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Collagen type XI α1 may be involved in the structural plasticity of the vertebral column in Atlantic salmon (*Salmo salar* L.)

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SUMMARY

Atlantic salmon (*Salmo salar* L.) vertebral bone displays plasticity in structure, osteoid secretion and mineralization in response to photoperiod. Other properties of the vertebral bone, such as mineral content and mechanical strength, are also associated with common malformations in farmed Atlantic salmon. The biological mechanisms that underlie these changes in bone physiology are unknown, and in order to elucidate which factors might be involved in this process, microarray assays were performed on vertebral bone of Atlantic salmon reared under natural or continuous light. Eight genes were upregulated in response to continuous light treatment, whereas only one of them was upregulated in a duplicate experiment. The transcriptionally regulated gene was predicted to code for collagen type XI α1, a protein known to be involved in controlling the diameter of fibrillar collagens in mammals. Furthermore, the gene was highly expressed in the vertebrae, where spatial expression was found in trabecular and compact bone osteoblasts and in the chordoblasts of the notochordal sheath. When we measured the expression level of the gene in the tissue compartments of the vertebrae, the collagen turned out to be 150 and 25 times more highly expressed in the notochord and compact bone respectively, relative to the expression in the trabecular bone. Gene expression was induced in response to continuous light, and reduced in compressed vertebrae. The downregulation in compressed vertebrae was due to reduced expression in the compact bone, while expression in the trabecular bone and the notochord was unaffected. These data support the hypothesis that this gene codes for a presumptive collagen type XI α1, which may be involved in the regulatory pathway leading to structural adaptation of the vertebral architecture.

Key words: vertebral deformity, spinal deformity, photoperiod, circadian rhythm, osteoblast, bone.

INTRODUCTION

In Atlantic salmon (*Salmo salar* L.), continuous light (Fjelldal et al., 2005) and pinealectomy (Fjelldal et al., 2004) are associated with lower vertebral mineral content and mechanical strength. In salmon, the response to change in photoperiod is thought to be primarily mediated by the pineal gland, and continuous light suppresses nocturnal melatonin production from this gland (Porter et al., 1999). Furthermore, the growth endocrinology of salmon vertebra change in response to continuous light (Nordgarden et al., 2006), and the expression of genes involved in proliferation, mineralization and osteoid incorporation are altered (Wargelius et al., 2009). These results clearly indicate that photoperiod has a substantial effect on the molecular mechanisms that govern composition and mechanical properties of the salmon vertebral bone. However, the regulatory proteins that trigger this change in bone composition and strength have not been identified.

Low mineral content and mechanical strength of the vertebrae have been associated with the development of vertebral anterior–posterior compression (Fjelldal et al., 2006; Fjelldal et al., 2007b; Fjelldal et al., 2009), which is the most common deformity in farmed Atlantic salmon and is usually restricted to the tail region of the vertebral column (Witten et al., 2005; Berg et al., 2006; Fjelldal et al., 2008). A compressed vertebra has a normal central part where the angle between the wall of the cone of the vertebra and the anterior–posterior axis is approximately 45 deg. In a deformed vertebra this angle increases to approximately 90 deg. in the anterior and posterior parts (Berg et al., 2006; Witten et al., 2005). This malformation seem to develop late in ontogeny (Berg et al., 2006; Witten et al., 2006; Fjelldal et al., 2007b), and has been associated with factors such as mineral malnutrition (Fjelldal et al., 2009), and vaccination (Berg et al., 2006). Compressed vertebrae have been characterized morphologically (Kvellestad et al., 2000; Witten et al., 2005; Fjelldal et al., 2007a; Fjelldal et al., 2007b), however, the molecular pathways underlying the pathogenesis have yet to be identified.

The primary aim of this study was to investigate which factors in vertebral bone are affected by light manipulation. A microarray analysis was performed and the gene for collagen type XI α1 was found to be upregulated by light. In order to further characterize the properties of this gene, tissue-specific expression was assessed, the effect of photoperiod on expressional level was determined, and the expression levels in compressed and normal vertebrae were investigated.

MATERIALS AND METHODS

Trials

Samples were taken from three separate experiments (numbered 1 to 3). In experiment 1, Atlantic salmon smolts in sea water (mass ~50 g) were reared in six outdoor cages, three of which received continuous light (LL) between 13th January and 19th June, and three received natural light (NL) during the same period. Sampling

for microarray analysis was performed 3 weeks after the onset of continuous light (3rd February) and a detailed description of the rearing conditions is given by Fjelldal et al. (Fjelldal et al., 2005).

In experiment 2, Atlantic salmon underyearling smolts, hatched in February 2005, were transferred to nine indoor seawater tanks in September 2005 at the Institute of Marine Research, Matre (60°N, 50°E), Western Norway. Each tank was illuminated with two 18 W fluorescent daylight tubes, giving a light intensity of 960 lux measured under water at the centre of the tank. The fish were fed a commercial diet continuously for 12 h a day (Skretting Nutra Olympic, Skretting, Stavanger, Norway). Water flow was kept above 2.5 l min^{-1} k^{-1} fish. The oxygen saturation of the outlet water was maintained above 70%. Seawater temperature was 10.2±0.3 throughout the study. From seawater transfer (13th September), fish in three tanks were reared under continuous light (LL), while six were reared under simulated natural light (12 h:12 h L:D photoperiod). Sampling was performed after 3 weeks (4th October). In experiments 1 and 2, vertebra nos 38–42 (counted in the cranial to caudal direction), which are located in the caudal region of the vertebral column, were sampled. For gene expression analysis the vertebrae were dissected and immediately frozen in liquid nitrogen.

Experiment 3 used Atlantic salmon at harvest size. The fish had been reared under natural light, receiving a commercial diet (Classic, Biomar AS, Myre, Norway) in a 12 m×12 m×12 m sea-cage that contained a total of 1000 salmon. At harvest (1.9 kg), the fish were palpated and normal individuals and individuals with vertebral deformaties in the tail region were selected for further radiographic evaluation (Fig. 6A). Whole vertebral columns were dissected and immediately frozen on liquid nitrogen. Frozen vertebrae from normal individuals (N=3), and fish exhibiting compressed vertebrae (N=4) in the tail region were selected from radiography. In order to investigate possible differences in expression, trabecular bone (TB), compact bone (CB) and notochord (NC) were microdissected. In order to assess the RNA of each sub-tissue within the vertebrae (N=3 fish), one vertebra from each fish was dissected into TB, CB and NC (Fig. 5D), and total RNA content was measured in each sub-tissue (Fig. 8).

