MMP-13 (Matrix MetalloProteinase 13) expression might be an indicator for increased ECM remodeling and early signs of vertebral compression in farmed Atlantic salmon (Salmo salar L.)

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Summary

Vertebral body compression is a common problem in commercial farming of Atlantic salmon. Although risk factors, such as vaccination and malnutrition, have been identified, the etiology is largely unknown. Histological studies of Atlantic salmon have shown that in a compressed deformity (platyspondyly) the length of the compact bone is reduced while the notochord start to form atypical chondrogenic structures. In mammals, similar remodeling activities have been linked to inflammatory processes in the tissue. Hence, we wanted to investigate whether the compressed vertebrae in Atlantic salmon showed presence of local (IL-1\textbeta, TNF-z1), systemic (IgM) and chronic (MMP-13, MMP-9) immune responses (measured with quantitative PCR). Unvaccinated groups of Atlantic salmon that would later develop high or low prevalence of vertebral compression during on-growth in seawater were sampled at seawater transfer, and 3 and 6 weeks after seawater transfer. In addition, compressed and normal vertebrae from the high deformity prevalence group were sampled 44 weeks after transfer to seawater. MMP-13 was significantly up-regulated in the group that developed a high prevalence of deformity, and also significantly up-regulated in compressed vertebrae, 44 weeks after seawater transfer. In compressed vertebrae, MMP-13 was equally up-regulated in the notochord, compact bone and trabecular bone. The results of the present study suggest that MMP-13 may serve as an early indicator for bone remodeling which may lead to vertebral compression, and that there is a relationship between the development of vertebral compression and increased remodeling activities in farmed Atlantic salmon.

Introduction

Vertebral deformities are a common pathology in farmed Atlantic salmon and may affect 11–73\% of the harvest sized fish (Witten et al., 2006; Fjelldal et al., 2009). The most common deformity is vertebral compression (platyspondyly, Witten et al., 2005; Fjelldal et al., 2007). It has previously been suggested that the compressed vertebrae of Atlantic salmon shows infiltration of inflammatory cells in the bone tissue (Kvellestad et al., 2000). Vaccination can increase the incidence of deformities and trigger an autoimmune response (Berg et al., 2006; Koppang et al., 2008). Likewise, bone pathologies in mammals can show presence of inflammatory cells, where a whole cascade of inflammatory cells and factors are observed in the common bone disorder rheumatoid arthritis (RA, Okada, 2009). On the other hand, the mammalian bone disease osteoarthritis (OA) is characterized by bone degeneration and remodeling without presence of inflammatory cells. Inflammatory factors and cells in fish have been characterized, whereas the immune response in fish seems to involve both innate and adaptive response to infection (Koppang et al., 2007). The innate response is characterized by the release of local cytokines at the site of inflammation. Local cytokines in teleosts has been identified upon homology to mammalian, where they have shown to be regulated at the gene expression level, as a direct response to inflammation (Randelli et al., 2008). Long term inflammation can lead to a chronic inflammatory response characterized by both bone tissue degeneration and remodeling. In these cases in the mammalian system it is possible to detect upregulation of matrix metalloproteinases, which form a family of proteins involved in the degradation of the extracellular matrix (ECM, Takaishi et al., 2008). Salmon lice (Lepeophtheirus salmonis) infested Atlantic salmon shows upregulation of both MMP-13 and MMP-9 in the skin (Skugor et al., 2008).

The aim of the present study was to evaluate innate, adaptive and chronic immune responses associated with vertebral compression by measuring gene expression levels of various immune factors such as; Interleukin 1 beta (IL-1\beta, Lindenstrom et al., 2006), Tumor necrosis factor alpha-1 (TNF-z1, Haugland et al., 2007), IgM heavy chain (IgM, Hordvik et al., 1997) and Matrix metalloproteinase 13 and 9 (MMP-13, MMP-9, Skugor et al., 2008) in groups of fish which would later develop high prevalence of deformity and in fish which contained vertebral compression.

