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# Local immune depression in Baltic cod (*Gadus morhua*) liver infected with *Contracaecum osculatum*

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**Research Paper**

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## Abstract

Third-stage larvae of the anisakid nematode *Contracaecum osculatum* infecting cod (*Gadus morhua*) liver elicit a host immune response involving both innate and adaptive factors, but the reactions differ between liver and spleen. Inflammatory reactions occur in both liver and spleen, but a series of immune effector genes are downregulated in liver infected with nematodes whereas these genes in spleen from the same fish are upregulated. A series of novel primer and probe sets targeting cod immune responses were developed and applied in a real-time quantitative polymerase chain reaction set-up to measure the expression of immune-relevant genes in liver and spleen of infected and uninfected cod. In infected liver, 12 of 23 genes were regulated. Genes encoding cytokines associated with inflammatory reactions (IL-1β, IL-6, IL-8) were significantly upregulated, whereas genes encoding effector molecules, assisting the elimination of pathogens, C-reactive protein (CRP)-PII, hepcidin, lysozyme G1, lysozyme G2, C3 and IgDm, were significantly downregulated. The number of downregulated genes increased with the parasite burden. In spleen, 14 of 23 immune genes showed significant regulation and nine of these were upregulated, including genes encoding CRPI, CRPII, C3, hepcidin and transferrin. The general gene expression level was higher in spleen compared to liver, and although inflammation was induced in nematode-infected liver, the effector molecule genes were depressed, which suggests a worm-induced immune suppression locally in the liver.

## Introduction

Parasitic nematodes within the family Anisakidae are parasites of invertebrates and vertebrates, and occur worldwide (Mattiucci *et al*., 2015; Gay *et al*., 2018; Shamsi, 2019). During the latest decades, third-stage larvae of one species within the family, *Contracaecum osculatum*, have become increasingly common in Baltic cod, a substock of the Atlantic cod (*Gadus morhua*). This benthopelagic fish plays a central role in the Baltic sea ecosystem, where it is divided into two populations occurring west and east of the island of Bornholm in the Southern Baltic (Sick, 1965). The final host of *C. osculatum* is the grey seal, *Halichoerus grypus*, and following a marked population expansion east of Bornholm during the latest two decades, the infection of *C. osculatum* with parasite larvae in eastern Baltic cod has increased to high levels (Eero *et al*., 2015; Zuo *et al*., 2018). The infection levels in the western Baltic, the Sound and Kattegat are still low (Sokolova *et al*., 2018). Small invertebrates (such as copepods and amphipods) and fish (sprat, herring, sand eel, cod) act as transport hosts carrying third-stage larvae (Køie & Fagerholm, 1995). It has been discussed to what extent the infection may influence Baltic cod (Haarder *et al*., 2014; Eero *et al*., 2015; Buchmann & Mehrdana, 2016; Zuo *et al*., 2016). The cod liver, the target organ of the parasite, is an important element in the fish immune system, and excretory-secretory (ES) compounds released by *C. osculatum* larvae regulate immune reactions in zebrafish and rainbow trout (Bahlool *et al*., 2013; Mehrdana *et al*., 2017), which suggests that the infection may affect cod immunity. The cod genome lacks certain immune genes, including CD4 and major histocompatibility complex (MHC) II (Star *et al*., 2011), and it is, therefore, worthwhile to elucidate how the cod immune system is stimulated by *C. osculatum* infection. We have performed a comparative study of immune gene regulations in cod with and without infection, and here we describe a differential immune response in liver and spleen of the host. Due to limited previous studies on the cod immune system we had to develop new tools for the work, and for this purpose we developed a series of novel real-time quantitative polymerase chain reaction (RT qPCR) tools (primer and probe sets).

## Materials and methods

### Fish and sampling

Twelve live infected Baltic cod, caught by local fishermen located along the eastern coastline of the Bornholm island (high infection area), were brought ashore (Nexø, Bornholm island, Denmark)



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and stocked in fish tanks (volume = 8 $m^3$) supplied with running saltwater from the Baltic (salinity = 8 ppt; temperature = 10°C). For comparison, 12 live uninfected cod, caught by a local fisherman along the coastline of Zealand (low infection area), were brought to the fish-keeping facility at the Blue Planet Aquarium (Kastrup, Denmark) and kept in similar fish tanks. Following seven days of acclimatization, cod were anesthetized by MS222 (cat. no. A5040, Sigma-Aldrich, Denmark) immersion (300 mg/L), euthanized by a subsequent blow to the head and dissected (Buchmann, 2007). Tissue samples from liver and spleen were aseptically sampled from each fish and immediately placed into 1.5 mL tubes containing RNAlater (cat. no. R0901, Sigma-Aldrich, Denmark), pre-stored at 4°C for 24 h and then stored at −20°C until further processing for gene expression analysis. Livers were subsequently examined for infection by nematode larvae using the glass plate compression method with light microscopic examination (magnification × 4–40) (Leica MZ125, Leica, Denmark), while the rest of the cod was examined for other parasites (Buchmann, 2007).

### Identification of isolated parasites

Nematode larvae isolated from cod livers were transferred to 96% ethanol, whereafter identification to the genus level was performed based on morphological characters (Fagerholm, 1982). Species and subspecies identification was performed by molecular methods (PCR and sequencing of rDNA and mitochondrial gene *cox*2) according to Zuo *et al.* (2018).