RNA isolation

All sampled tissues were immediately frozen in liquid nitrogen. Total RNA was extracted from vertebral tissue (10–20 mg each) using the FastRNA Pro Green Kit (Q-biogene, Illkirch cedex, France). The RNA used for microarray analysis was treated with DNAse (Promega, Madison, WI, USA) at 37°C for 30 min, and then extracted once more with phenol (pH 4.5), whereas that used for real-time PCR was treated with DNAse using TURBO DNA-*free*™ (Ambion, Austin, TX, USA) according to the manufacturer's recommendations. The amount and quality of RNA was verified by the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DA, USA). An Agilent 2100 Bioanalyzer (Agilent Technologies, South Queensferry, UK) was used for the microarray samples and RNA integrity number (RIN) values for all samples were between 9.8 and 10.0.

Microarray hybridization

The GRASP (Genomics Research on All Salmon Project) consortium's salmon 16K (16 thousand) cDNA array was used in the study (von Schalburg et al., 2005). Six samples (individual fish) from each light-exposure group were compared, including two replicate dye swaps (N=8 total arrays). For each array 10 μg of RNA from each group (NL or LL) was used, and cDNA was synthesized using Superscript II enzyme (Invitrogen, Oslo, Norway) together with Cy3 (cyanine fluorescent dye 3) or Cy5 (Genisphere, USA). Cy3- and Cy5-labeled cDNA were then mixed and concentrated using Microcon Spin columns (YM-30, Millipore). Microarrays were hybridized and washed according to the Genisphere Array 50™ (version 2) labeling protocol, dual-channel experiment (Protocol version 3 July 2004; Koop Lab, University of Victoria, Canada). Chemiluminescent signal-detection image acquisition and image analysis of the microarray analyses were performed on the Agilent G2567AA Microarray Analyzer and measurements were made and raw data sorted using GenePix^{R}Pro 6.0 Software (Agilent, South Queensferry, UK).

Microarray analysis

The raw data extracted from this program were analyzed using J-Express Pro V2.6 software (Molmine, Norway) (Dysvik and Jonassen, 2001). Genes were normalized using Global Lowess normalization. The data were compiled using arrays that were replicated using a medium combination method. Results were presented as log ratio. In order to further sort differently expressed genes, the following conditions were set: 0% missing values allowed, maximum standard deviation <0.3, deviation from zero difference >1.5, 83 genes were left to analyze. Since any gene expressional differences were expected to be found in all arrays, 0% missing values was allowed. K-means cluster analysis of the 83 genes revealed that eight genes were significantly upregulated in the vertebrae of the fish exposed to continuous light. In order to verify that the analysis was correct the color of the upregulated eight spots from each array are presented in Fig. 1.

RT-PCR

Gene expression analysis was performed in order to confirm the microarray results and to assay differences in gene expression in response to light and within a vertebral deformity. All reactions were run on a 7900 HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The conditions for PCR were as follows for all the reactions: 50°C for 2 min followed by 95°C for 10 min, thereafter 40 cycles of 95°C for 15 s followed by 60°C for 1 min. The primers used for the real-time analysis are shown in Table 1. The efficiency of targets in relation to the reference gene *elongation factor 1 alpha* (*e1α*) (Olsvik et al., 2005) was determined using a standard curve method together with a validation experiment (ABI User Bulletin #2 for ABI 7700 sequence detections system). Real-time analysis was performed with cybergreen for genes except for *collagen type XI α1* which was run with a Taqman probe (Applied Biosystems). The primer pair for *collagen type XI α1* was designed to flank an intron–exon boundary found in mouse, fugu and zebrafish, and PCR with the primer pairs from salmon (listed in Table 1) amplified a longer fragment when running the PCR on genomic DNA compared (about 200 bp) to PCR cDNA (80 bp). A probe covering the predicted intron–exon boundary (Table 1) showed that the PCR was unable to pick up any signal from genomic DNA using the probe. In the validation experiment, 500, 250, 125, 62.5, and 31.1 ng of RNA were used for cDNA synthesis, and the slope of the log input amount of RNA *versus* ΔC_t is shown in Table 1. All genes used showed approximately equal efficiency between target and reference. The relative expression level was calculated using the Comparative C_t method (ABI User Bulletin #2 for ABI 7700 sequence detection system). In all experiments no-template controls were run together with the samples.

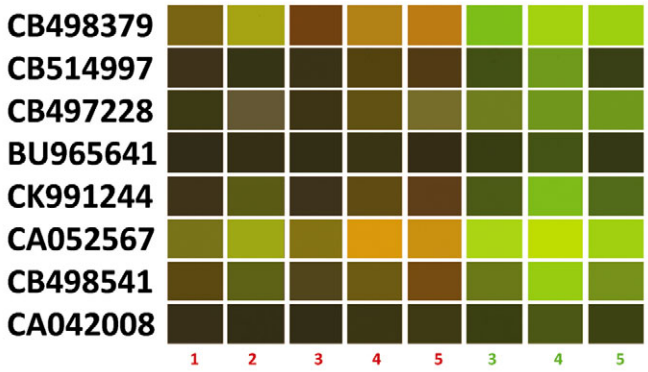


Fig. 1. Microarray results. Eight genes with higher expression in the vertebrae of fish reared under continuous light (experiment 1). Accession numbers are listed in the left-hand column. At the bottom, individual microarray comparisons are numbered (1–5). Each color box on the horizontal axis reflects the hybridization signal in individual comparisons. Higher expression in the continuous light group is shown with more of the red channel signals in comparisons 1–5, numbered in red. Comparisons 3–5, numbered in green, represent dye-swap comparisons, where the green channel signal indicates overexpression in the continuous light group.

Northern blot

Samples for RNA analysis were isolated from vertebrae around vertebra 40 of 50–100 g Atlantic salmon grown in sea water. Total RNA was isolated using the FastRNA Pro Green Kit (Q-biogene) following the manufacturer's recommendations, and RNA integrity was evaluated on an Agilent 2100 Bioanalyzer (Agilent Technologies, Foster City, CA, USA). RNAs were treated with DNAse using TURBO DNA-*free*^{TM} (Ambion) according to the manufacturer's recommendations. Northern blotting was performed using ~3 µg total RNA per lane and sequence-specific ^{32}P-labeled DNA probes against *collagen type XI α1*, a 513 bp sequence [nucleotides (nt) 1017–1529; Fig. 3B] corresponding to a part of the conserved collagen C-propeptide sequence.