Materials and methods

Experimental design

On September 04th 2006, 968 parr with a mean weight of 36 g were randomly allocated between eight square, white and covered indoor tanks (\(1 \times 1 \times 0.6\) m) at the Institute of Marine Research (IMR), Matre (Western Norway), leaving 121 fish per tank. In the period between September 04th and November 27th, four tanks were given 6 weeks with 10\(^\circ\)C freshwater followed by 6 weeks with 10\(^\circ\)C seawater (10–10), while the remaining four tanks were given 6 weeks with 16\(^\circ\)C freshwater followed by 6 weeks 16\(^\circ\)C seawater (16–16). During this period, the photoperiod was 24 h continuous lights, in all tanks. For illumination, two 18 Watt fluorescent daylight tubes (OSRAM L 18W / 840 LUMILUX, OSRAM GmbH, Aisburg, Germany) were used to produce 960 lux measured under water in the centre of the tank. The oxygen saturation in
the outlet water was kept above 75%. The fish were fed (2.0 mm; Nutra Olympic, Skretting AS, Stavanger, Norway) to excess (feeding 24 h per day) by automatical feeders (ARVO-TEC T Drum 2000; Point Four Systems Incorporated, Coquitlam, Canada), regulated by PC operated system (Normatic AS, Nordfjordeid, Norway). On November 30th 2006, all remaining fish were fin clipped according to temperature regime, and transferred to a common sea cage (12 × 12 × 14 m) and reared for a following 36 weeks under a natural photoperiod and ambient temperature. During seagecage rearing, the average temperature between 1 and 10 m of depth varied between 8 and 10°C from November 2006 to mid March 2007, decreased to 6–8°C in mid May, increased to 14°C in late June, and ranged between 14 and 16°C in July and August. During this period, the fish were fed twice a day (Bio Optimal and Classic, BioMar AS, Trondheim, Norway), with increasing pellet size from 5 mm to 12 mm. The salinity in the indoor tanks was kept stable at 34 ppt while in the sea cage the salinity was above 32 ppt below 4 m of depth (total depth of sea cage 15 m).

**Tissue sampling**

Vertebrae (number 40–43) were sampled at transfer to seawater on September 16th 2006, and 3 and 6 weeks after transfer to seawater (n = 9 fish from each temperature, including at least two fish per tank from four replicate tanks). At 44 weeks on September 16th 2006, all remaining fish were fin clipped according to temperature regime, and transferred to a common sea cage (12 × 12 × 14 m) and reared for a following 36 weeks under a natural photoperiod and ambient temperature. The salinity in the indoor tanks was kept stable at 34 ppt while in the sea cage the salinity was above 32 ppt below 4 m of depth (total depth of sea cage 15 m).

**RNA extraction, DNase treatment and cDNA synthesis**

Total RNA was extracted from the samples using FastPrep and TRI reagent® (Sigma-Aldrich Norway AS, Oslo, Norway) according to the manufacturer's instruction. Genomic DNA was eliminated from the samples by RQ DNase I (Promega GmbH, Mannheim, Germany) treatment. RNA samples were stored at −80°C until further analysis. Quantity and quality of the isolated RNA's were assessed by NanoDrop® spectrophotometer (NanoDrop Technologies, Wilmington, DE). Only samples with a 260/280 nm absorbance ratio of 1.8–2.0 were approved. The RNA integrity was evaluated by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) using RNA 6000 Nano LabChip® kit (Agilent Technologies, Palo Alto, CA), and only samples showing no sign of RNA degradation were used. First strand cDNA was reversely transcribed from 250 ng RNA using a Reverse Transcription Core Kit (Eurogentec, Seraing, Belgium).

**Real-time PCR**

Primers for amplification and detection of IgM, MMP-13 and MMP-9 in addition to the control gene elongation-factor alpha (ef1a, Olsvik et al., 2005) were designed using the PRIMER EXPRESS 2.0 software (Applied Biosystems, Foster city, CA), and are listed in Table 1. The real time assays for IL-1β, TNF-α1 was constructed according to McBeath et al., 2007. Real-time PCR was carried out on an ABI 7700 (Applied Biosystems, Oslo, Norway) system using SYBR® Green PCR Master Mix (Applied Biosystems, Foster city, CA), with the following thermal cycling conditions: 50°C for 2 min followed by 98°C for 10 min, and then 40 cycles of 95°C for 15 s followed by 60°C for 1 min. No-template controls (NTC) for each gene were run on each PCR plate. To determine the efficiency of targets in relation to reference (ef1a) we used the standard curve method and a validation experiment described in ABI User Bulletin #2 (ABI 7700 sequence detection system).

