineralization increased in fish on continuous light towards the end of the experiment. This is also reflected in the *per diem* rate of mineral incorporation, which rose significantly during the last phase (April to June) of the experiment. This was not the case in the normal light group, in which the mineral incorporation per day increased gradually throughout the whole experimental period (February to June).

In the continuous light group, stiffness increased between February and March, while in the normal light group the increase occurred between February and April. The more rapid increase in mineral incorporation in the bone is not reflected in the molecular markers used in this study. However, a previous growth study showed that IGF-1 gene expression in bone was significantly higher in the continuous light group 2 weeks after onset of the light (Nordgarden et al., 2006). In mice, IGF-1 promotes mineralization of trabecular bone (Zhang et al., 2002). The earlier increase in stiffness in the vertebral bone of continuous-light-treated fish can perhaps be explained by an increase in local IGF-1 production, which then results in a temporary higher mineralization rate of the vertebral bone. Also a growth-promoting effect of light was observed at the whole fish level, for which there was higher specific growth rates in response to continuous light (Nordgarden et al., 2006). This observation implies that in salmon the stiffness (mineral content) of the bone is altered in response to the higher growth rates induced by the continuous light, while the osteoid incorporation is delayed.

Plasma levels of melatonin in Atlantic salmon are under diurnal regulation, and increase during the dark phase (Iigo et al., 1997; Porter et al., 2001). It is possible that the effects of constant light on the vertebral bone have been mediated by the action of melatonin on bone, through the suprachiasmatic nucleus in setting the circadian rhythm, and further through sympathetic signaling directly to osteoblastic cells, resulting in changes in the bone-specific circadian clock (Patel and Elefteriou, 2007). In mice, an interrupted circadian clock increases the number of osteoblasts in bone and the bone formation rate (Fu et al., 2005). The opposite results were obtained in this experiment, with a decrease in osteoid production (reduced *col I* expression, reduced yield-load) and a potential reduction in osteoblast numbers (reduced *shh* expression) in response to constant light. Our results therefore suggest that the circadian rhythm of the osteoblasts might have been altered, in that they showed loss of a higher rate of bone formation at night, which implies that circadian rythmicity may have been temporarily lost, resulting in the delayed induction of osteoblast proliferation and organic matrix production.

Exposure to constant light alters the pattern of vertebral growth in Atlantic salmon, by delaying proliferation and bone formation while advancing mineralization. However, by the end of the experiment, close to the summer solstice, the fish had managed to balance out the changes induced by the continuous light treatment, thereby displaying similar levels of both yield-load and stiffness. The light-induced change in bone properties during the spring did not result in any bone deformities, implying that continuous light treatment does not promote vertebral deformities. In addition it would appear that *col I*, *shh*, *mgp* and *alp* can be used as markers of changes in bone properties related to both the organic and inorganic extracellular matrices of salmon vertebrae. To conclude, continuous light exposure has a large impact at the whole organism level in salmon, where it affects the growth rate, timing of smoltification and reproduction (Berg et al., 1992; Krakenes et al., 1991; Saunders and Henderson, 1988; Stefansson et al., 1991). Little is known about how light affects the physiology at the specific tissue level, but it is known that other tissues than bone are affected by light, such as the composition and cellularity of muscle (Johnston et al., 2003; Nordgarden et al., 2003). Results from this study and previous reports clearly indicate that light has a substantial impact both at the tissue as well as on the whole organism level.

LIST OF ABBREVIATIONS

alp	alkaline phosphatase gene
col I	collagen type I α2 gene
ECM	extracellular matrix
e1α	elongation factor 1α gene
mgp	matrix Gla protein gene
shh	sonic hedgehog gene

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