Cloning

The gene corresponding to the *collagen type XI α1* in rainbow trout (acc. no. CB497228.1) was identified by homology searching using BLAST (NCBI database, 97% identity) in Atlantic salmon (acc. no. CO470370.1). This sequence turned out to code for a *collagen type XI α1* 3′ sequence in salmon. The gene was cloned using forward primer 5′-CATCCAGTCTCCCAAGAAGAA-3′ and reverse primer 5′-ACCACAGACTGATGGCAGTTA-3′, producing a 513 bp sequence that corresponded to a sequence homologous to the collagen domain of this gene (nt 1017–1529; Fig. 3B). The sequence was further cloned into the pCRII-TOPO vector (Invitrogen) and used for both *in situ* hybridization and northern blotting. In order to further identify the 3′ and 5′ regions of this gene, we employed the SMART RACE cDNA amplification kit (Takara Bio Europe/Clontech, Saint Germain en Laye, France). One variant of the 5′ end was identified using the RACE 5′ primer 5′-GCCTGCATGTCTGCGTGTCTGCGGTTC-3′ and a 3′ end variant was identified using the following primer 5′-ACGTTGACGCAGCCGGCAACACA-3′. This produced a *collagen type XI α1*-like 3′ end of 445 bp and a 5′ end of 1061 bp, which together with the original sequence produced a sequence corresponding to a 1826 bp sequence (acc. no. FJ790236). However, it is uncertain whether this is the whole protein, and due to the difficulty of the RACE on the 5′ end we terminated further cloning of the 5′ end.

Table 1. Primers and probes used for real-time PCR

Experiment	Gene	Acc. no.	Primer, forward	Primer, reverse	Probe/Cybergreen	Validation	Predicted intron–exon location
1, 2	1. Unknown	CB498379	5′-TTTGCCCTTCAAGACCATAGGT-3′	5′-TGTGCTGGTGAGAGTCTTCCA-3′	Cybergreen	–0.1096	–
1, 2	2 and 5. Unknown 2=5	CB514997	5′-TTGATGACCTCAATGGCTAAACA-3′	5′-CACCTTACCGGTCAATTCTCAAT-3′	Cybergreen	0.0923	–
1, 2, 3	3. *collagen type XI α1*	FJ790236	5′-CGGTCCAGAAGGTCTGCG-3′	5′-TTGTCCAGAAGAACCAGGAAGTC-3′	5′-GGAATCCCTGGC-CCTGTTGGTGA-3′	0.06	Nt 614–625
1, 2	4. Ig-4 domain	BU965641	5′-GCCAGGCATACACACAGCTAGA-3′	5′-GGAGTCTCACCTGGTCTGGTAGA-3′	Cybergreen	–0.008	–
1, 2	5. Unknown 5=2	CK991244			Cybergreen	0.092	–
1, 2	6. Unknown	CA052567	5′-CAGAGAGTACGTGGCAGGTTGTA-3′	5′-TTTGAGACGCAACCGAGATCT-3′	Cybergreen	–0.0098	–
1, 2	7. *Cyto-keratin K19*	CB498541	5′-ACAGAAGTTATCCAAACGTCCAGAT-3′	5′-CGTACGTTTGACTTCTGAGATTTCA-3′	Cybergreen	0.071	–
1, 2	8. Unknown	CA042008	5′-CAATTGCACGAGAGCAGCAA-3′	5′-AAGTGCTTCTTGATTCCTGCATT-3′	Cybergreen	0.081	–

Table 2. Identities and sequence accession numbers for sequences used for phylogenetic analysis (Fig. 3C)

Sequence name in phylogenetic tree	Acc. no. (NCBI)	% Identity with salmon colXI	Protein length	Sequence used for tree (length of sequence)
Salmon ColXI	FJ790236	100	588	588 (588aa)
Zebra fish ColXIα1	NP_001077313	87	1736	1148–1736 (588aa)
Zebra fish ColXIα2	CAK04962	63	1877	1278–1877 (599aa)
Red sea bream ColV/XIα1	BAB03287	76	1820	1227–1820 (593aa)
Skate ColXI/Vα1	BAE16360	73	1860	1268–1860 (592aa)
Chicken ColXIα1	AAA48707	86	888	299–888 (607aa)
Chicken ColVα1	AAF28099	77	1835	1243–1835 (592aa)
Mouse ColXIα1	EDL12408	83	1779	1090–1779 (589aa)
Mouse ColVα1	EDL08374	77	1838	1307–1838 (591aa)
Mouse ColXIα2	Q64739	56	1736	1175–1736 (561aa)
Human ColXIα1	BAD92081	84	1017	428–1017 (589aa)
Human ColVα1	AAH08760	76	599	1–599 (599aa)

Phylogenetic analysis

The search for collagen type XI α1 protein identity between Atlantic salmon and other species was performed with ExPASy BLAST2 using the NCBI BLASTP 2.2.17 program. The following organisms were used for phylogenetic analysis: zebrafish, red sea bream, skate, chicken, mouse and human. The sequences and lengths used for the phylogenetic analysis are listed in Table 2. Phylogenetic analysis was carried out using the CLUSTAL X2 program and NJplot program using the neighbor-joining method (Larkin et al., 2007). The longer sequences were cropped to contain only the region which was identical to the isoform cloned in salmon (Fig. 3B).

In situ hybridization

Salmon vertebrae from 50–100 g salmon were collected and fixed overnight in freshly made 4% paraformaldehyde and 1× PBS. After fixation, the vertebrae were washed four times for 15 min in 1× PBS, followed by decalcification for 8 weeks at 4°C in a solution of 10% EDTA and 0.5 mol l^{-1} Tris-HCl pH 7.5. After decalcification, specimens were washed four times for15 min in 1× PBS before overnight incubation in a solution of 25% sucrose, 1× PBS and 25% Tissue-Tek® (Sakura, Zoetherwoude, Netherlands). Samples were frozen in 100% Tissue-Tek® and kept at –80°C until sectioning. The tissues were sectioned into 14 μmol l^{-1} sections and dried for 2 h at room temperature. *In situ* hybridization was performed according to Krossoy et al. (Krossoy et al., 2009) at a hybridization temperature of 60°C using the DIG labeling reagents (Roche, Oslo, Norway). For *collagen type XI α1* a 513 bp sequence (corresponding to nt 1017–1529; Fig. 3B) was used for *in situ* probes.