**Radiology**

Radiographs were made using a portable X-ray apparatus (HI-Ray 100; Eickenmeyer Medizintechnik für Tierärzte e.K., Tuttlingen, Germany) and 30×40 cm film (AGFA D4 DW ET). The film was exposed twice for 50 mA s and 72 kV (90 cm), and developed using a manual developer [Cofar Cemat C56D, Arcore (MI), Italy] with Kodak Professional manual fix and developer (KODAK S.A., Paris, France). The pictures were digitalised by scanning (Epson Expression 10 000 XL, Seiko Epson Corp., Nagano-Ken, Japan).

**Statistical analysis**

All gene expression data was subjected to Kolmogorov-Smirnov test for Gaussian distribution. None of the gene

<table>
<thead>
<tr>
<th>Gene</th>
<th>Reference, genbank acc.no.</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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</thead>
<tbody>
<tr>
<td>IgM</td>
<td>(Hordvik et al., 1997), Y12457, AY617117</td>
<td>5'-TTGGGAAAATGTCAATGGGAAGA-3'</td>
<td>5'-GTTAGTGTCACATATGCGGAACTTGT-3'</td>
</tr>
<tr>
<td>IL-1β</td>
<td>(Lindenstrom et al., 2006), CAC42769</td>
<td>5'-GGCTGAGGTGAGGAGGAGT-3'</td>
<td>5'-GGCGGCCGCTCCTCAA-3'</td>
</tr>
<tr>
<td>TNF-α1</td>
<td>(Haugland et al., 2007), DQ787157</td>
<td>5'-AGTCTACGGTAGCAGCAA-3'</td>
<td>5'-CGTCAAAGGCTGGTGGAGACGTA-3'</td>
</tr>
<tr>
<td>MMP-13</td>
<td>(Skugor et al., 2008), CA42769</td>
<td>5'-CCTTCGTTCTTGGGCTCTCGAGG-3'</td>
<td>5'-AGTCTACGGTAGCAGCAA-3'</td>
</tr>
<tr>
<td>MMP-9</td>
<td>(Skugor et al., 2008), CA42769</td>
<td>5'-CCTTCGTTCTTGGGCTCTCGAGG-3'</td>
<td>5'-AGTCTACGGTAGCAGCAA-3'</td>
</tr>
<tr>
<td>ef1a</td>
<td>(Olsvik et al., 2005), AF321836</td>
<td>5'-CCTTCGTTCTTGGGCTCTCGAGG-3'</td>
<td>5'-AGTCTACGGTAGCAGCAA-3'</td>
</tr>
</tbody>
</table>

IgM, Immunoglobulin M; IL-1β, Interleukin-1 beta; TNF-α1, Tumour Necrosis Factor-alpha 1; MMP-13, Matrix Metallo Proteinase 13; MMP-9, Matrix Metallo Proteinase 9; ef1a, elongation-factor alpha (reference gene).
expression data were confirmed to have Gaussian distribution and 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). A P value < 0.05 indicates statistical significance.

Results
Prevalence of deformities
In the experiment the prevalence of vertebral deformities was stable over time at approximately 25% in the 10°C group, while it increased to 92% in the 16°C group 44 weeks after transfer to seawater. The dominate type of deformity was vertebral body compression (80%, Fig. 1a), showing chondrogenic tissue in the intervertebral region, instead of notochordal body compression (80%, Fig. 1a), showing chondrogenic tissue in the intervertebral region, instead of notochordal body compression (80%, Fig. 1a), showing chondrogenic tissue in the intervertebral region, instead of notochordal body compression (80%, Fig. 1a), showing chondrogenic tissue in the intervertebral region, instead of notochordal tissue (Fig. 1b).

