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Transcriptomic analysis of Baltic cod (*Gadus morhua*) liver infected with *Contracaecum osculatum* third stage larvae indicates parasitic effects on growth and immune response

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ABSTRACT

High infection levels due to third-stage larvae of the anisakid nematode *Contracaecum osculatum* have been documented in cod from the eastern part of the Baltic sea during the latest decades. The nematode larvae mainly infect the liver of Baltic cod and prevalence of infection has reached 100% with a mean intensity up to 80 parasites per host in certain areas and size classes. Low condition factors of the cod have been observed concomitant with the rise in parasite abundance suggesting a parasitic effect on growth parameters. To investigate any association between parasite infection and physiological status of the host we performed a comparative transcriptomic analysis of liver obtained from *C. osculatum* infected and non-infected cod. A total of 47,025 predicted gene models showed expression in cod liver and sequences corresponding to 2084 (4.43%) unigenes were differentially expressed in infected liver when compared to non-infected liver. Of the differentially expressed unigenes (DEGs) 1240 unigenes were up-regulated while 844 unigenes were down-regulated. The Gene Ontology (GO) enrichment analysis showed that 1304 DEGs were represented in cellular process and single-organism process, cell and cell part, binding and catalytic activity. As determined by the Kyoto Encyclopedia of Gene and Genomes (KEGG) Pathways analysis, 454 DEGs were involved in 138 pathways. Ninety-seven genes were related to metabolic pathways including carbohydrate, lipid, and amino acid metabolism. Thirteen regulated genes were playing a role in immune response such as Toll-like receptor signaling, NOD-like receptor signaling, RIG-I-like receptor signalling and thirty-six genes were associated with growth processes. This indicates that the nematode infection in Baltic cod may affect on molecular mechanisms involving metabolism, immune function and growth.

1. Introduction

Baltic cod, a common name for the substock of the Atlantic cod *Gadus morhua* (Linnaeus 1758) inhabiting the Baltic Sea, is an economic commodity on the international market and high focus is placed on quality and health of the stock. It is known that Baltic cod may act as host for a variety of parasites, but a few of these have exhibited a marked increased occurrence during recent decades [1–3]. The anisakid nematodes of the genera *Anisakis, Pseudoterranova* and *Contracaecum* have been recorded previously in the Baltic cod [4,5] but especially infection with third-stage larvae of *Contracaecum osculatum* has increased recently [1–3]. The prevalence of infection of third-stage larvae of *C. osculatum* has reached 100% in certain areas and the mean intensity more than 80 parasites per host [6]. It has been hypothesized that the parasite, due to its location in a central organ, may affect the physiological status, general health and survival of the Baltic cod [7] and it is noteworthy that declining fitness, increasing mortality and reduced growth of Baltic cod [2,3,8,9] has been recorded concomitantly with the increased infection pressure.

Third stage larvae of *C. osculatum* reside mainly in liver of Baltic cod and the parasite load is positively correlated with fish size [7]. The liver has multiple functions in metabolism [10], detoxification [11], and immune regulation [12,13] and it is therefore hypothesized that the infection may directly influence liver function with secondary effects on metabolism, growth and immunity. However, systematic investigations of the Baltic cod liver in response to parasite infection, focusing on overall pathways relevant to metabolism, immunity and growth of the host, have not been conducted. Recent development of a sequenced and annotated genome of Atlantic cod [14] has paved the way for functional studies and studies on liver transcriptomics of cod exposed to

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environmental pollutants [15,16]. Next generation high-throughput RNA sequencing technology (RNA-Seq) permits genome-wide transcriptomic analysis at a higher resolution and this technology has been applied to highlight several differentially expressed genes (DEGs) and pathways involving parasite infections in other fish species [17–20]. In the present investigation we have combined these approaches and conducted a comparative RNA-Seq study to elucidate and characterize gene expression in liver of Baltic cod infected or not infected by *C. osculatum*. The results will contribute to understanding of parasite-host interactions in Baltic cod and possible patho-physiological effects of this anisakid nematode larva.

2. Materials and methods

2.1. Fish and sampling

Samples for transcriptomic analyses were collected from cod caught by local fishermen. Infected cod: Live cod (ten specimens) obtained by trawling along the coastline of Bornholm were brought ashore (Nexø, Bornholm island, Denmark) and stocked in fish tanks (volume 8 cubic meter) supplied with running saltwater from the Baltic (salinity 8 ppt, temperature 10 °C). Non-infected cod: Live cod (ten specimens) were caught on the coastline of Zealand and stocked in similar fish tanks at the fish keeping facility (Blue Planet, Kastrup, Denmark). After one week of acclimatization in the facilities cod were euthanized by MS222 (tricaine methane sulphonate) immersion (300 mg/L) and a subsequent blow to the head whereafter dissection was performed [21]. Stomach content analysis showed that all cod from both areas had been feeding. Liver samples were immediately recovered and preserved in 1.5 mL tubes containing RNAlater (Sigma Aldrich, USA), pre-stored at 4 °C for 24 h and then stored at −20 °C until further processing for RNA purification and transcriptome library preparation analysis. Following sampling for transcriptomic analysis livers were recovered and the number of third stage larvae counted in each fish [3]. In addition, full examination of a subsample of cod was performed to evaluate if other parasite types could influence the results [21].

2.2. RNA extraction, library construction, and sequencing

Liver samples were transferred to lysis solution with 2-mercaptoethanol and homogenized using TissueLyser II (Qiagen, USA). Total RNA of each sample was isolated using RTN350 (Sigma-Aldrich), according to the manufacturer's protocol and subsequently DNase treated with DNase I (Thermo Scientific, USA). Quality and integrity parameters of the total RNA were confirmed on an Agilent Bioanalyzer 2100 total RNA Nano series II chip (Agilent, Amstelveen, Netherlands) and using 2% agarose gel electrophoresis with ethidium bromide (EtBr) staining. Illumina RNAseq libraries were prepared from 500 ng total RNA using the Illumina TruSeq™ Stranded mRNA LT Sample Prep Kit according to the manufacturer's protocols (Illumina Inc. San Diego, CA, USA). All RNAseq libraries (150–750 bp inserts) were sequenced on an Illumina HiSeq2500 sequencer as 1 × 50 nucleotides single-end reads according to the manufacturer's instruction. Image analysis and base calling were performed using the Illumina pipeline.

2.3. Transcriptomic data analysis

Reads were initially aligned to the Atlantic cod reference genome https://figshare.com/articles/Transcript_and_genome_assemblies_of_Atlantic_cod/3408247 [22] using TopHat (version 2.0.5) [23]. To exclude secondary alignment of reads, the resulting files were filtered using SAMtools (version 0.1.18) [24]. Aligned fragments per predicted gene were counted from SAM alignment files using the Python package HTSeq (version 0.5.3p9) [25] for statistical comparison of gene expression levels between groups. To make comparisons across samples possible, these fragment counts were corrected for the total amount of sequencing performed for each sample. We employed library size estimates determined using the R/Bioconductor (release 2.11) package DESeq [26] as a correction scaling factor. Read counts were normalized by dividing the raw counts obtained from HTSeq by its scale factor. Correction for false positives is included in the statistical analysis of gene expression through DESeq. The cut-off for significance was set to adjusted $p < 0.05$ and at least 2-fold change. However, due to the relatively limited annotation found in this database we applied a zebrafish platform. Thus, by using a similar strategy Gene Ontology (GO) annotation and KEGG analysis were performed by DAVID Bioinformatics Resources 6.8 (https://david.ncifcrf.gov) using the official gene symbol from the BLASTx results against zebrafish proteome from ensembl via ftp://ftp.ensembl.org/pub/release-96/fasta/danio_rerio/pep/link. The annotation result was categorized with respect to Biological Process, Molecular Function, and Cellular Component. Fisher Exact test with p-value < 0.05 as a threshold was used to select significant GO categories and KEGG pathways. Manual literature reviews were also performed in order to gain an overview of gene pathway networks, especially with regard to metabolism, immune-related pathways and growth of Baltic cod.