### Ribonucleic acid (RNA) extraction and reverse transcription

Liver and spleen samples were removed from RNAlater and cut into small pieces and processed in TissueLyser II (Qiagen, USA). Total RNA was extracted using the GenElute™ total RNA kit (cat. no. RTN350, Sigma-Aldrich, Denmark). The extracted RNA was subsequently treated with deoxyribonuclease (DNase) (DNase I, cat. no. EN0521, Fermentas, Denmark) to remove genomic DNA contamination. RNA concentrations and purity were measured spectrophotometrically on a NanoDrop reader (Saveen & Werner ApS, Denmark) and the integrity and purity of RNA were examined by 1.5% agarose gel electrophoresis. The RNA was stored at −80°C. For cDNA production, RNA was reversely transcribed using random hexamers (TaqMan® Reverse Transcription, cat. no. 4311235, Applied Biosystems, Denmark). Reactions were performed in a T100™ Thermo Cycler (BioRad, USA) with 20 µl reaction volumes each containing 1000 ng of RNA. The reactions were carried out under the following conditions: 25°C for 10 min, 37°C for 60 min and 95°C for 5 min. A volume of 20 µl of resulting cDNA was diluted ten times by adding 180 µl of ribonuclease (RNase)-free water (cat. no. 10977, Invitrogen, Denmark) and stored in −80°C until further analysis.

### Design of primer and probes

We designed new primers and probes due to the limited availability in the literature of probe-based qPCR assays for immune-relevant genes in the Atlantic cod, *G. morhua*. A series of TaqMan probe qPCR assays were designed, including four reference genes (actin-related protein 2, elongation factor 1$\alpha$ (EF-1 α), ribosomal protein L4 (RPL4), ubiquinone), using the Internet-based tool Primer3Plus (Untergasser *et al.*, 2007). Table 1 presents the sequences of primers, probes, GenBank accession numbers and references. A melting curve analysis was performed using Brilliant III Ultra-Fast SYBR QPCR MM (cat. no. 600882, AH diagnostics, Denmark) for the exclusion of primer combinations forming primer/dimers and for specificity confirmation in combination with 3% ethidium bromide-stained agarose gel electrophoresis. Based on the dilution series of templates and analysis of the cumulative fluorescence curves using the software LinRegPCR version 2014.6 (Ruijter *et al.*, 2009), all assays were demonstrated to have efficiencies within 100% ± 5%, which makes it relevant to apply the simplified $2^{-\Delta\Delta Cq}$ method (Schmittgen & Livak, 2008) for analysing the relative gene expression. All the qPCR assays performed well at an annealing temperature of 60°C.

### RT qPCR

RT qPCR assays were performed using an AriaMx Real-Time PCR system (Agilent technology, USA). The cDNA was used as a template for qPCR reactions with a primer and probe designed for particular genes (table 1). Reactions were run in ready-made master mix (Brilliant® II QPCR master mix, Stratagene, USA) with 5.5 µm magnesium chloride concentration. A 12.5 µl setup was used: 6.25 µl of Brilliant® II QPCR master mix (Agilent Stratagene, USA), forward primer and reverse primer (0.8 µM each), TaqMan probe (0.4 µM), 1.75 µl DNase/RNase-free $H_2O$ and 2.5 µl of cDNA template. The cycling conditions were 94°C for 10 min followed by 40 cycles of 94°C for 10 s and 60°C for 15 s. Control (without template) and reverse transcriptase minus negative control were run for each assay. RT qPCR was used to monitor the changes in the expression of different immune-relevant genes of Baltic cod following infection with *C. osculatum*. The regulation of genes encoding the following molecules were examined: Cytokines (interleukin (IL)-1β, IL-6, IL-10, IL-12, IL-22, IL-8 and IFN-γ), immunoglobulins (IgMs, IgDm), acute-phase proteins (hepcidin, C-reactive protein (CRP)-PI, CRP-PII and transferrin), complement factor C3, antimicrobial peptides (cathelicidin), lysozyme (lysozyme G1 and lysozyme G2), neurotransmitter signalling (S100A), toll-like receptor (TLR3), hematopoietic factors (GATA-binding protein 3 (GATA-3; full length), GATA-3 sv (splice variant)), peptide presentation (MHC I) and signal transducer and activator of transcription 1 (STAT1). EF-1 α, actin-related protein-2 (ARP-2), RPL4 and ubiquitin (ubi) genes were used as reference genes.

### Data analysis

For the relative expression of candidate genes, the $2^{-\Delta\Delta Ct}$ method was used (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008). The data were normalized for each gene against an average of the four applied reference genes (encoding EF-1 α, ARP-2, RLP4 and ubi). The normalized gene expression data for infected and uninfected groups were compared at each time point using the Student's *t*-test. Data were considered significantly different when $P < 0.05$ and fold change was at least 2. The data were presented as the mean of fold increase/decrease at each time point. Gene expression was analysed with one-way analysis of variance using GraphPad Prism version 6.00 for Windows (GraphPad Software, Inc., La Jolla, CA, USA).