Radiology

Radiographs were taken with a portable X-ray apparatus (HI-Ray 100, Eickenmeyer Medizintechnik für Tierärzte e.K., Tuttlingen, Germany) on 30 cm×40 cm sheet film (AGFA Structurix D4 DW ETE, Agfa-Gevaert N.V., Mortsel, Belgium). The film was exposed twice for 50 mAs and 72 kV, and developed using a manual developer [Cofar Cemat C56D, Arcore (MI), Italy] with Kodak Professional manual fixer and developer (KODAK SA, Paris, France). The radiographs were digitized using an A3 positive scanner (Epson Expression 10000 XL, Seiko Epson Corp., Kagano-Ken, Japan).

Statistics

All gene expression data were subjected Kolmogorov–Smirnov tests for Gaussian distribution. None of the gene expressional data was confirmed to have Gaussian distribution and they were thus 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 indicates statistical significance.

RESULTS

Gene expression response to continuous light

Increased expression of eight genes was found in response to continuous light. The accession numbers for these and the spot color in each array are presented in Fig. 1. Each color on the horizontal axis reflects the hybridization signal in each individual comparison. Higher expression in the continuous light group is shown in the first five rows with more red (Fig. 1, numbered 1–5 in red), while the last three rows represent dye-swap comparisons, where the green channel signal indicates overexpression in the continuous light group (Fig. 1, numbered 3–5 in green). Sequence analysis using BLAST (NCBI) revealed that CB514997 and CK991244 turned out to be sequences arising from the same gene, which yielded a total of seven genes for downstream analysis. In order to verify the upregulation of genes in response to continuous light, real-time PCR was performed on samples from experiment 2. Comparison of gene

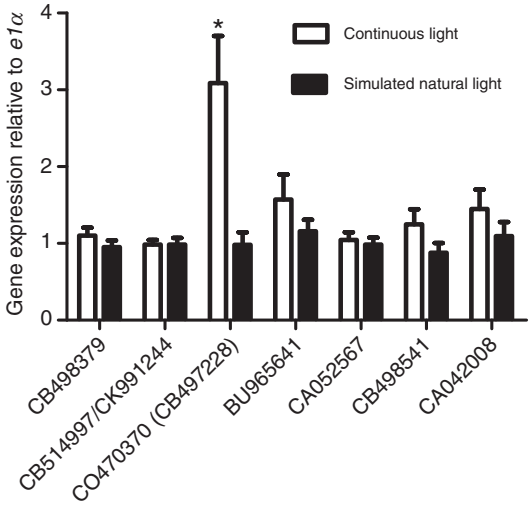
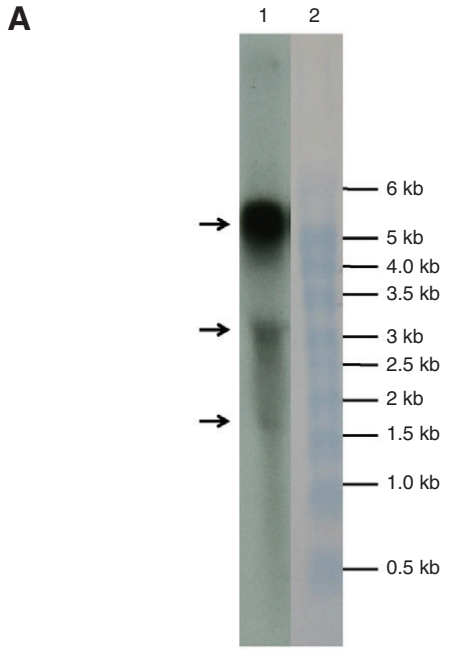
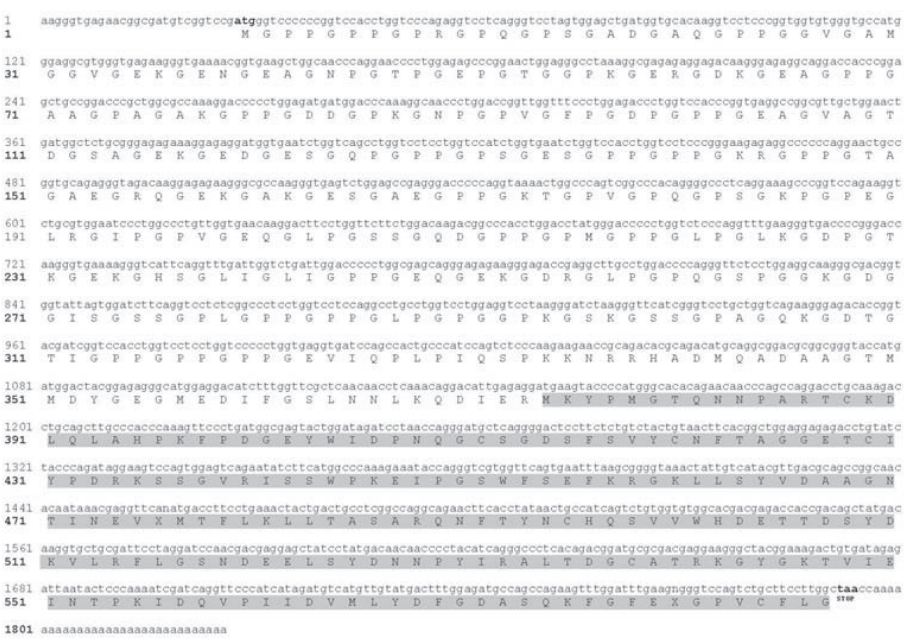


Fig. 2. Candidate gene analysis. Gene expression of eight candidate genes in experiment 2, 3 weeks after the onset of continuous light. Accession numbers of the assayed genes are listed on the x-axis. The abundance of transcripts relative to *elongation factor 1 alpha* is shown on the Y-axis. Simulated natural light means that fish were exposed to a 12 h light and 12 h dark photoperiod. Significant upregulation is indicated by an asterisk (*P<0.05). Data are presented as means ± s.e.m.