2.4. Quantitative real-time PCR

RNA-seq data were validated by quantitative real time PCR (qRT-PCR) using four selected genes including complement factor c3 (c3), protein crp p1, protein crp p2, and immunoglobulin D (igd). Real time PCR assays were performed using an AriaMx Real-Time PCR system (Agilent technology, USA). The cDNA was used as a template for qPCR reactions with primer and probe designed for particular genes (Supplementary Table S1). Reactions were run in ready-made master mix (Brilliant® II QPCR master mix, Stratagene, USA) with 5.5 µM $MgCl_2$ concentration. In order to verify that only one product was produced, and no primer dimer was formed all qPCR assays were assessed by SYBR Green qPCR assay and subsequent melting curve analysis. 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. The qRT-PCR data were calculated using $2^{-\Delta\Delta Ct}$ [27] method with the average of four reference genes consist of elongation factor 1α (ef-1α), actin-related protein-2 (arp-2), ribosomal protein L4 (rlp4) and Ubiquitin (ubi) as the internal control.

3. Results

3.1. Number of third stage Contracaecum osculatum in Baltic cod liver

All Baltic cod from the east coast of the Bornholm island, Denmark (infected group) were infected with third stage larvae *C. osculatum* (100% prevalence) with intensities from 37 to 74 parasites per fish. The infection level was positively correlated to fish liver size (Spearman rank correlation coefficient 0.65) (Table 1). No *C. osculatum* larvae were found in cod from the coastline of Zealand (non-infected group). Low infections of the trematode *Lepidapedon elongatum* (pyloric caecum

Table 1
Number of third stage *Contracaecum osculatum* nematode larvae in Baltic cod livers of different size.

Number of fish (n)	Weight of Baltic cod liver (g)	Mean number of parasites per liver (± SD)
6	20–40	37.1 ± 21.4
2	41–60	52.0 ± 4.2
2	61–80	74.5 ± 3.5

lumen) and the acanthocephalan *Echinorhynchus gadi* (intestinal lumen) were found in both groups.

3.2. Sequencing and annotation of prediction proteins

A total of twenty cDNA libraries were constructed based on total liver RNA from ten non-infected and ten infected cod. The sequenced transcriptome of each library consisted of an average of 17,133,731 raw reads with a single-read 50-nt run. The raw data mapped to the reference genome was approximately 60% (10,188,558) for each library. The reads have been deposited in the NCBI GEO repository under accession number GSE125868. Detailed information on data quality and mapping statistics are presented in Table S2. A total of 47,025 predicted genes models showed expression in cod liver. Of these were 12,053 (25.63%) found to represent distinct sequences of unigenes annotated to the zebrafish proteome databases using BLASTx algorithm with a cut-off-E-value $\leq 1.0 \times 10^{-5}$.

3.3. Differentially expressed genes

A total of 2084 (4.43%) unigenes showed significant differential expression in the liver with the criteria of adjusted p-value < 0.05 and the absolute value of fold changes greater than 2. Among the differentially expressed unigenes (DEGs), 1240 unigenes were up-regulated while 844 unigenes were down-regulated in parasite-infected liver samples (Fig. 1). Moreover, a total of 313 (15.02%) DEGs were unknown genes, 198 of which were up-regulated whereas 115 were down-regulated.

3.4. Gene Ontology

The possible functions of DEGs were determined using the Gene Ontology (GO) classification system. Distributions of DEGs were annotated at level 1 to 51 functional groups. In total 1304 out of 2084 DEGs could be assigned to functional groups, 1121 (53.79%), 1065 (51.10%), and 1002 (48.08%) of which were assigned to the GO domains molecular function, biological process, and cellular component, respectively. GO enrichment analysis of the DEGs indicated that 22, 17 and 12 GO terms in the biological process, cellular component, and molecular function, respectively, were enriched (Fig. 2). A large number of putative unigenes were annotated in the cellular process (883 unigenes), single-organism process (809 unigenes), and cell (761 unigenes). Other prominent functional groups included the categories cell part (756 unigenes), binding (755 unigenes), and metabolic process (586 unigenes). Immune-related unigenes were found within the biological process category, including 399 unigenes, 309 unigenes, and 69 unigenes enriched in response to stimulus, signaling, and immune system process, respectively. We found 22 enriched GO term (Fisher exact $P < 0.05$), with 11 GO terms corresponding to biological process, 6 GO terms corresponding to cellular component and 5 GO term corresponding to molecular function. Enriched term form biological process, included metabolic process (GO:0008152), and growth (GO:0040007) (Table 2).

3.5. KEGG pathway

KEGG is a bioinformatics resource for linking genomes to life and the environment [28] and it is useful to build the network of genes according to the relationship among genes, proteins, and compounds in the database [29]. GO annotation and KEGG enrichment analysis were applied to study the global biological change in infected liver. Through functional classification of the genes by exploring the pathways in the liver of Baltic cod, a total of 454 DEGs were found involved in 138 KEGG Pathways. The pathways were assigned to six main categories; these included metabolism which contained 449 unigenes, followed by Environmental Information Processing (231 unigenes), Cellular Processes (216 unigenes), Organismal System (130 unigenes), Genetic Information Processing (75 unigenes), and Human Diseases (55 unigenes). Among 30 pathways in hierarchy 1, the highest number of unigenes contributed in global and overview maps (173 unigenes), followed by signal transduction (170 unigenes), and cellular community-eukaryotes (85 unigenes) (Fig. 3). Total 69, 53, and 56 unigenes were involved in carbohydrate metabolism, lipid metabolism, and amino acid metabolism pathways, respectively (Table S3). Moreover, 22 DEGs were involved in 5 immune system pathways, including Cytosolic DNA-sensing pathway (2 unigenes), Intestinal immune network for IgA production (2 unigenes), NOD-like receptor signaling (7 unigenes), RIG-I-like receptor signaling pathway (6 unigenes), and Toll-like receptor signaling pathway (5 unigenes) (Table S4). Further, we found 13 enriched KEGG term (Fisher exact < 0.05). The DEGs were engaged in several pathways, such as Cysteine and methionine metabolism, Propanoate metabolism, and Metabolic pathways (Table 3).

Based on enrichment analysis, annotation and manual literature search, the genes associated with parasitic infection of Baltic cod were grouped into three categories including metabolism, immune system and growth.