## Results

### Infection of cod

Cod caught along the east coast of the island Bornholm were all infected by third-stage *C. osculatum* larvae in the liver, whereas

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**Table 1.** Primers and probes used for RT qPCR.

| Gene | GenBank acc. no. | Primers and probe, 5′ end to 3′ end | Reference |
|---|---|---|---|
| ARP-2 | EX741634 | Fwd: TCTGCTCCGTGTGGAAGTTG<br>Rev: CGAGAAGATCCTCTGCCACAA<br>Probe: TGCGCTTCATGATGATGAGAGCGGA | Olsvik *et al*. (2008)<br>Probe: this study |
| C3 | AY739672 | Fwd: AGTGGGAAACTACGCACCTT<br>Rev: TCGACCAACTTTCTGTGCAT<br>Probe: TGCGAGCTATCTCATCATGGGCA | Audunsdottir *et al*. (2012) |
| Cathelicidin isoforms 1, 2 and 3 | EU707291<br>KJ831369<br>KJ831350 | Fwd: AGATGATCCCGGACCCCTTT<br>Rev: ATTGCTGTCTGGGCATGGAA<br>Probe: ACTTCCGCCCCCTGCTGGACCA | This study |
| CRP-I | ES240144 | Fwd: AGATGATCCCGGACCCCTTT<br>Rev: ATTGCTGTCTGGGCATGGAA<br>Probe: ACTTCCGCCCCCTGCTGGACCA | This study |
| CRP-II | ES771793 | Fwd: GGCTACTCGCACCCGTATAA<br>Rev: CATGTGCCACAGATGGAGAC<br>Probe: CGTGGCTGATTTCCCCGAGC | This study |
| ELF 1α | EF093581 | Fwd: AAAGGGAAGCGTGAGGTCATC<br>Rev: CGCGCCACACGTTGAGT<br>Probe: CGCACGCGGGACTTCGACG | Olsvik *et al*. (2008)<br>Probe: this study |
| GATA-3 | HQ596576 | Fwd: CGGTATCCTCAAGCCCAACA<br>Rev: GTCAGAGACTCGTGGTG<br>Probe: CGCCCCCGCCAACGTCACCA | This study |
| GATA-3sv | HQ596575 | Fwd: AGCATGGCATCTATCGGAGC<br>Rev: CGGGGTCGACGTAGCTC<br>Probe: TGCCCACCACCCCATCGCCACA | This study |
| Hepcidin | EU334514 | Fwd: AGCATGGCATCTATCGGAGC<br>Rev: GTAATGTAAATAAAGCGCACGCC<br>Probe: TGCCCACCACCCCATCGCCACA | This study |
| IFNγ | FJ356235 | Fwd: GAGGTGGAGGAGGTGACAAG<br>Rev: GACAGCAGTTGCAACACCAG<br>Probe: CATTCGAGGCAGAAGAGGCA | This study |
| IgDm | AF155203 | Fwd: GGGTCCTTCTTCCCCAGAGA<br>Rev: CTGGGTTCTCCTCAGCAGTG<br>Probe: TCAGCCGCCAACCGACAGCCA | Seppola *et al*. (2009)<br>Probe: this study |
| IgMs | X58870 | Fwd: TGGAGTAACGCTGTGGATGG<br>Rev: TGCTTTGTCGGAGCAAATAGTC<br>Probe: TGCGTTTCTTGTCCTGACTGAAAGCCA | This study |
| IL-6 | JF309111 | Fwd: ACTCGTCTGCTCGACCAATG<br>Rev: GGAGACAGCAGATCTCAGCG<br>Probe: ACGCTGACACGTATCAGGCGGCCC | This study |
| IL-8 | AJ535731 | Fwd: TGACGCACGTGAAGAAGGAG<br>Rev: TGCGGTGACCTTCCTTTGAA<br>Probe: GCGCCAGCTCACTCGTGTCTGCCC | This study |
| IL-10 | EU004087 | Fwd: CTTCTACCCCGCGGACATTT<br>Rev: ACACTCTGCCGTCTTTCCAA<br>Probe: ACGGCGACTTGTTTTCCACTTATGGCC | This study |
| IL-12 | EX188149 | Fwd: GTGCAACAACCAGTGCTGTC<br>Rev: GTCTTGGCGCAGTCTCCTAA<br>Probe: TCGTGGAGGGATCCCCGGTCAGG | This study |
| IL-1β | EU007443 | Fwd: TGCGCACACACAGGATTTTG<br>Rev: TCGTACTTCCCCCTGGTCTC<br>Probe: TGTTTCGGCCCGTGCAGTTTCCACA | This study |
| IL-22 | FM207988 | Fwd: CCACGGACCATGAAGTGAGT<br>Rev: GTGCTGATGTACCAACCGGA<br>Probe: GCGCCAGCTCACTCGTGTCTGCCC | This study |
| Lysozyme G1 | EU377606 | Fwd: GGAGATCATTCCGGCCAATTCACACTG<br>Rev: CCGTCCTTTTCAGAGTGGCA<br>Probe: GGAGATCATTCCGGCCAATTCACACTG | Larsen *et al*. (2009)<br>Probe: this study |
| Lysozyme G2 | AY614594 | | |

(*Continued*)