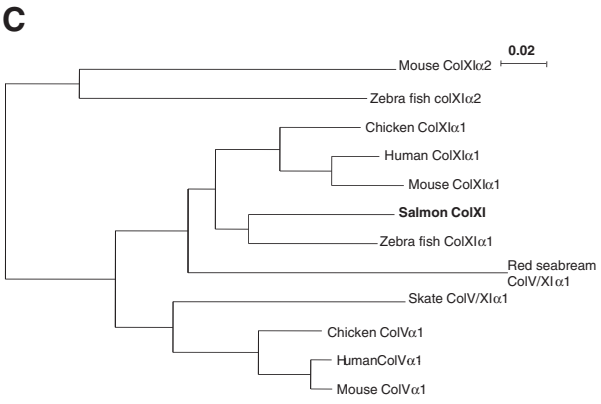


Fig. 3. Northern blot and sequence of an isoform of *collagen type XI α1* in salmon. Northern blot of salmon vertebrae using a probe for *collagen type XI α1* (A). The left lane shows the presence of three variants of the gene (arrows). The right lane shows an RNA ladder. (B) Nucleotide and amino acid sequence of the cloned *collagen type XI α1* in salmon. The translational initiation codon and termination codon are indicated in bold type. Grey shading of amino acids sequence demarcates the location of the conserved collagen C-propeptide domain (acc. no. FJ790236). (C) Phylogenetic tree showing that the cloned and translated collagen type XI α1 (ColXI) clusters together with the collagen type XI α1 proteins identified in zebrafish, chicken, mouse and human (accession numbers listed in Table 2).

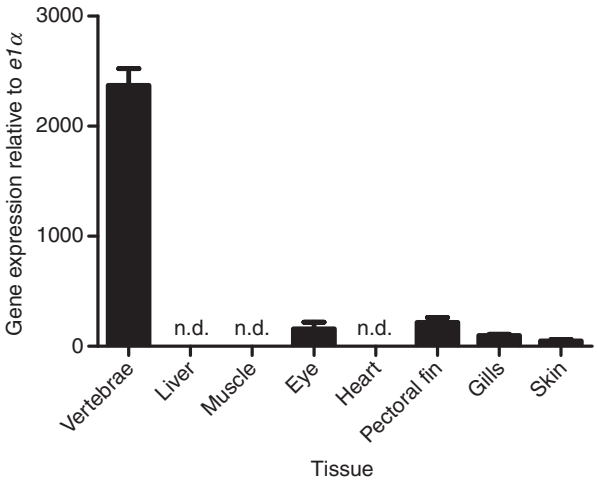


Fig. 4. Tissue expression level of *collagen type XI α1*. Expression of *collagen type XI α1* in selected tissues of salmon. The Y-axis shows the abundance of the transcripts relative to the normalization factor, *elongation factor 1α* (*e1α*). In the liver, muscle and heart no expression was detected (n.d.). Data are presented as means ± s.e.m.

expression recorded in experiments 1 and 2 revealed that one gene was induced in both experiments (P<0.05; Fig. 2). Sequence analysis revealed that the upregulated gene (Fig. 2; acc. no. CB497228) showed a predicted translation into a type XI collagen, as previously identified in rainbow trout. On sequence homology analysis, a homologous sequence was found in Atlantic salmon (97% homology; CO470370).

Number of isoforms and cloning

Northern blot analysis revealed three bands of about 1.8, 3.2 and 5.5 kb (Fig. 3A), which would suggest that *collagen type XI α1* has three variants in Atlantic salmon (Fig. 3A). However, it is not possible to predict this when sequences have not been cloned. One isoform or partial sequence of the gene was cloned and turned out to be an 1826 bp long cDNA which coded for a 588 amino-acid (aa) long protein (Fig. 3B; acc. no. FJ790236). The cloned collagen contained a part of the conserved collagen C-propeptide sequence which is typical of all structural collagens (gray area in Fig. 3B).

Homology searches of the collagen type XI α1 protein revealed that it has the highest identity to collagen type XI α1 in zebrafish, chicken, mouse and human (84–87%; see Table 2). The protein had a lower identity to the intermediate type V/XI collagen α1 chains found in red seabream and skate (73–77%; Table 2), and to Collagen α1(V) chain in chicken and mouse (77%; Table 2). The translated gene displayed 55% identity to the predicted collagen type XI α2 chain in salmon (COL11A2; acc. no. EF210363), and 63% identity to the α2 chain in zebrafish. In order to monitor whether the percentage of identity was reflected in the relatedness of the proteins, a phylogenic analysis was performed, using the homologous part of the salmon sequence. The phylogenetic analysis showed that the translated salmon collagen type XI (acc. no. FJ790236) grouped together with zebrafish, chicken, mouse and human collagen of type XI α1 (Fig. 3C).

Tissue-specific expression

In order to further identify which tissues expressed *collagen type XI α1*, we performed real-time PCR on vertebrae, liver, muscle, eye, heart, pectoral fin, gills and skin. The gene turned out to be highly expressed in the vertebrae but, it was also present in the eye, pectoral fin, gills and skin (Fig. 4). In order to determine which cells expressed *collagen type XI α1*, *in situ* hybridization was performed using the 513 bp sequence, which codes for a part of the conserved C-propeptide (same sequence used for the northern blot, corresponding to nt 1017–1529; Fig. 3B). This probe was selected since it is likely to pick up all the splice variants of the gene, being located in the conserved C-propeptide. In the vertebrae, the gene was found to be expressed in osteoblasts lining the trabecular (TB) and compact bone (CB), and also within the chordoblasts of the notochord (NC; Fig. 5A). Inside the gills, expression was found within the cartilage of the gill arches (GA; Fig. 5B) and in the supportive hyaline cartilage of the gill filament (GF; Fig. 5C). Within the pectoral fin, expression was found in osteoblasts lining the dermal bone of the lepidotrichia (DB; Fig. 5H). Expression was also detected in the dermis (D; Fig. 5I). Inside the eye expression was found in the scleral cartilage (SC; Fig. 5J). In the vertebrae, gill, fin, skin and eye no expression was found using the sense probe for the gene (Fig. 5E–G,K–M).

Compressed vertebrae

Compared with normal vertebrae, *collagen type XI α1* expression was significantly lower in the compressed vertebrae [P=0.02(3) and P=0.05(4); Fig. 6]. When we compared expression in vertebrae with apparently normal morphology that had been sampled cranially and caudally, to that of vertebrae from a deformed region, no differences were detected (Fig. 6B). Gene expression level was normalized to *elongation factor 1 alpha* (*e1α*) (Olsvik et al., 2005).