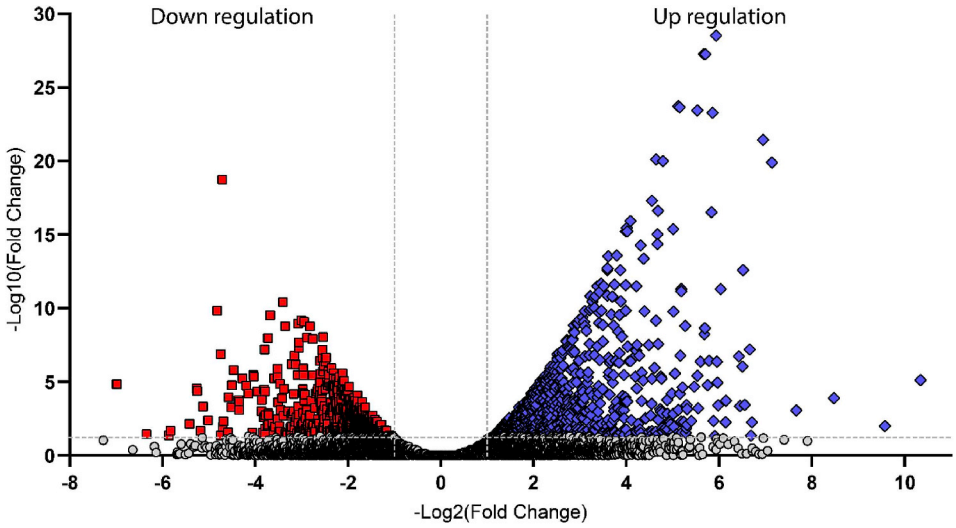

Fig. 1. The volcano plot of DEGs from the liver of Baltic cod infected by *C. osculatum*. Blue diamonds, red squares, and grey dots denote up-regulated, down-regulated, and non-regulated genes, respectively. The horizontal dotted line depicts the adjusted p-value equal to 0.05 and the vertical dotted lines depict two times up-regulation and down-regulation, respectively. The value of log2 fold change for all genes was analyzed as log2 (infected/control). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

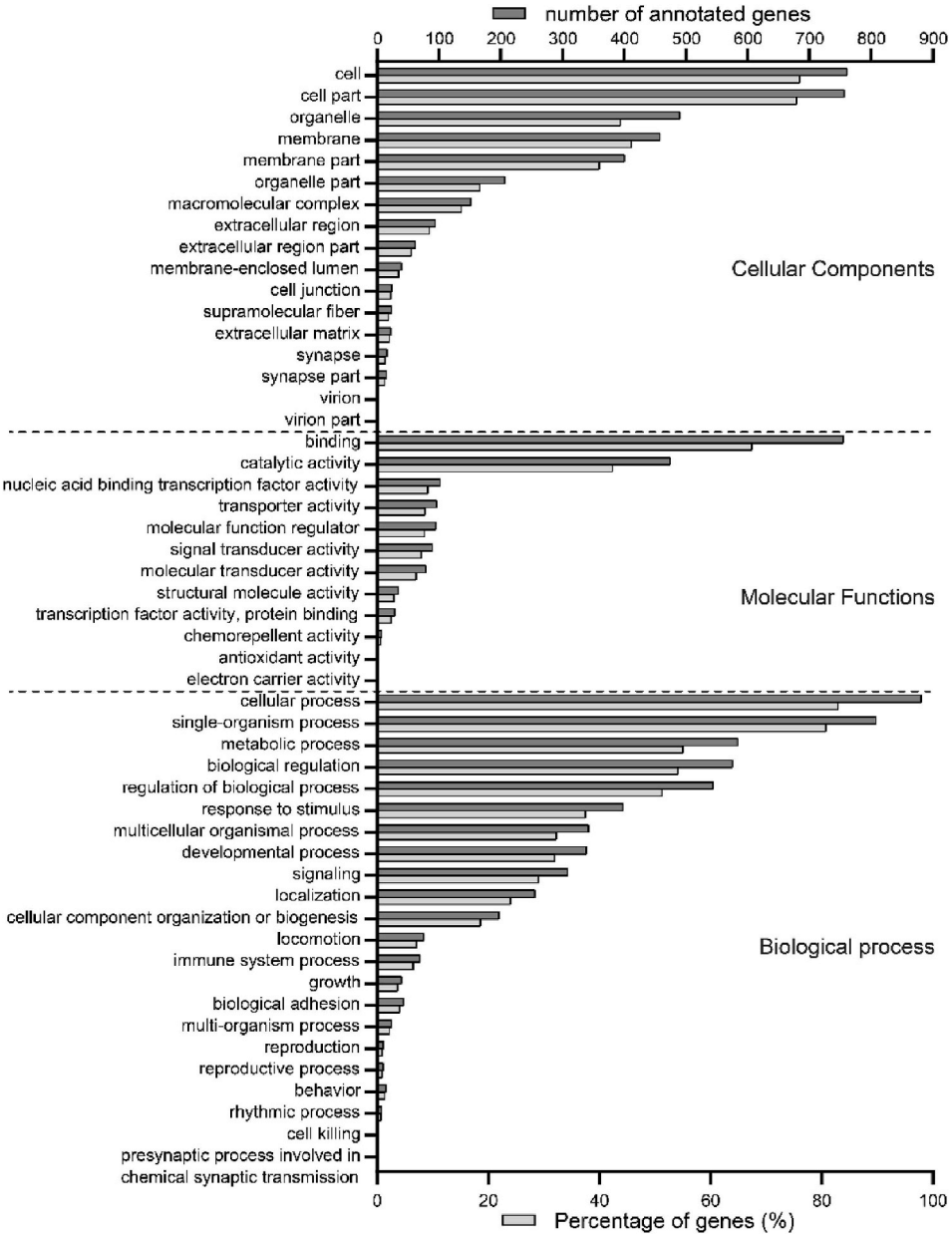

Fig. 2. GO annotation of DEGs from the liver of Baltic cod infected by third stage larvae of *C. osculatum* with emphasis on GO terms for cellular component, molecular function and biological process.

3.6. Differential expression of metabolism-related genes in the Baltic cod liver

Expression data for key metabolism-associated genes related to carbohydrate metabolism, amino acid metabolism, and lipid metabolism are listed in Table 4. For these entities 30, 35 and 32 genes, respectively, were differentially expressed in the infected Baltic cod liver. Fourteen genes encoding gfpts2, pfkfb3, itpkcb, pfkpb, ippk, glula, ldha, g6pca.2, pi4kb, ldhba, pi4k2b, ldhbb, cyb5r4, aco2 were significantly up-regulated (carbohydrate metabolism), fourteen genes encoding gfpt2, th, tph1a, hdc, cyp1b1, glula, Idha, bbox1, arg2, Idhba, tdh, Idhba, dnmt1, and mtr were significantly up-regulated (amino acid metabolism), and eighteen genes encoding pla2g7, soat1, plpp3, cyp1b1, ch25h, pla2g12b, pisd, dgki, ptges, gba2, cers2a, hsd3b7, pla2g7, sqlea, ptdss2, elovl1b, sptlc2b, and pcyt2 were significantly up-regulated (lipid metabolism). The gfpt2 gene encoding glutamine-fructose-6-phosphate aminotransferase 2 was the highest up-regulated gene in the metabolic pathways and it was involved in both carbohydrate and protein metabolism. Moreover, pla2g12b gene was up-regulated in all part metabolism in lipid metabolism pathways, such as alpha-linolenic acid, arachidonic acid, ether lipid, glycerophospholipid, and linoleic acid.