Gene expression of inflammatory factors in vertebrae from fish groups which will later develop high incidence of bone deformities
IL-1β was not detected in any samples from the vertebrae in the early sea water phase (Fig. 2). For IgM, TNF-α and MMP-9 it was not detected any differences in gene expression over time and in between temperatures. MMP-13 showed a significant increase in gene expression between 6 and 9 weeks in the 16°C group of fish. MMP-13 gene expression was significantly (P < 0.05) higher at 6 and 9 weeks in the 16°C compared to 6 and 9 weeks respectively in the 10°C group.

Gene expression in a compressed deformity
Gene expression in compressed contra normal looking vertebrae was measured in the middle of deformity, numbered 3 and 4 in Fig. 3a, in the outskirts of the deformity (2 and 5) and on the outside of the deformity (1 and 6, vertebrae numbered from anterior to posterior, n = 3 control vertebrae and n = 4 compressed vertebrae). In these samples, gene expression was measured and neither TNF-α or IL-1β could be detected in any of the samples (Fig. 3b). For IgM, no differences could be detected along the vertebral compression (1–6) or between normal and compressed vertebrae. MMP-13 showed significantly higher expression in the vertebrae 1, 2 and 3 of the compressed deformity compared to the control vertebrae (P < 0.05), while there were no differences between the posterior vertebrae (4, 5 and 6).

Gene expression in the compartments of a compressed deformity
To determine which major compartment of the vertebrae contributed most to the increase in MMP-13 expression, dissected samples of compressed and normal vertebrae were sampled from the same fish (n = 3 fish). Vertebrae were dissected into notochord (NC), compact bone (CB) and trabecular bone (TB). Gene expression analysis showed no difference between control and deformed fish in any if the tissues concerning IgM, TNF-α1or MMP-9 while IL-1β could not be detected (Fig. 4). The dissected samples all showed a higher MMP-13 expression in all measured compartments of the vertebrae (P < 0.05).

Discussion
In this study MMP-13 gene expression was upregulated in fish groups kept at 16°C around smoltification. These results suggest that there is higher ECM turnover in fish kept at higher temperatures during smoltification and at early sea water phase. It is possible that increased ECM matrix turnover in early sea water phase could contribute to the high prevalence of bone deformities one year later at slaughter. Increased MMP-13 gene expression does not explicitly mean that remodeling is occurring at higher rate since it is only the gene expression and not the protein activity we are measuring. However increased MMP-13 gene expression is found in most mammals displaying arthritis which then in turn have increased bone remodeling and ECM degradation (Takashi et al., 2008). Atlantic salmon skin shows an increased MMP-13 expression over time, in response to salmon lice infestation (Skugor et al., 2008), further implying that the response we see

Fig. 1. X-ray (a) and histology (b) of compressed Atlantic salmon vertebrae from fish from the 16°C incubation group after 44 weeks in sea water. The vertebrae shown (a,b) were taken from the region in the middle of the deformity (numbered 3, 4 according to Fig. 3). (a) X-ray of the compressed deformity, with the area used for histology (b) indicated by the red box. (b) shows a haematoxylin eosin stained sagittal section through the same two vertebrae shown in (a). (c) Haematoxylin eosin stained sagittal section of a normal vertebra displaying both notochord (N) and notochordal sheath (NS). Scale bars: 2.5 mm (a, b in white), 1 mm (c in black) and Abbreviations: CB, compact bone; *C, chondrocytes which has replaced normal notochordal tissue in the compressed deformity; TB, trabecular bone; N, notochord; NS, notochordal sheath.
Fig. 2. Gene expression of IgM, TNF-α1, IL-1β, MMP-13 and MMP-9 relative to the normalisation gene elongation-factor alpha (ef1α) in vertebral bone of Atlantic salmon. The figure shows gene expression 6, 9 and 12 weeks after temperature treatment started (10 and 16°C). The time points represents sea water transfer (SWT, 6 weeks), 3 weeks after SWT (9 weeks) and 6 weeks after SWT (12 weeks). The x-axis presents the time, and the temperature group and gene used are indicated above the bars. The y-axes show the abundance of transcripts relative to the reference gene (ef1α). n.d. = not detectable. Data are given as mean ± standard error of mean (SEM), n = 7–9 samples per bar, as indicated in the box below the IgM bars. The letters a, b and c demarcates significant differences in gene expression (P < 0.05).