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**Table 1.** (*Continued.*)

| Gene | GenBank acc. no. | Primers and probe, 5′ end to 3′ end | Reference |
|---|---|---|---|
| | | Fwd: GGGAATTAAAGAGTTCAAGCCAATCTC<br>Rev: ACGTTTCTACCTGCGTGATGTC<br>Probe: CCCTACAGAATGAATTCC | Larsen *et al.* (2009)<br>Probe: this study |
| MHC I | AF414216 | Fwd: GAAATCACAACTTCTTCTGCAAGGAAT<br>Rev: ACGTTTCTACCTGCGTGATGTC<br>Probe: CCGTACCCCATTTTAG | This study |
| RPL4 | EX725958 | Fwd: TGGTGTGCCATGCTACAGG<br>Rev: GTCGTGGTTGGGGAGGAC<br>Probe: GGCCAGGAGCTCCATGAGCAGGTG | Olsvik *et al.* (2008)<br>Probe: this study |
| S100A1 | EL612304 | Fwd: GGTGCCATACAGCTGATCCA<br>Rev: CCAGGCATCACACTGCAGAA<br>Probe: CGGAAAGCGCTCTCGGTCC | This study |
| STAT1 | HM046443 | Fwd: CGCGTCCAAGGATAGCAAGA<br>Rev: CGGGGTCCTTCTGAGACTTG<br>Probe: GCTTAGCCGGCGGGAGCTGAGAG | This study |
| TLR3 | ES476110 | Fwd: CATGGAGGAGGGGAATGCAG<br>Rev: CGTCGGTCAGTCGGTCATTA<br>Probe: CCTGGAGCAGTTCGTCAGTGTGCCC | This study |
| Transferrin | L40370 | Fwd: GACGCTGTGATCCTGGTCTT<br>Rev: CAGCTCTTCACTGGGGACAAT<br>Probe: GGGGCATGCTGCAGCTGCGC | Audunsdottir *et al.* (2012) |
| Ubi | EX735613 | Fwd: GAGCTCCCATCGACAGCTAC<br>Rev: CAAACCCAGCAGAGGAGAAG<br>Probe: CACGCTGTGGTTAGCCGCGT | Olsvik *et al.* (2008)<br>Probe: this study |

All the primers in these qPCR assays have annealing temperature at 60°C.
ARP-2, actin-related protein-2; C3, complement factor 3; CRP-I, C-reactive protein PI; CRP-II, C-reactive protein PII; ELF, 1α elongation factor 1α; GATA-3, GATA-binding protein 3; GATA-3sv, GATA-binding protein 3 (splice variant); IFNγ, interferon; IgDm, immunoglobulin δ (membrane-bound); IgMs, immunoglobulin μ (secreted); IL, interleukin; MHC I, major histocompatibility complex I; RPL4, ribosomal protein L4; S100A1, S100 calcium-binding protein A1; STAT1, signal transducer and activator of transcription 1; TLR3, toll-like receptor 3; Ubi, ubiquinone.

the cod from Zealand were all uninfected. The cod were grouped into four infection categories: uninfected, low infection, medium infection and high infection (table 2). The cod were also examined for other parasites, but no other anisakid nematode species were found. In all cod examined, low intensities of the intestinal acanthocephalan *Echinorhynchus gadi* and the digenean *Lepidapedon elongatum* in the pyloric caeca were detected.

### Overall expression in liver and spleen

The expression of 23 immune-related genes in *C. osculatum*-infected Baltic cod was quantified using qPCR analysis and compared to expression in uninfected cod (fig. 1). When the overall expression of genes was analysed (all time-points), it was seen that 14 out of 23 genes showed higher expression in spleen compared to liver. Only nine genes (including the innate immune genes STAT 1, hepcidin, CRP-PI, CRP-PII, lysozyme G1, lysozyme G2, C3, TLR3 and transferrin) were expressed at a higher level in the liver (supplementary table S1).

### Significant regulation of immune genes in infected cod

Following infection, it was found that a total of 16 genes were regulated compared to uninfected cod. Of these were 12 genes (52.17% of all tested genes) in liver and 14 (60.86%) in spleen (fig. 2). A significant upregulation of genes encoding inflammation-associated cytokines IL-1β, IL-6 and IL-8 in both liver and spleen of Baltic cod was recorded in infected cod when compared to uninfected cod. Th2-associated genes, such as the regulatory cytokine IL-10 gene, were significantly downregulated in liver but upregulated in spleen, whereas other Th2-associated genes (encoding transcription factors GATA-3 and GATA-3sv) were significantly downregulated in both organs. The expression of the gene encoding IL-22, which is associated with the Th17 pathway in mammals, was significantly downregulated in both liver and spleen when compared to uninfected cod. Similarly, we noted downregulation of the gene IFN-γ, which is associated with the Th1 pathway in mammals, in both liver and spleen. With regard to genes encoding effector molecules with direct activity towards invading microorganisms, it was found that lysozyme G1 and G2 genes were significantly downregulated only in the liver. Immunoglobulin IgD (membrane-bound, IgDm) was significantly downregulated in both liver and spleen but the IgMs gene was not regulated. A significant upregulation of genes encoding acute-phase proteins CRP-PI, CRP-PII, hepcidin, transferrin and complement component 3 (C3) was observed in spleen. In contrast, in liver no significant changes in the expression levels for CRP-PI and transferrin genes were seen, and the expression of CRP-PII, hepcidin and C3 genes was significantly downregulated. No significant changes in the expression of the genes encoding TLR3, STAT1, S100A, antigen processing and peptide presentation (MHC I) and antimicrobial peptides (cathelicidin) were recorded in either the liver or spleen.