3.7. Differential expression of immune related genes in the Baltic cod liver

Differential expression of immune-related genes in Baltic cod liver (Table 5) involved eight genes encoding tnfaip3, ccr9a, rela, jun, mapk14a, ripk2, cxcr4b, and casp8 which were significantly up-regulated whereas five genes encoding traf2b, caspa, tradd, pik3r1, and dhx58 were significantly down-regulated. The tnfaip3 gene encoding TNF Alpha Induced Protein 3 was the highest up-regulated whereas the strongest down-regulated immune gene encoded traf2b.

3.8. Differential expression of growth-related genes in the Baltic cod liver

Thirty-nine growth-related genes were differentially expressed in

Table 2
List of enriched GO terms in DEGs.

GO_ID	Class	Term	Fisher exact
GO:0032502	GOTERM_BP_1	Developmental process	5,6E-5
GO:0008152	GOTERM_BP_1	Metabolic process	5,7E-4
GO:0008150	GOTERM_BP_1	Single-organism process	8,6E-4
GO:0050789	GOTERM_BP_1	Regulation of biological process	1,0E-3
GO:0065007	GOTERM_BP_1	Biological regulation	1,6E-3
GO:0032501	GOTERM_BP_1	Multicellular organismal process	5,0E-3
GO:0040007	GOTERM_BP_1	Growth	7,4E-3
GO:0040011	GOTERM_BP_1	Locomotion	9,6E-3
GO:0023052	GOTERM_BP_1	Signaling	1,6E-2
GO:0050896	GOTERM_BP_1	Response to stimulus	1,9E-2
GO:0051179	GOTERM_BP_1	Localization	5,0E-2
GO:0043226	GOTERM_CC_1	Organelle	2,6E-3
GO:0005576	GOTERM_CC_1	Extracellular region	5,7E-3
GO:0044421	GOTERM_CC_1	Extracellular region part	1,3E-2
GO:0044464	GOTERM_CC_1	Cell part	5,2E-2
GO:0005623	GOTERM_CC_1	Cell	6,8E-2
GO:0099512	GOTERM_CC_1	Supramolecular fiber	4,9E-2
GO:0003700	GOTERM_MF_1	Nucleic acid binding transcription factor activity	3,9E-8
GO:0098772	GOTERM_MF_1	Molecular function regulator	2,8E-4
GO:0003824	GOTERM_MF_1	Catalytic activity	2,0E-3
GO:0000988	GOTERM_MF_1	Transcription factor activity, protein binding	2,8E-3
GO:0045499	GOTERM_MF_1	Chemorepellent activity	1,1E-2

Baltic cod liver infected with *C. osculatum* (Table 6). Several genes involved in key growth were down-regulated, including among others krt91, igfbp2a, grna, bamp 10, bamp 4, extl3, and rbbp4, while other genes might associate with growth of immune cell were up-regulated such as, sema3gb, sema3h, m17 and junbb.

3.9. RNA sequencing validation using real-time PCR

RNA-seq results were mainly consistent with the qRT-PCR results, indicating that the gene expression profile generated by RNA-seq were reliable, thereby confirming the observed changes in the expression of genes responsive to *C. osculatum* infection. As shown in Fig. 4, all the selected genes, gene encoding c3, crp p1, crp p2, and igd were down-regulated.

4. Discussion

The present study was performed to extend our understanding of the physiological changes in Baltic cod liver when exposed to infection with third stage larvae of *C. osculatum.* This parasite has become abundant during recent years due to the expanding population of grey seals in the Baltic Sea. The seal is the final host of the parasite, which uses small crustaceans as the first transport host, small fish as the second transport host and cod as the third transport host [6,30]. Our investigation showed that the number of third stage larvae of *C. osculatum* in the liver was correlated to the size of the liver, but in all cod the infection was as high as recently reported [3,7,8]. Cod is a non-model species with a very limited number of sequences annotated so far and we could therefore contribute with a large number of new annotated genes. The comparison of the transcriptomic libraries constructed from the two experimental groups enabled the identification of hundreds of differentially expressed genes. RNA-Seq studies on Atlantic cod liver mainly focusing on toxicology have been conducted in recent years [15] and the present work supplements these studies by addressing parasitic effects. It was demonstrated that genes related in particular to metabolism, immune system, and growth were differentially expressed in liver of infected cod compared to non-infected. The liver has an important role in many biochemical and physiological processes, whereby it plays a regulatory role in nutrient absorption, food digestion, and immune response [31]. The knowledge on the reorganization of Baltic cod metabolism and immune response in the liver during nematode infection is limited and the transcriptomic analysis presented in the present study fills in gaps in our understanding of these pivotal processes. A total of 47,025 predicted gene models were expressed in cod liver corresponding to 12,053 (25.63%) showing strong similarity to the zebrafish proteome using BLASTX algorithm with a cut-off-E-value $\leq 1.0 \times 10^{-5}$. Furthermore, the mapping quality of our sequenced reads was favorable compared to corresponding studies [15,32–34]. In this study, 1304 DEGs were enriched to Gene Ontology in Baltic cod liver infected by *C. osculatum*. The total number of DEGs identified in our study was higher than in the study on cod liver exposed to pollutants [15], and genes upregulated in other cod organs [33,34]. The majority of genes were up-regulated in our study corresponding to investigations in similar transcriptome studies. However, considering that infected samples were collected from naturally infected cod (non-experimental infection) and control cod were obtained from a different location, it should be emphasized that genetic and environmental differences between host groups can contribute to the transcriptome profile.

The GO annotation and KEGG enrichment analyses suggest that the infection had a major impact on Baltic cod liver physiology (cellular processes, metabolic pathways, immune system, growth and reproduction). The majority of DEGs in the liver of Baltic cod was involved in metabolism associated with nitrogen compound metabolic process, cellular metabolic process, primary metabolic process, single-organism metabolic process and organic substance metabolic process. This confirms that the cod liver plays a central role in the metabolic related to energy metabolism, synthesis, secretion of serum proteins, and immune response [35]. We showed that metabolic DEGs were involved in amino acid (protein), carbohydrate, and lipid metabolism. Infected fish require energy for optimal immune function [36] and are dependent on the physiological pathways including protein and lipid metabolism. This may be important during an inflammatory response, where the liver produces high levels of acute phase proteins in order to neutralize an invading pathogen such as a *C. osculatum* larva. It was also indicated that the infection might cause suppression of metabolic process such as carbohydrate, lipid and amino acid metabolism, which may counteract an effective immune response. In this context it is noteworthy that Mehrdana et al. [37], applying a zebrafish model, showed that immune gene expression was significantly affected by excretory-secretory (ES) proteins from *C. osculatum* which will add to the notion that the worm larva affects the host immune response of the fish host.

The gfpt2 is a rate-limiting enzyme in the hexosamine biosynthesis pathway [38] converting D-fructose-6-phosphate (Fru-6-P) and L-glutamine to D-glucosamine-6-phosphate (GlcN-6-P) and L-glutamate, which is the obligatory source of essential amino sugars for the synthesis of glycoproteins, glycolipids, and proteoglycans [39,40]. It was reported that gfpt2 expression was stimulated by alkaline pH and cadmium stress in white shrimp *Litopenaeus vannamei* [41], may reduce H_2O_2-induced toxicity in HT-22 in mammals [42], and reduce effects of methyl-mercury in *Saccharomyces cerevisiae* [43]. It could be hypothesized that *C. osculatum* stimulates the expression of the gfpt2 gene. It is up-regulated during hepatocellular injury [44], is an important pathway for cellular glucose sensing [45] and related to regenerating liver cell types [46]. In mammals, the gene is over-expressed in association with insulin resistance, postprandial hyperglycemia and oxidative stress in type II diabetes [47–49]. Several genes which were highly expressed in the infected Baltic cod liver were associated with glucose control such as itpkcb, pfkpb, ippk, and glula in the carbohydrate metabolism pathway. Infected fish may invest more energy to maintain cellular homeostasis, and the increased expression of those genes could be a result of an increased response of glucose supply in the liver, in order to compensate for the energy expenditure. However, verification of this hypothesis will require additional studies.