Comparison of expression of *collagen type XI α1* in the notochord and compact and trabecular bone

Expression of *collagen type XI α1* was 150 and 25 times higher in the notochord and compact bone, respectively, than in trabecular bone (Fig. 7A). In deformed vertebrae there was a significant reduction in expression in compact bone (Fig. 7B; P<0.03, N=3). RNA quantification of one vertebra revealed that trabecular bone contributes 78%, compact bone 15%, and notochord 7% (Fig. 8) of the total RNA pool extracted from one vertebra.

DISCUSSION

Considering the complex effect that continuous light has on the vertebrae (Fjelldal et al., 2005; Nordgarden et al., 2006; Wargelius et al., 2009), the induction of eight genes must be regarded as a low number, a number that was even lower in experiment 2. However, much of the effect may have been masked by the circadian rhythm of bone tissue, and as is known in mouse bone, 26% of the transcriptome exhibits a circadian pattern (Zvonic et al., 2007). Candidate pathways may therefore have a circadian rhythm; for example, bone formation increases during the night in mammals (Pandi-Perumal et al., 2006), a process that would not have been detected by our sampling regime (sampling was performed during the day). Another possibility could be that only one type of bone tissue (either compact bone, trabecular bone or notochord) responds to the light stimuli and if this the case, expressional changes in the notochord and compact bone could have been masked by the substantial contribution of RNA from the trabecular bone (78%; Fig. 8).

The cloned salmon *collagen type XI α1* contained a region that was predicted to code for a part of the conserved collagen C-propeptide, and protein identity searches in other species showed a high resemblance to the collagens type XI and V (Table 2). These

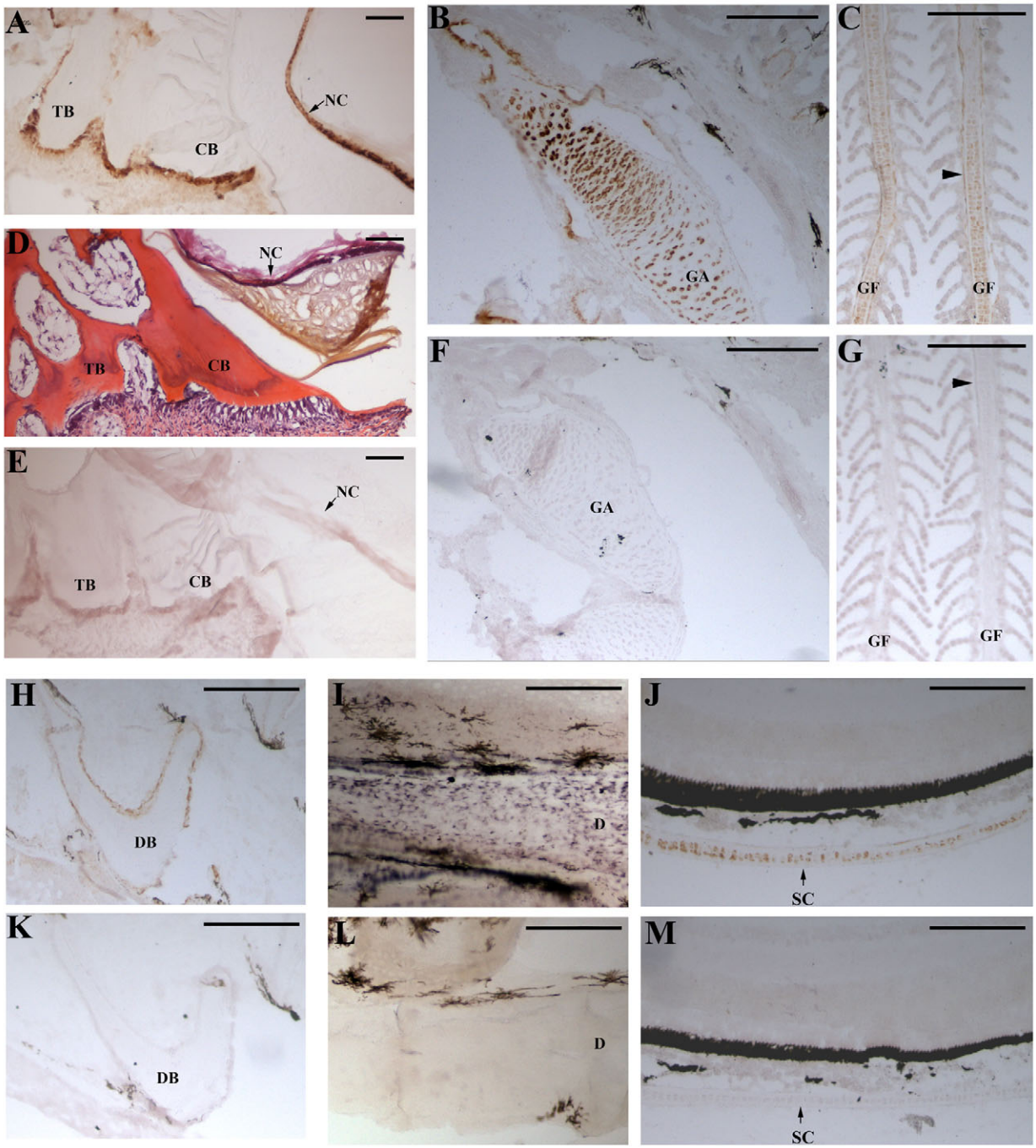


Fig. 5. Spatial expression of *collagen type XI α1* in the vertebrae, gill, fin ray and skin. Tissue-specific expression of *collagen type XI α1* in (A) the vertebrae, (B,C) gill, (H) fin, (I) skin and (J) eye. In the vertebrae (A), expression was limited to the trabecular bone, compact bone and notochordal sheath (arrow). In the gill (B), expression was limited to the chondroid tissue of the gill arch. In the gill filaments (C), expression was restricted to the chondroid tissue of the gill filament (arrowhead). Within the fin rays (H), expression was restricted to the layer of osteoblast surrounding the dermal bone of the lepidotrichia. In the skin (I), expression was limited to the dermis. Within the eye (J), expression was restricted to the scleral cartilage (arrow). (D) Hematoxylin–eosin staining of the vertebrae. (E–G,K–M) The sense probe control on the vertebrae, gill arch, gill filament, fin, skin and eye, respectively. TB, trabecular bone; CB, compact bone; SC, notochordal sheath; GA, gill arch; DB, dermal bone; D, dermis and SC, scleral cartilage. Scale bar, 200 μm.