### Immune gene expression related to infection intensity

Infection was associated with increased expression, both in liver and spleen, of inflammatory genes such as those encoding IL-1β, IL-6 and IL-8, but several effector molecule genes were

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**Table 2.** Number of cod with different parasite intensities (number of *Contracaecum osculatum* larvae per cod liver).

| Number of fish | Number of *Contracaecum osculatum* larvae | Body length | Nematode infection category | *Echinorhynchus gadi* | *Lepidapedon elongatum* |
|---|---|---|---|---|---|
| 12 | 0 | 28–37 cm | Uninfected | + | + |
| 4 | 1–34 | 30–40 cm | Low | + | + |
| 4 | 35–64 | 30–45 cm | Medium | + | + |
| 4 | >65 | 35–45 cm | High | + | + |

*Echinorhynchus gadi* (acanthocephalan in intestinal lumen) and *L. elongatum* (digenean in pyloric caeca) were present at low levels in all examined cod (+). No other nematodes than *C. osculatum* were recovered.

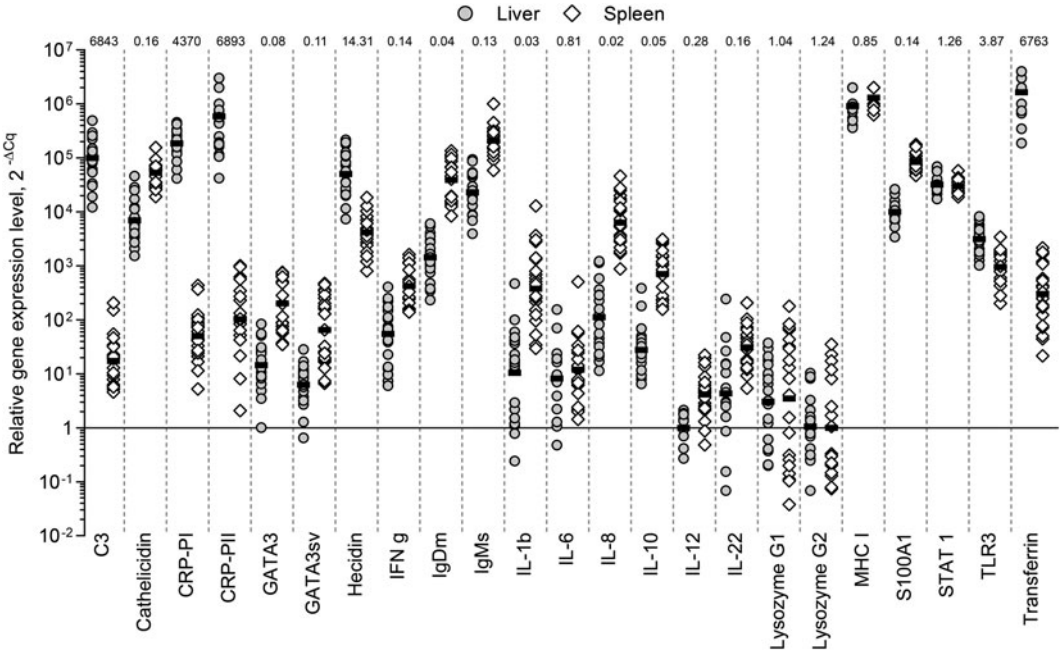


**Fig. 1.** Overall gene expression levels in the liver and spleen of Baltic cod based on all recordings, including infected and control groups. The expression levels were calculated as $2^{-\Delta Cq}$ and normalized to the least expressed gene, IL-12 in liver and lysozyme G2 in spleen. At the top of the graph, the ratio between the expression level in liver and spleen is shown for each gene.

highly downregulated in cod liver when the parasite burden increased (fig. 3). Livers carrying a low, medium and high infection showed downregulation of four, six and nine genes, respectively. At all infection levels, genes encoding CRP-PII, lysozyme G1, interferon γ (IFNγ) and IL-22 were downregulated. Further, two genes (C3, hepcidin) were depressed in the medium infection, and in the most heavily infected cod, IL-10, lysozyme G2 and GATA-3 genes also became depressed. In spleen, several of these genes were upregulated and the expression levels of genes encoding transferrin, CRP-PI, CRP-PII, C3, IL-10 and hepcidin were high at all infection levels, whereas genes encoding IgDm, GATA-3 and GATA-3sv were downregulated. Genes encoding MHC1, S100 calcium-binding protein A1, TLR3, STAT1, IL12, cathelicidin and IgMs remained stably expressed in both organs (fig. 3).

## Discussion

*Contracaecum osculatum* is a parasitic anisakid nematode, which in its larval stage often establishes a long-lasting infection in the fish host despite the presence of a marked cellular response (Buchmann, 2012; Buchmann & Mehrdana, 2016). Survival of this larval stage is a prerequisite for allowing reproduction of the species as it must be transferred to the final host by predation in order to reach the adult reproductive stage in the seal stomach (Mehrdana *et al.*, 2017). The larva will, therefore, benefit from depression of host immunity and it is noteworthy that ES-compounds from this nematode regulate immune reactions in zebrafish (Mehrdana *et al.*, 2017). Baltic cod is the natural host and obtains significant liver infections (Nadolna & Podolska, 2014; Horbowy *et al.*, 2016; Sokolova *et al.*, 2018; Zuo *et al.*, 2018), but it is not known how the parasite and the cod interact at the molecular level. We have conducted a comparative study on infected and uninfected cod – with special focus on the differential expression of immune genes in different organs of the host – and the present study contributes to our understanding of how the cod immune system copes with *C. osculatum* larvae in the host spleen and liver. Due to limited previous focus on cod immune responses and, thereby, lack of available molecular methods, we developed new tools based on the available cod genome sequences (Star *et al.*, 2011; Tørresen *et al.*, 2017), comprising 18 novel primer/probe sets and five novel probes for existing primer sets for use in our RT qPCR. We have shown that genes encoding molecules involved in the inflammation and expulsion