The present study identified both pla2g7 and pla2g12b were up-regulated in lipid metabolism. The pla2g7 is one of the phospholipase A

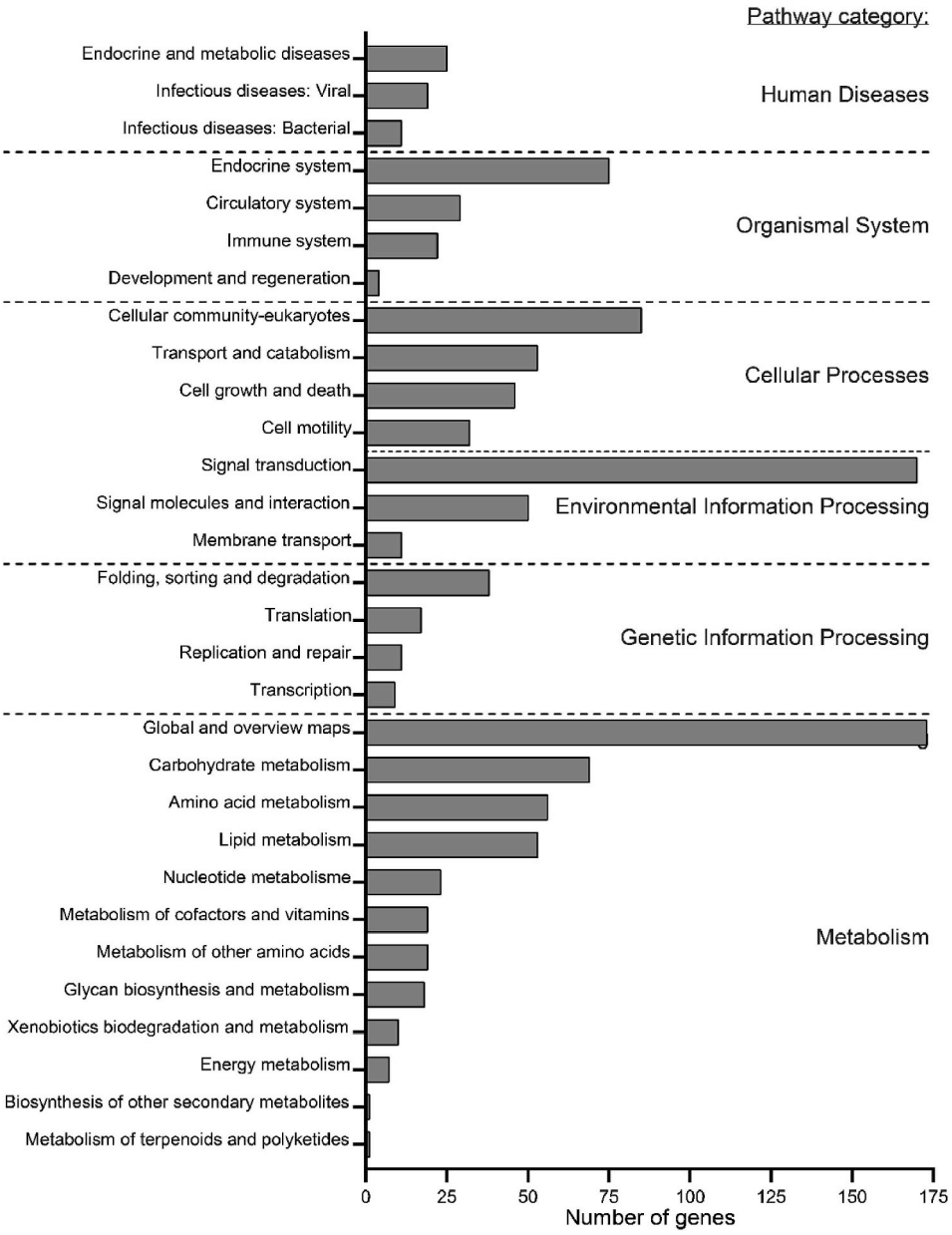

Fig. 3. KEGG classification of DEGs from the liver of Baltic cod infected by *C. osculatum*.

Table 3
List of enrichment KEGG pathways in DEGs.

Pathways ID	Pathways	Fisher exact
Ko00270	Cysteine and methionine metabolism	1,9E-4
Ko00670	One carbon pool by folate	1,1E-3
Ko02010	ABC transporters	3,2E-3
ko04115	p53 signaling pathway	4,5E-3
ko00640	Propanoate metabolism	2,9E-3
ko04510	Focal adhesion	9,7E-3
ko04068	FoxO signaling pathway	9,1E-3
Ko01130	Biosynthesis of antibiotics	1,4E-2
Ko04931	Insulin resistance	1,3E-2
ko04210	Apoptosis	1,7E-2
ko01100	Metabolic pathways	5,6E-2
ko04120	Ubiquitin mediated proteolysis	4,0E-2
ko04530	Tight junction	4,6E-2

(PLA) 2 family was high up-regulated among other genes involved in this pathway and its function as potent pro- and anti-inflammatory molecule has been implicated in multiple inflammatory disease processes. Moreover, it plays a crucial role in a number of physiological activities [50–52]. Upregulation of the pla2g12b gene has been reported in virus infected zebrafish [53] and in the liver of fasting fish [54,55]. It is associated with lipid metabolism (fatty acid biosynthesis) [55,56] and knockout and mutagenesis of the gene in mice cause decreasing serum lipids and increased liver fatty droplets [57]. Due to the important role of lipids for growth and fish meat quality [58,59] it is noteworthy that cod infected with *C. osculatum* showed a significant upregulation of the gene.

Differential expression of genes related to both the innate and the adaptive immune system was measured in infected cod but most genes were associated with pathways playing fundamental roles in innate immunity. These included 1) Cytosolic DNA-sensing pathway [60], 2)

Table 4
Representative key metabolism-related genes differentially expressed in *C. osculatum* infected cod liver.