types of collagens have a unique function during fibrillogenesis as they keep and use their propeptide domain to cause steric hindrance of further growth of the fibril (Blaschke et al., 2000; Wenstrup et al., 2004). Low concentrations of both collagen V or XI thus lead to thicker and fewer collagen fibers (Fernandes et al., 2007; Wenstrup et al., 2004). It has also been shown that a specific amount of collagen XI is required to form thin collagen fibrils (10+4 microfibrils), similar to those found in cartilage (Holmes and Kadler, 2006). In salmon, we detected changes in *collagen type XI α1* expression level, although we did not measure protein level, in response to light in deformed vertebrae; whether these changes in gene expression can affect fibrillar properties is unknown. Within deformed vertebrae, however, there

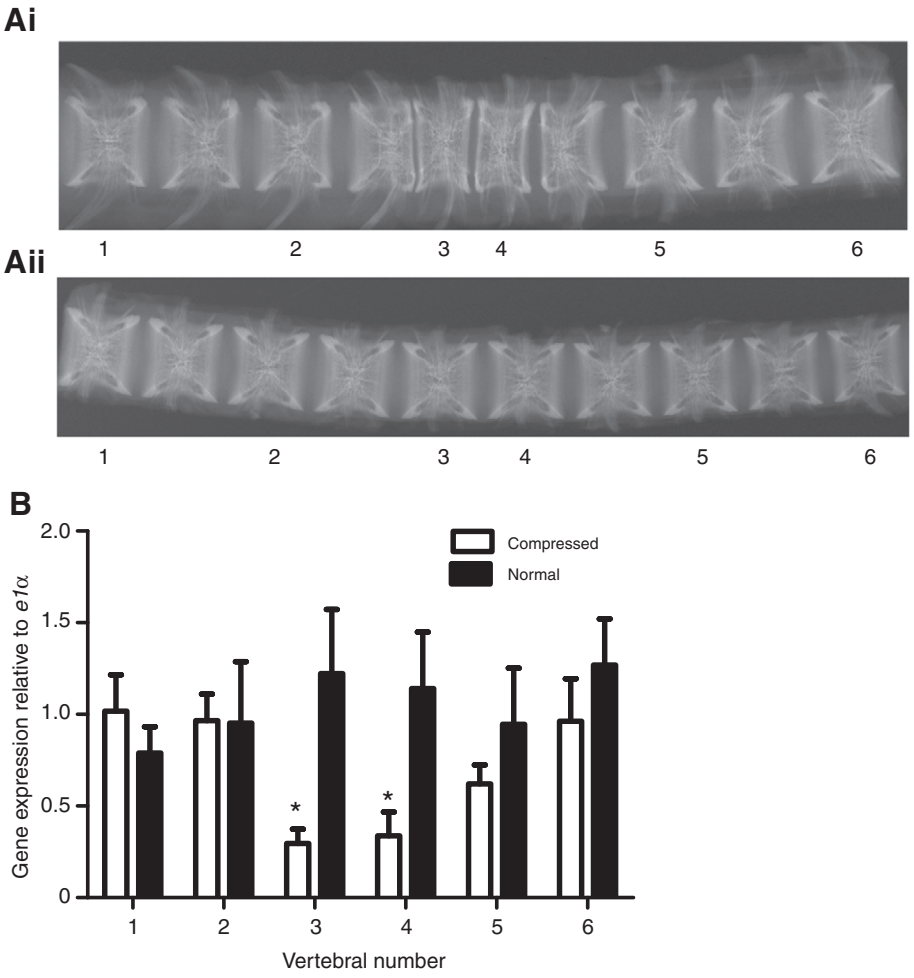


Fig. 6. X-ray and gene expression of *collagen type XI α1* in compressed and normal vertebrae. (A) The two images show compressed (Ai) and normal (Aii) vertebrae. The numbers 1 to 6 under each image indicate the vertebrae that were sampled for RNA. (B) The expression of *collagen type XI α1* in the middle of the deformity and on the edge of the deformity. The expression of type *collagen type XI α1* relative to *elongation factor 1α* is given for each vertebra. Data are presented as means ± s.e.m. Significant differences in gene expression between deformed and normal vertebrae are indicated by an asterisk (*P<0.05).

are severe lesions that affect bone morphology; it is therefore possible that the fibrillar properties are altered.

In mammals, the genes for type V and XI collagens share high nucleotide identity; however, they are differently distributed in tissue, as type *V* and *XI* expression is higher in bony tissue and cartilage, respectively. Likewise, we found much higher expression of the salmon *collagen type XI α1* in the notochord, which is a more cartilage-like tissue. In mammals, *collagen XI* and *V* are closely related, and in the teleost red sea bream an intermediate form between *collagen types XI* and *V* has been detected: *colXI/V α-1* (Touhata et al., 2001). In order to determine which phylogenetic group the salmon *collagen type XI α1* belonged to, we performed a phylogenetic analysis of collagen type V, XI/V and XI, which revealed that salmon collagen XI clustered together with collagen XI α1 of zebrafish, chicken, mouse and human and is probably therefore a collagen type XI α1.

In humans, three splice variants of *collagen type XI α1* have been characterized; A (5421 nt, acc. no. AAF04725), B (5457 nt, acc. no. AAF04726) and C (3054 nt, acc. no. BAD92081) (Annunen et al., 1999). In salmon, we likewise detected three lengths of mRNA on the northern blot using *collagen type XI α1* probes of about 5500, 3200 and 1800 nt in length. We were able to clone only one variant of the gene of 1826 bp (Fig. 3B). However, it is uncertain if this is the short band observed on the northern blots. Phylogenetic analysis revealed that the cloned sequence (FJ790236; Fig. 3B) groups together with the longer isoforms of α1s found in zebrafish, chicken and mouse, further showing that it is probably a splice variant of this gene (Fig. 3C). If this is the case, it is very likely that the gene expression level and spatial distribution of *collagen type XI α1* measured reflects the level of all splice variants as a pool so the possible significance of regulation of splicing has not been analyzed in this paper.