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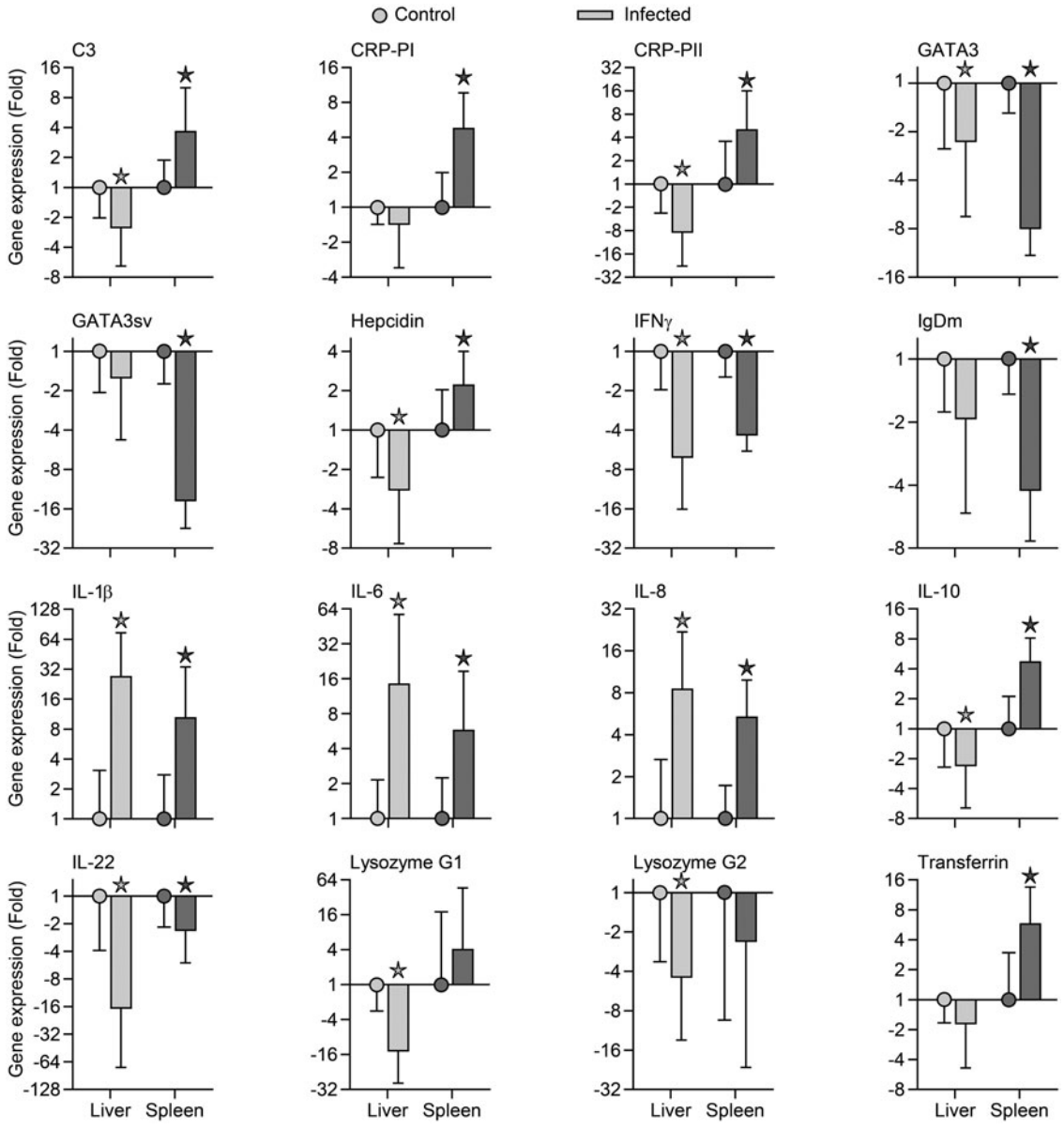


**Fig. 2.** Expression of immune-related genes in cod infected with *Contracaecum osculatum* third-stage larvae in relation to uninfected cod. Only significantly regulated genes ($P < 0.05$) are shown. Details on fold changes for each gene are provided in supplementary file Table S1.

of pathogens (effector molecules) are differentially expressed in liver (reflecting the local response) and spleen (reflecting the systemic response) of the infected host. The immune genes upregulated in the affected liver were associated with inflammation (IL-1β, IL-6, IL-8) and the downregulated genes were mainly involved in the expulsion of pathogens (lysozyme, CRP, IgD, C3, hepcidin, IL-22). This may be interpreted as a parasite-related switch of the host immune response, but it does not adhere to the general concept of Th1/Th2 switching seen in many other helminth–host systems (Artis, 2006; Buchmann, 2012). One of the reasons for this may be found in the unique genome of Atlantic cod, which lacks central immune elements, allowing this switch to be affected. Cod lack genes encoding CD4 and MHCII (Star *et al.*, 2011), making the classical switch impossible, and it may be hypothesized that cod apply other pathways, as seen in this work, allowing the survival of worm larvae in cod.