Pathways	Query sequence ID	Gene encoding	Definition	Log2 fold change
Carbohydrate metabolism	GAMO_00057048-RA	abat	4-aminobutyrate aminotransferase(abat)	−1.93
	GAMO_00064747-RA	aco2	aconitase 2, mitochondrial(aco2)	1.30
	GAMO_00005925-RA	acss2	acyl-CoA synthetase short-chain family member 2(acss2)	−1.45
	GAMO_00068820-RA	aldh6a1	aldehyde dehydrogenase 6 family, member A1(aldh6a1)	−1.66
	GAMO_00025652-RA	amt	aminomethyltransferase(amt)	−2.05
	GAMO_00021249-RA	cyb5r4	cytochrome *b*5 reductase 4(cyb5r4)	1.30
	GAMO_00077030-RA	eno3	enolase 3, (beta, muscle)(eno3)	−1.43
	GAMO_00061627-RA	enosf1	enolase superfamily member 1(enosf1)	−1.43
	GAMO_00066200-RA	g6pc3	glucose 6 phosphatase, catalytic, 3(g6pc3)	−1.58
	GAMO_00066626-RA	g6pca.2	glucose-6-phosphatase a, catalytic subunit, tandem duplicate 2(g6pca.2)	1.91
	GAMO_00050310-RA	gfpt2	glutamine-fructose-6-phosphate transaminase 2(gfpt2)	6.47
	GAMO_00027519-RA	glula	glutamate-ammonia ligase (glutamine synthase) a(glula)	2.20
	GAMO_00057027-RA	hao1	hydroxyacid oxidase (glycolate oxidase) 1(hao1)	−2.75
	GAMO_00039665-RA	ippk	inositol 1,3,4,5,6-pentakisphosphate 2-kinase(ippk)	2.54
	GAMO_00083243-RA	itpkb	inositol-trisphosphate 3-kinase B(itpkb)	−2.07
	GAMO_00011619-RA	itpkcb	inositol-trisphosphate 3-kinase Cb(itpkcb)	4.69
	GAMO_00007420-RA	kl	klotho(kl)	−2.05
	GAMO_00045721-RA	ldha	lactate dehydrogenase A4(ldha)	1.94
	GAMO_00046125-RA	ldhba	lactate dehydrogenase Ba(ldhba)	1.59
	GAMO_00046126-RA	ldhbb	lactate dehydrogenase Bb(ldhbb)	1.30
	GAMO_00045381-RA	mlycd	malonyl-CoA decarboxylase(mlycd)	−1.20
	GAMO_00003493-RA	pfkfb3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3(pfkfb3)	4.79
	GAMO_00032325-RA	pfkpb	phosphofructokinase, platelet b(pfkpb)	2.65
	GAMO_00015171-RA	pi4k2b	phosphatidylinositol 4-kinase type 2 beta(pi4k2b)	1.56
	GAMO_00072625-RA	pi4kb	phosphatidylinositol 4-kinase, catalytic, beta(pi4kb)	1.75
	GAMO_00059691-RA	plcd3a	phospholipase C, delta 3a(plcd3a)	−1.65
	GAMO_00024891-RA	tktb	transketolase b(tktb)	−1.95
	GAMO_00014749-RA	ugdh	UDP-glucose 6-dehydrogenase(ugdh)	−2.51
	GAMO_00012090-RA	ugp2a	UDP-glucose pyrophosphorylase 2a(ugp2a)	−1.28
	GAMO_00012088-RA	ugp2b	UDP-glucose pyrophosphorylase 2b(ugp2b)	−1.47
Amino acid metabolism	GAMO_00057048-RA	abat	4-aminobutyrate aminotransferase(abat)	−1.93
	GAMO_00039030-RA	ahcyl1	adenosylhomocysteinase-like 1(ahcyl1)	−1.44
	GAMO_00068820-RA	aldh6a1	aldehyde dehydrogenase 6 family, member A1(aldh6a1)	−1.66
	GAMO_00025652-RA	amt	aminomethyltransferase(amt)	−2.05
	GAMO_00064827-RA	arg1	arginase 1(arg1)	−1.33
	GAMO_00055749-RA	arg2	arginase 2(arg2)	1.85
	GAMO_00019079-RA	bbox1	butyrobetaine (gamma), 2-oxoglutarate dioxygenase (gamma-butyrobetaine hydroxylase) 1(bbox1)	1.90
	GAMO_00060001-RA	bcat2	branched chain amino-acid transaminase 2, mitochondrial(bcat2)	−1.42
	GAMO_00042251-RA	bhmt	betaine-homocysteine methyltransferase(bhmt)	−3.31
	GAMO_00058981-RA	colgalt1	collagen beta(1-O)galactosyltransferase 1(colgalt1)	−1.97
	GAMO_00037893-RA	comtb	catechol-O-methyltransferase b(comtb)	−1.33
	GAMO_00036692-RA	cyp1b1	cytochrome P450, family 1, subfamily B, polypeptide 1(cyp1b1)	3.42
	GAMO_00002174-RA	dbh	dopamine beta-hydroxylase (dopamine beta-monooxygenase)(dbh)	−1.38
	GAMO_00021551-RA	ddc	dopa decarboxylase(ddc)	−1.66
	GAMO_00058893-RA	dnmt1	DNA (cytosine-5-)-methyltransferase 1(dnmt1)	1.27
	GAMO_00053644-RA	dnmt3ab	DNA (cytosine-5-)-methyltransferase 3 alpha b(dnmt3ab)	−1.32
	GAMO_00024119-RA	dnmt3ba	DNA (cytosine-5-)-methyltransferase 3 beta, duplicate a(dnmt3ba)	−2.67
	GAMO_00005935-RA	dnmt3bb.1	DNA (cytosine-5-)-methyltransferase 3 beta, duplicate b.1(dnmt3bb.1)	−1.96
	GAMO_00000103-RA	ftcd	formimidoyltransferase cyclodeaminase(ftcd)	−4.75
	GAMO_00028185-RA	gamt	guanidinoacetate N-methyltransferase(gamt)	−1.33
	GAMO_00050310-RA	gfpt2	glutamine-fructose-6-phosphate transaminase 2(gfpt2)	6.47
	GAMO_00065235-RA	glud1b	glutamate dehydrogenase 1b(glud1b)	−1.23
	GAMO_00027519-RA	glula	glutamate-ammonia ligase (glutamine synthase) a(glula)	2.20
	GAMO_00016008-RA	hdc	histidine decarboxylase(hdc)	4.69
	GAMO_00052442-RA	hnmt	histamine N-methyltransferase(hnmt)	−1.59
	GAMO_00036810-RA	kmo	kynurenine 3-monooxygenase(kmo)	−1.60
	GAMO_00045721-RA	ldha	lactate dehydrogenase A4(ldha)	1.94
	GAMO_00046125-RA	ldhba	lactate dehydrogenase Ba(ldhba)	1.59
	GAMO_00046126-RA	ldhbb	lactate dehydrogenase Bb(ldhbb)	1.30
	GAMO_00063596-RA	mtr	5-methyltetrahydrofolate-homocysteine methyltransferase(mtr)	1.19
	GAMO_00019934-RA	setdb1b	SET domain, bifurcated 1b(setdb1b)	−1.54
	GAMO_00044738-RA	tat	tyrosine aminotransferase(tat)	−1.56
	GAMO_00068239-RA	tdh	L-threonine dehydrogenase(tdh)	1.35
	GAMO_00043954-RA	th	tyrosine hydroxylase(th)	4.88
	GAMO_00016000-RA	tph1a	tryptophan hydroxylase 1 (tryptophan 5-monooxygenase) a(tph1a)	4.86

(*continued on next page*)

Table 4 (*continued*)