In mammals, the gene for type XI collagens are usually found in adult cartilage, but have also been detected in bone during fetal development. In the developing mouse, the gene was expressed in the vertebrae, in the osteoblasts of the trabecular bone and in the chondroid tissue of the intervertebral disc (Yoshioka et al., 1995). In salmon it was found in the osteoblast of the trabecular and compact bone, even though expression was much higher in notochordal chordoblasts (Fig. 7A). It is uncertain whether the gene expression we observe in the trabecular bone can be compared with fetal expression in the mouse. However, the fish are actively growing during this life phase, which may be more comparable to fetal bone growth than to adult bone remodeling in mouse, where *collagen type XI* is expressed in the skin, limbs and tail, but not in the heart and liver. We likewise identified salmon *collagen type XI α1*-like gene expression in skin and fins, but not in heart or liver. In salmon it was also located in the cartilage and osteoblast-like cells of the gills and in the scleral cartilage of the eye. In the red sea bream an intermediate between *collagen type XI* and *V* exist and this *collagen type XI/V* is expressed in the skin similar to the salmon *collagen type XI α1* we found, but its expression has also been detected in muscle, which is not the case for salmon (Touhata et al., 2001). In mammals, collagen type V protein is present in bone, the interstitial

matrix in muscle, liver, lungs and placenta (reviewed by Gelse et al., 2003). Salmon *collagen type XI α1* was not expressed in muscle or liver. The tissue-specific expression, gene identity and phylogenetic analysis results suggest that the gene identified in salmon is more closely related to the type *XI collagen*.

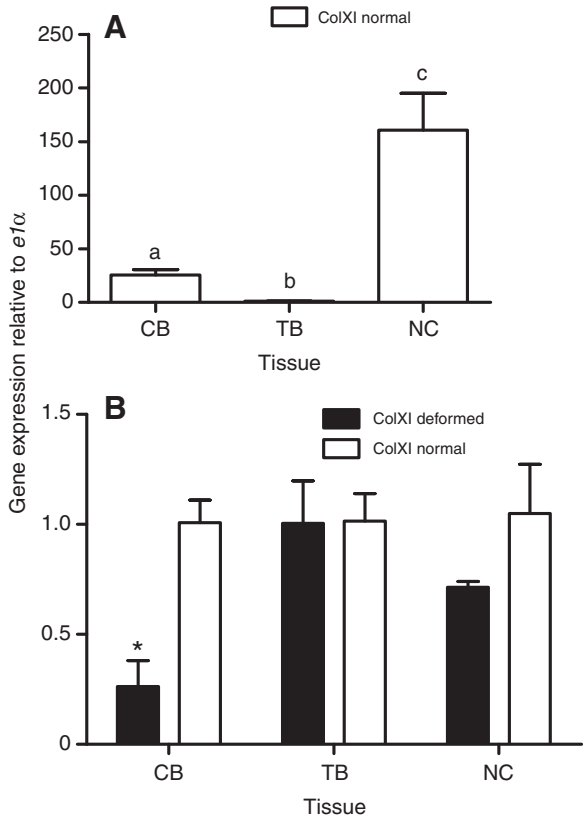


Fig. 7. Expression of *collagen type XI α1* in the compartments of normal and compressed vertebrae. Expression of *collagen type XI α1* (relative to the normalization factor, *elongation factor 1α*) in the compact bone (CB), trabecular bone (TB) and notochord (NC) of normal vertebrae (A) and in each tissue type in normal and deformed vertebrae (B). Data are presented as means ± s.e.m.

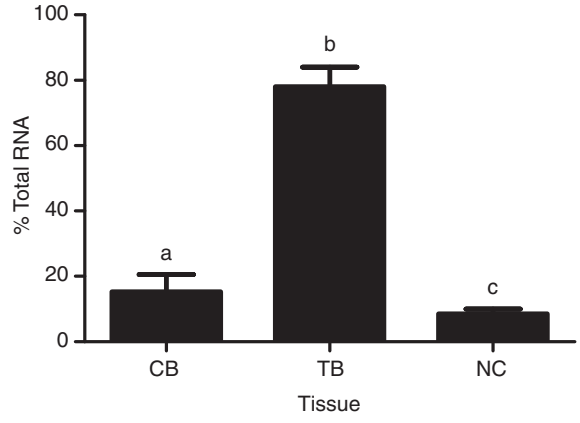


Fig. 8. The percentage of total RNA derived from each compartment of a normal vertebra. Contribution of RNA from the different compartments of a normal vertebra. Abbreviations: CB, compact bone; TB, trabecular bone; NC, notochord. Data are presented as means ± s.e.m.

The etiology of compressed vertebral deformation is unknown. However, the disease is progressive because the number of adjacent compressed vertebra rise over time (Fjelldal et al., 2007b; Witten et al., 2006). In the normal vertebra neighboring the deformed region, no differences in *collagen type XI α1* expression were observed, which indicated that its reduced expression in the compact bone of compressed vertebrae was not related to the etiology of the deformity. However, *collagen type XI α1* expression is reduced in the compact bone after the vertebral compression is manifest. As collagen is the major structural protein of bone, constituting more than 95% of the organic phase, the quality of the extracellular matrix is highly dependent on collagen properties. Since collagen type XI restricts fibril diameter, reduced secretion may lead to higher prevalence of thicker fibrils, which may affect mineralization and mechanical properties. In salmon suffering from vertebral deformations, the expression of *collagen type XI α1* in the notochord tended to be lower, which may reflect a reduction in notochord tissue, since this type of deformity may be due to lesions in the intervertebral joint (Witten et al., 2005; Fjelldal et al., 2007b). In comparison, humans with intervertebral disc degeneration display lower expression of both the gene and the protein of collagen type XI (Mio et al., 2007). Finally, the structure of the trabecular bone in the compressed vertebrae appeared to be normal, and there were no differences in gene expression.

Mammals may involve the same molecular mechanisms in the development and growth of supportive tissues as teleosts (Mari-Beffa et al., 2007). The Wnt pathway, where *collagen type XI α1* participates, plays a key role in skeletal development and in maintaining bone homeostasis (Baron et al., 2006; Glass and Karsenty, 2007; Westendorf et al., 2004). The downstream target of the Wnt pathway, the transcription factor Lef-1, suppresses the expression of *collagen type XI α1* (Galceran et al., 2004). The possibility cannot be excluded that the gene expression levels observed in normal and pathological bone of salmon is regulated *via* the Wnt pathway. If so, the Wnt pathway might also be modulated by photoperiod.

Future studies should include functional characterization of the factors of the Wnt pathway in teleosts, in order to determine whether the pathway is involved in the adaptive remodeling of teleost bone. Furthermore, molecular mechanisms important to teleost bone biology need to be elucidated. This could be done by studying the unique skeletal features of teleosts, such as bone formation through direct ossification, acellular bone and exoskeletal structures (fins, scales).

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