### *Host genetics and other infections as influential factors*

The two fish groups examined in the present study belong to two subpopulations east and west of the island of Bornholm in the Baltic Sea (Sick, 1965). This is not optimal, but due to the high infection pressure east of Bornholm it was not possible to obtain uninfected cod from the eastern Baltic. The different origin of the two fish groups may, consequently, represent a bias, but in the present study we compared the expression of genes in two organs of the same host and the uninfected cod group was only used as a reference. In addition, it cannot be excluded that parasites other than *C. osculatum* larvae in the examined fish may influence the expression of the immune genes in question, but the relatively small cod examined were only infected by a few intestinal acanthocephalans and digenean pyloric parasites, which are regarded of minor importance in this context.

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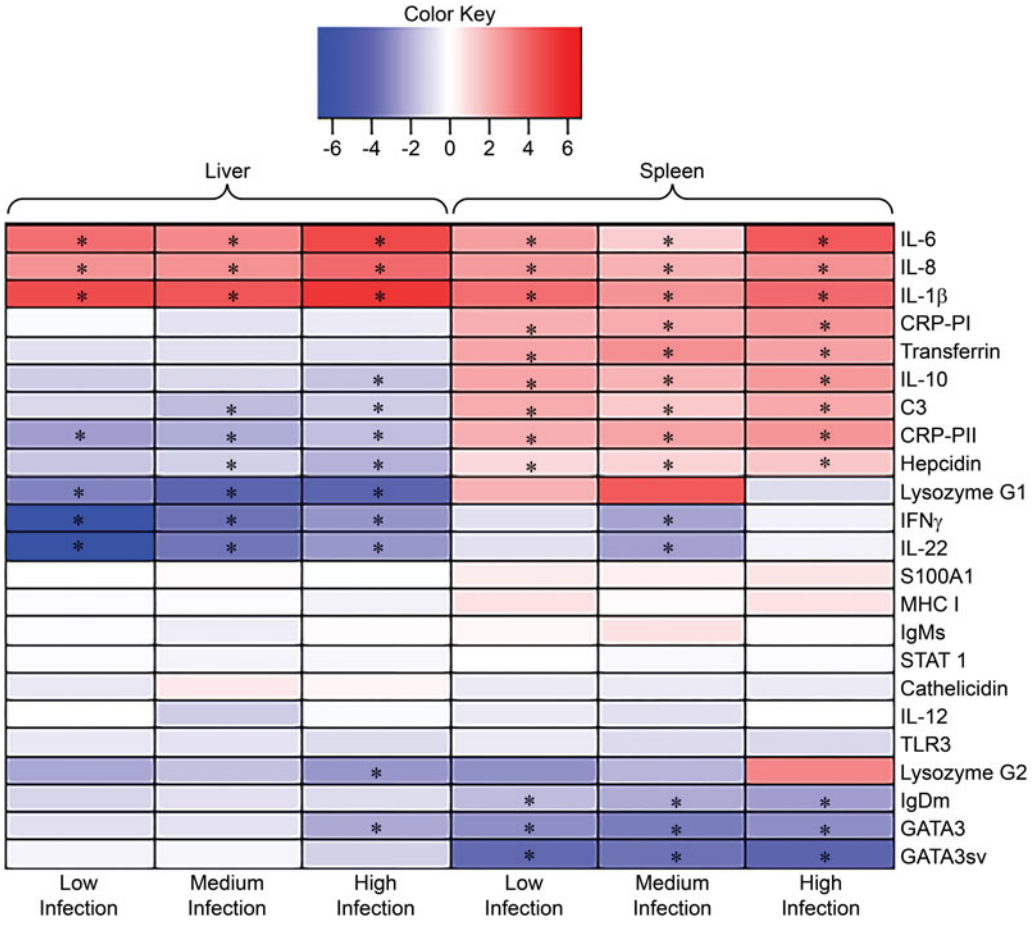


**Fig. 3.** Heat map analysis showing the relative gene expression of immune-associated genes in Baltic cod carrying different loads of *Contracaecum osculatum* nematode larvae compared to uninfected fish. Red and blue shading indicates upregulation and downregulation, respectively.*Gene expression is significantly different from the uninfected group ($P < 0.05$).

### *Immune gene expression in liver with and without infection*

Infection was found to be associated with the increased expression of proinflammatory cytokines both in liver and spleen. First, IL-1β, a cytokine stimulating phagocytosis, lymphocyte activation, migration of leucocytes and regulation of MHC (Zou & Secombes, 2016), was one of the first to be expressed, as in other infections (Dinarello, 1997; Bird *et al.*, 2002; Huising *et al.*, 2004). This was associated with the increased expression of the inflammatory cytokine IL-6 assisting immunoglobulin synthesis, haematopoiesis, T-cell differentiation, acute-phase reaction and neuro-endocrine processes (Øvergård *et al.*, 2012; Norris *et al.*, 2014; Grayfer *et al.*, 2018; Schett, 2018). The significantly upregulated IL-8 expression in liver and spleen, probably co-expressed with IL-1β (Seppola *et al.*, 2008), acts as a chemotactic stimulus for neutrophils to the site of infection (van der Aa *et al.*, 2010). The activation of these genes indicates that the cod's immune system, both locally and systemically, recognizes the presence of a foreign element and, subsequently, activates various granulocytes.