Pathways	Query sequence ID	Gene encoding	Definition	Log2 fold change
Lipid metabolism	GAMO_00069519-RA	pla2g7	phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)(pla2g7)	4.55
	GAMO_00029459-RA	soat1	sterol O-acyltransferase 1(soat1)	4.24
	GAMO_00030827-RA	plpp3	phospholipid phosphatase 3(plpp3)	4.03
	GAMO_00036692-RA	cyp1b1	cytochrome P450, family 1, subfamily B, polypeptide 1(cyp1b1)	3.42
	GAMO_00064822-RA	ch25h	cholesterol 25-hydroxylase(ch25h)	3.37
	GAMO_00036328-RA	pla2g12b	phospholipase A2, group XIIB(pla2g12b)	3.27
	GAMO_00073023-RA	pisd	phosphatidylserine decarboxylase(pisd)	2.25
	GAMO_00074218-RA	DGKI	diacylglycerol kinase, iota(dgki)	2.21
	GAMO_00073044-RA	ptges	prostaglandin E synthase(ptges)	2.12
	GAMO_00076792-RA	gba2	glucosidase, beta (bile acid) 2(gba2)	1.64
	GAMO_00019939-RA	cers2a	ceramide synthase 2a(cers2a)	1.58
	GAMO_00059889-RA	hsd3b7	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase(hsd3b7)	1.50
	GAMO_00069518-RA	pla2g7	phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)(pla2g7)	1.48
	GAMO_00020863-RA	sqlea	squalene epoxidase a(sqlea)	1.47
	GAMO_00046453-RA	ptdss2	phosphatidylserine synthase 2(ptdss2)	1.43
	GAMO_00029213-RA	elovl1b	ELOVL fatty acid elongase 1b(elovl1b)	1.38
	GAMO_00068213-RA	sptlc2b	serine palmitoyltransferase, long chain base subunit 2b(sptlc2b)	1.30
	GAMO_00015519-RA	pcyt2	phosphate cytidylyltransferase 2, ethanolamine(pcyt2)	1.19
	GAMO_00058492-RA	tmem86b	transmembrane protein 86B(tmem86b)	−1.28
	GAMO_00005864-RA	faxdc2	fatty acid hydroxylase domain containing 2(faxdc2)	−1.30
	GAMO_00037893-RA	comtb	catechol-O-methyltransferase b(comtb)	−1.33
	GAMO_00049064-RA	gpx3	glutathione peroxidase 3(gpx3)	−1.40
	GAMO_00005525-RA	pcyt1bb	phosphate cytidylyltransferase 1, choline, beta b(pcyt1bb)	−1.46
	GAMO_00022078-RA	gpx8	glutathione peroxidase 8 (putative)(gpx8)	−1.48
	GAMO_00029352-RA	lpl	lipoprotein lipase(lpl)	−1.60
	GAMO_00002439-RA	ggt1b	gamma-glutamyltransferase 1b(ggt1b)	−1.76
	GAMO_00027344-RA	cyp2p8	cytochrome P450, family 2, subfamily P, polypeptide 8(cyp2p8)	−1.90
	GAMO_00014351-RA	srd5a2a	steroid-5-alpha-reductase, alpha polypeptide 2a(srd5a2a)	−2.47
	GAMO_00009526-RA	ache	acetylcholinesterase(ache)	−2.59
	GAMO_00045250-RA	pnpla3	patatin-like phospholipase domain containing 3(pnpla3)	−2.73
	GAMO_00039594-RA	cyp27b1	cytochrome P450, family 27, subfamily B, polypeptide 1(cyp27b1)	−4.57
	GAMO_00014349-RA	srd5a2a	steroid-5-alpha-reductase, alpha polypeptide 2a(srd5a2a)	−4.85

RIG-I-like receptor signaling pathway [61,62], 3) Toll-like receptor signaling pathway [63], 4) NOD-like receptor signaling pathway [64]. DEGs associated with adaptive immunity were particularly associated with the Intestinal immune network for IgA known from mammals [65].

The present study identified casp8, jun, rela, and mapk14a component of TLRs pathway which were up-regulated. TLRs are the most important class of pattern recognition receptors. TLRs recognize a broad range of pathogens, including parasites, viruses, bacteria, and fungi, and essential for the activation of innate immunity [66,67]. Casp8, the gene encodes a member of the cysteine-aspartic acid protease (caspase) family, is involved in the apoptosis in human-induced by AIP (apoptosis-inducing protein) resulted by protein purified and cloned from *Chub mackerel* infected with the larval nematode, *Anisakis simplex* from *Chub mackerel* [68], and it was involved in the apoptosis in fish [69–71]. In line with those studies, up-regulated of casp8 in Baltic cod liver was induced by apoptosis inducing-protein activity after *C. osculatum* infection.

The tnfaip3, ripk2, casp8, mapk14a, and rela are involved in the NOD-like receptor-signaling pathway. Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) consist of a family of intracellular pattern-recognition receptors and they have been widely investigated as signaling platforms playing important roles in the regulation of inflammatory signaling in response to pathogen signals and cellular stress [72]. The tnfaip3 gene, encoding A20 protein, is a zinc finger protein induced during TLR stimulation that has two enzymatic activities, acting as an E3 ubiquitin ligase and a de-ubiquitinase [73]. It inhibits activation of NF-κB in various cell types to prevent inflammatory by restricting signaling of NF-kB, from Toll-like receptors, the receptor Nod2 and other receptors of the innate immune system [74,75]. The tnfaip3 gene was upregulated significantly relative to the control in the catfish following columnaris bacterial infection [76], in

Table 5
Representative key immune-related genes differentially expressed after *C. osculatum* infection.

Query sequence ID	Gene encoding	Definition	Log2 fold change
GAMO_00001999-RA	pik3r1	phosphoinositide-3-kinase, regulatory subunit 1 (alpha)(pik3r1)	−1.26
GAMO_00002460-RA	traf2b	Tnf receptor-associated factor 2b(traf2b)	−2.54
GAMO_00015935-RA	tradd	tnfrsf1a-associated via death domain(tradd)	−1.30
GAMO_00030672-RA	ripk2	receptor-interacting serine-threonine kinase 2(ripk2)	1.62
GAMO_00030783-RA	jun	jun proto-oncogene(jun)	1.91
GAMO_00031753-RA	ccr9a	chemokine (C–C motif) receptor 9a(ccr9a)	2.13
GAMO_00032094-RA	casp8	caspase 8, apoptosis-related cysteine peptidase(casp8)	1.15
GAMO_00034470-RA	tnfaip3	tumor necrosis factor, alpha-induced protein 3(tnfaip3)	2.41
GAMO_00037324-RA	mapk14a	mitogen-activated protein kinase 14a(mapk14a)	1.89
GAMO_00052407-RA	cxcr4b	chemokine (C-X-C motif), receptor 4b(cxcr4b)	1.41
GAMO_00058626-RA	dhx58	DEXH (Asp-Glu-X-His) box polypeptide 58(dhx58)	−1.23
GAMO_00076429-RA	rela	v-rel avian reticuloendotheliosis viral oncogene homolog A(rela)	2.11
GAMO_00079257-RA	caspa	caspase a(caspa)	−1.41

Table 6
Representative key growth-related genes differentially expressed in cod liver infected with *C. osculatum*.