Fig. 3. (a) X-ray picture showing compressed (above) and normal (below) vertebrae, 44 weeks after sea water transfer in a fish group which had been exposed to 16°C for 12 weeks around smoltification. (a) shows numbering of the vertebrae where 3 and 4 represents the middle of the compression, 2 and 5 the outskirts of the compression and 1 and 6 the outside of the compression. Scale bars (white): 7 mm. (b) Relative expression of IgM, TNF-α1, IL-1β and MMP-13 in relation to elongation-factor alpha (ef1α), which was measured in numbered vertebral bodies from normal (n = 3, control) and compressed vertebrae (n = 4, def.). The x-axis indicates the vertebral position (1–6), with the type of tissue (control, def) and gene used indicated above bars. The y-axes show the abundance of transcripts relative to the reference gene (ef1α). Data are given as mean ± standard error of mean (SEM). * indicates significant differences in gene expression (P < 0.05) between control (vertebrae 1–6) and def. vertebrae. Abbreviations: def = compressed vertebrae, and n.d. = not detectable.
in vertebral bone is leading to ECM degradation and remodeling. None of the other markers of inflammation showed expression changes in response to temperature. Neither IL-1β nor TNF-α1 could be detected in any of the samples around smoltification. This implies that there is no primary immune response in the aetiology of the compressed vertebrae. However both cytokines are induced as a short-time primary response to infection in salmonids (Tafalla et al., 2005; Mulder et al., 2007) and it is possible we missed such a primary response window. Due to the tetraploidy of Atlantic salmon it is feasible that another isoform could be involved in a primary inflammatory response in bone. There was a constant low level of IgM expression in both temperature groups in the early sea water phase (16 and 10°C), which suggests no sign of increased antibody production and presence of adaptive immune in the vertebrae. Immunized salmon can have signs of autoimmunity which in mammals could affect the joints, and this could be a possible link to deformities in Atlantic salmon (Koppang et al., 2008). In Atlantic salmon it is a link between time of vaccination and the prevalence of deformities (Berg et al., 2006). In this experiment the fish were not vaccinated but still showed a high prevalence of compressed deformity at slaughter. These results suggest that the aetiology of bone deformities can be multifactorial, where both temperature and vaccination could be among the risk factors.

In rat osteoblasts the transcription of MMP-13 is directly driven by the parathyroid hormone (PTH) pathway (Selvamurugan et al., 2006; Boumah et al., 2009). PTH is involved in bone resorption and linked to calcium and phosphorus homeostasis in mammals. The function of PTH in fish is somewhat uncertain since fish lack the parathyroid gland. But recent studies in fish have revealed expression of this hormone in the pituitary gland, nerve tissues and ion-exchange tissues (gill tissues, Guerreiro et al., 2007). These studies have linked the hormone to calcium and phosphorus balance in fish. It may be possible that the gene expression changes we observe in MMP-13 could be linked to a requirement for calcium and phosphorus in the fish.

MMP-13 was induced in the compressed vertebrae of Atlantic salmon, while no other marker measured was affected (IL-1β, TNF-α1, IgM and MMP-9). In mammals the presence of MMP-13 has been related to both OA and RA, where RA is characterized by presence of inflammatory cells in addition to increased MMP-13 activity. We could not detect any changes in IgM expression in the compressed deformities or any obvious infiltration of inflammatory cells seen from the histological sections of the deformity (Fig. 1). It might be possible that vertebral compressions could be linked to OA, which is a bone degenerative disease with an aetiology which has been associated with micro-cracks and mechanical load (Takaishi et al., 2008). It has previously been suggested that the compressed vertebrae occurrence in Atlantic salmon could be linked to changes in mechanical load (Witten et al., 2005). Perhaps increase in MMP-13 expression during smoltification can result in changes in both structure and mineral content which might lead to microcracks and mechanical load changes in the vertebrae.

To conclude, increased MMP-13 expression could be linked to both development and presence of compressed vertebrae in Atlantic salmon. Also the presence of MMP-13, but no other inflammatory markers measured could possibly link the compressed deformity to a type of teleost osteoarthritis (OA).

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MMP-13 and ECM remodeling in salmon


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