Only a few cytokine genes were downregulated in liver, but the expression of the gene encoding the cytokine IL-22, associated with the Th17 response in mammals, was significantly downregulated. This may be of importance for the host integrity as it has been described that IL-22 deficient zebrafish embryos exhibit high mortality rates (Costa *et al.*, 2013). In addition, the loss of effector molecule production upon nematode infection is noteworthy. Lysozyme is produced by neutrophils/phagocytes and is a central molecule in the innate fish defence system (Jia *et al.*, 2018), and the downregulation of genes encoding lysozyme G1 and G2 in infected cod liver may contribute to increased pathogen survival.

The complement system is one of the main mechanisms to ward off any infection as part of the innate immune response as well as assisting the adaptive response (Magnadóttir, 2006; Alvarez-Pellitero, 2008). We saw a significantly downregulated C3 gene expression in the infected liver, implying a lowered innate immune response and decreased possibility for expulsion of the invading larva. Acute-phase protein genes are involved in acute-phase response (APR) to injury, trauma or infection (Magnadottir *et al.*, 2011; Audunsdottir *et al.*, 2012) and take part in local reactions such as increased accumulation of neutrophils and macrophages, permeability of blood vessels, systemic reactions and stimulation of fibroblasts (Bayne & Gerwick, 2001; Gruys *et al.*, 2005). The CRP-PI and CRP-PII in cod are pentraxins involved in APR (Gisladottir *et al.*, 2009) and the downregulation of both genes may increase survivability of *C. osculatum* larvae in the liver. Hepcidin, a liver-expressed antimicrobial peptide involved in APR (Park *et al.*, 2001; Álvarez

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et al., 2016), is involved in the innate immune response of fish against a variety of infectious agents. Iron sequestration is one of the main functions whereby the downregulation of the hepcidin gene in infected Baltic cod liver may positively affect parasite survival by improving better pathogen access to iron in the liver (Kovacevic et al., 2015).

The membrane-bound immunoglobulin IgDm, part of the adaptive immune system, was also found to be downregulated in infected cod liver. It may be speculated that a lowered level may affect parasite survival locally in the liver as antibodies are generally able to bind invading pathogens and assist expulsion. The observation complies with a previous study showing the decreased expression of another immunoglobulin, IgM, in the liver of *Anisakis* ES-injected rainbow trout (Bahlool et al., 2013).

### Immune gene expression in spleen with and without infection

A series of immune genes was upregulated in the spleen of infected cod – contrasting with reactions in the liver – and it suggests that the observed immune suppression by the nematode mostly acts at the local level in the liver. Thus, while immune genes were depressed locally in the parasite microhabitat – the liver – the immune level was elevated systemically. Following invasion by a nematode larva, basic immune cells recognize the pathogen and transmit signals to central immune organs, such as the spleen, which may subsequently assist a systemic response. The key function of the IL-10 gene, which is often associated with IL-1β gene expression, is to regulate the inflammatory response at the transcriptional level (Seppola et al., 2008; Iyer & Cheng, 2012), protecting hosts from exaggerated inflammatory and immune reactions (Matsumoto et al., 2018). In mammals, IL-10 suppresses nitric oxide production in macrophages and cytotoxic activity of CD8+ T cells by inhibiting IFN-γ (Ito et al., 1999), and it is noteworthy that we found a corresponding IL-10 gene upregulation concomitantly with decreased IFN-γ expression in both spleen and liver.

We also showed upregulation of hepcidin and transferrin in the spleen, suggesting that the cod's immune system has recognized an infection and applies these APRs to protect itself via the regulation of iron distribution (Ellis, 2001; Langston et al., 2001; Solstad et al., 2008; Dietrich et al., 2010; Lü et al., 2012). In line with the general response in spleen, the cathelicidin genes were also active in signalling an elevated innate response (Maier et al., 2008). It is noteworthy that two variants of the GATA-3 gene were downregulated in spleen of infected cod. This molecule is part of a family of six zinc-finger transcription factors (GATA-1 to GATA-6) (Zheng & Flavell, 1997), promoting Th2 in mammals (Zhu et al., 2006). Increased expression of the GmGATA-3S gene (splice variant) after phorbol myristyl acetate (PMA) injection in cod was previously described by Chi et al. (2012). The downregulation in the liver and spleen of *C. osculatum*-infected cod of both GATA-3 (full length) and GATA-3sv (splice variant) suggests that ES compounds of the nematode may depress the expression of this gene, as was seen in zebrafish (Mehrdana et al., 2017). Due to a lack of CD4 and MHCII genes in cod (Star et al., 2011), the role of GATA variants and a possible association with Th2 responses in this host needs further clarification.

In conclusion, the infection of *C. osculatum* in cod liver induced a basic inflammatory reaction both locally in liver and systemically (as reflected by the spleen expression). It is noteworthy that a series of genes encoding important effector immune molecules (CRP, C3, lysozyme, hepcidin and IgD) involved in pathogen expulsion were downregulated in the infected liver. In addition, the more parasites recorded in the liver, the more genes became downregulated. In contrast, corresponding genes (encoding C3, CRP, hepcidin, transferrin) were upregulated in the spleen of the same fish. It is suggested that worm products released at the infection site in the liver induced a level of immune gene suppression.

**Supplementary material.** To view supplementary material for this article, please visit https://doi.org/10.1017/S0022149X19001111.

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**Ethical standards.** The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

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