Query sequence ID	Gene encoding	Definition	Log2foldchange
GAMO_00009322-RA	serpinh1b	serpin peptidase inhibitor, clade H (heat shock protein 47), member 1b(serpinh1b)	−3.75
GAMO_00081271-RA	krt91	keratin 91(krt91)	−3.39
GAMO_00060008-RA	grna	granulin a(grna)	−2.24
GAMO_00022755-RA	bmp10	bone morphogenetic protein 10(bmp10)	−1.99
GAMO_00055339-RA	bmp4	bone morphogenetic protein 4(bmp4)	−1.97
GAMO_00051808-RA	zeb2a	zinc finger E-box binding homeobox 2a(zeb2a)	−1.85
GAMO_00049478-RA	gpc4	glypican 4(gpc4)	−1.84
GAMO_00000273-RA	igfbp2a	insulin-like growth factor binding protein 2a(igfbp2a)	−1.71
GAMO_00054427-RA	rbbp4	retinoblastoma binding protein 4(rbbp4)	−1.28
GAMO_00067819-RA	extl3	exostosin-like glycosyltransferase 3(extl3)	−1.23
GAMO_00053918-RA	setd3	SET domain containing 3(setd3)	−1.14
GAMO_00046627-RA	dusp6	dual specificity phosphatase 6(dusp6)	1.15
GAMO_00033665-RA	vangl2	VANGL planar cell polarity protein 2(vangl2)	1.30
GAMO_00011131-RA	yap1	Yes-associated protein 1(yap1)	1.38
GAMO_00052407-RA	cxcr4b	chemokine (C-X-C motif), receptor 4b(cxcr4b)	1.41
GAMO_00079489-RA	chd3	chromodomain helicase DNA binding protein 3(chd3)	1.43
GAMO_00042303-RA	sema4c	sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4C(sema4c)	1.51
GAMO_00070530-RA	sema4ab	sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4Ab(sema4ab)	1.53
GAMO_00029366-RA	tax1bp3	Tax1 (human T-cell leukemia virus type I) binding protein 3(tax1bp3)	1.55
GAMO_00071829-RA	pard6gb	par-6 family cell polarity regulator gamma b(pard6gb)	1.74
GAMO_00074964-RA	sema3aa	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3Aa(sema3aa)	1.78
GAMO_00037324-RA	mapk14a	mitogen-activated protein kinase 14a(mapk14a)	1.89
GAMO_00069102-RA	akap12b	A kinase (PRKA) anchor protein 12b(akap12b)	1.93
GAMO_00071832-RA	pard6gb	par-6 family cell polarity regulator gamma b(pard6gb)	1.97
GAMO_00049080-RA	hbegfa	heparin-binding EGF-like growth factor a(hbegfa)	2.05
GAMO_00024188-RA	sema3ga	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3Ga(sema3ga)	2.09
GAMO_00005002-RA	fgf1a	fibroblast growth factor 1a(fgf1a)	2.21
GAMO_00064367-RA	admp	anti-dorsalizing morphogenic protein(admp)	2.38
GAMO_00023795-RA	acvr1ba	activin A receptor, type IBa(acvr1ba)	2.59
GAMO_00055251-RA	ppp2r3a	protein phosphatase 2, regulatory subunit B″, alpha(ppp2r3a)	2.75
GAMO_00034724-RA	fam53b	family with sequence similarity 53, member B(fam53b)	2.79
GAMO_00057797-RA	junbb	jun B proto-oncogene b(junbb)	2.95
GAMO_00002264-RA	m17	IL-6 subfamily cytokine M17(m17)	4.12
GAMO_00025718-RA	sema3h	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3H(sema3h)	4.59
GAMO_00054519-RA	crim1	cysteine rich transmembrane BMP regulator 1 (chordin-like)(crim1)	4.65
GAMO_00061806-RA	klf6a	Kruppel-like factor 6a(klf6a)	4.71
GAMO_00025953-RA	sema3gb	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3 Gb(sema3gb)	5.10

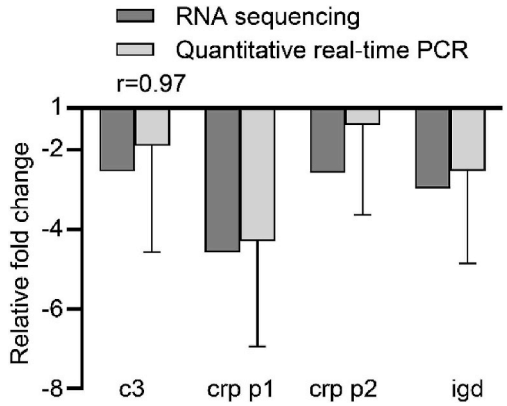

Fig. 4. Confirmation of the RNA-seq data with qPCR analysis on selected genes in Baltic cod. Complement factor c3 (c3), Protein crp p1, protein crp p2, and immunoglobulin D (igd). The genes presented here were both in the transcriptomic and the gene expression analysis significantly regulated (Fold > ± 2, and $p < 0.05$) upon infection. The r indicates Pearson's correlation factor between the transcriptomic and the gene expression analysis. Gene expression values are shown as relative fold change (mean ± SEM) when comparing the infected group to the control group (N = 10).

the Asian seabass infected with NNV virus [77] and in the *Schizothorax prenanti* during *Aeromonas hydrophila* infection [78]. Deletion of tnfaip3 gene in mice lead to die prematurely due to severe inflammation and cachexia and are more responsive to LPS and TNF [79]. Furthermore, tnfaip3 maintains immune homeostasis [80,81] and its expression is important for limiting inflammatory responses and the damage those responses cause in multiple tissues [79,82] and it seems to be associated with the inflammatory response to *C. osculatum* infection in cod. Another gene involved in this pathway was ripk2, which is a receptor-interacting protein family of serine/threonine protein kinases, mediating stress responses that lead to the activation of casp, NF-κB, and MAP kinases [83,84] and apoptosis [85]. Furthermore, it is a critical downstream mediator of NOD1 and NOD2 signalling [86,87] and plays an essential role in the modulation of innate and adaptive immunity [88].

The present study also indicated that *C. osculatum* larvae may suppress several expression of key genes related to growth. We found that the igfbp2a gene, insulin-like growth factor binding protein-2, was down-regulated in nematode infected Baltic cod liver and as this gene regulates the availability of insulin-like growth factors (IGFs) in various tissues and modulate IGF binding to its receptors [89–91] the observed suppression may be involved in growth performance of the host. Expression of the igfbp2a was down-regulated in the adult zebrafish at the late stage of chronic tuberculosis due to *Mycobacterium marinum* infection [92]. Knockdown of igfbp2a gene in zebrafish resulted in delayed development, distruptions to cardiovascular development, and reduced body growth [91,93,94].

The gene encoding krt91 was also down-regulated in infected cod. It has function in the keeping of cellular architecture and in giving mechanical resistance against stress [95,96] and is downregulated in osteoporotic zebrafish [97]. The results suggest a parasitic effect on the cod in line with the general notion that host performance and growth

decrease due to infection [98] and a more specific demonstration from the 1950s (during a previous period with high *C. osculatum* larva infection of Baltic cod) of an association between nematode infection and a low condition of the host [99,100].

In conclusion, we performed a high-throughput sequencing of liver from Baltic cod infected by *C. osculatum*. A total of 2084 DEGs were identified, 1240 unigenes were up-regulated while 844 unigenes were down-regulated. We found that 97, 13 and 36 genes were involved in metabolism, immunity and growth, respectively. Infection with *C. osculatum* was associated with depression of some genes related to metabolism, immune system, and growth. Infected fish need to invest energy when coping with invading pathogens which indirectly may influence growth of the host. In addition, it cannot be excluded that the parasite or parasite products directly regulate gene expression of growth and immunity related genes. The present study gave a far better resolution of the metabolism pathways, immune response, and growth of Baltic cod than previously presented which will be useful in the future controlled studies related to physiological adaptation of Baltic cod in response to pathogens or other environmental factors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.08.034.

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