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Embryonic gene expression of *Coregonus palaea* (whitefish) under pathogen stress as analyzed by high-throughput RNA-sequencing

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ABSTRACT

Most fishes produce free-living embryos that are exposed to environmental stressors immediately following fertilization, including pathogenic microorganisms. Initial immune protection of embryos involves the chorion, as a protective barrier, and maternally-allocated antimicrobial compounds. At later developmental stages, host-genetic effects influence susceptibility and tolerance, suggesting a direct interaction between embryo genes and pathogens. So far, only a few host genes could be identified that correlate with embryonic survival under pathogen stress in salmonids. Here, we utilized high-throughput RNA-sequencing in order to describe the transcriptional response of a non-model fish, the Alpine whitefish *Coregonus palaea*, to infection, both in terms of host genes that are likely manipulated by the pathogen, and those involved in an early putative immune response. Embryos were produced *in vitro*, raised individually, and exposed at the late-eyed stage to a virulent strain of the opportunistic fish pathogen *Pseudomonas fluorescens*. The pseudomonad increased embryonic mortality and affected gene expression substantially. For example, essential, upregulated metabolic pathways in embryos under pathogen stress included ion binding pathways, aminoacyl-tRNA-biosynthesis, and the production of arginine and proline, most probably mediated by the pathogen for its proliferation. Most prominently downregulated transcripts comprised the biosynthesis of unsaturated fatty acids, the citrate cycle, and various isoforms of b-cell transcription factors. These factors have been shown to play a significant role in host blood cell differentiation and renewal. With regard to specific immune functions, differentially expressed transcripts mapped to the complement cascade, MHC class I and II, TNF-alpha, and T-cell differentiation proteins. The results of this study reveal insights into how *P. fluorescens* impairs the development of whitefish embryos and set a foundation for future studies investigating host pathogen interactions in fish embryos.

1. Introduction

Externally-fertilized fish embryos develop as free-living organisms in their aquatic environment and are hence exposed to microbes from very early developmental stages on. They are primarily protected by the physical barrier of their chorion [1,2] and by antimicrobial compounds that the mother allocated to her eggs before spawning [3–5]. At later stages of embryonic development, the importance of maternal environmental effects decreases [6–8], while the embryo itself starts expressing genes [2,7,9] and genetic effects become integral in determining embryonic performance [10–13]. However, only a few genes could so far be identified to affect embryonic performance during experimental pathogen infections [3,14–17]. Our understanding of the concerted expression of genes involved in the immune function of fish embryos is largely based on the zebrafish (*Danio* rerio) model [*e.g.*, [18–20]].

Until recently, genome-wide investigations of host-pathogen interactions were not possible in non-model organisms [21]. Pathogen resistance in different salmonid embryos at the late-eyed developmental stage has been linked to a small number of specific immune alleles [15,17], major histocompatibility complex (MHC) gene expression [9,22], and nucleotide diversity at MHC loci [23,24]. With the advent of massively parallel next generation

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sequencing techniques, studies have now become possible that facilitate the characterization of transcriptome level responses of natural populations in their ecological context.

Here we investigated the whole transcriptomic response in embryos of the Alpine whitefish *Coregonus palaea*, a non-model vertebrate, after experimental infection. Members of the *Coregonus* genus have been increasingly used to identify genes of adaptive significance in a changing environment [*e.g.*, [15,25,26]]. *C. palaea* is a pelagic-dwelling salmonid species of great economic importance [27,28]. The external fertilization of *Coregonus* spp. (and other salmonid species) allows employing a full-factorial breeding design where every dam is crossed *in vitro* with every sire [29]. Embryos may then be raised in individual wells, where environmental variation can be experimentally manipulated [10]. Maternal and paternal (*i.e.*, genetic) effects on embryonic performance can thereby be disentangled from environmental variation [16,29].

Pseudomonas fluorescens is an opportunistic, bacterial fish pathogen that is found in most aquatic environments [30]. It has been shown to develop its pathogenic properties in different fish species, such as *Aristichthys nobilis* [30], *Cyprinus carpio* [31], *Hypophthalmichthys molitrix* [30], and *Oncorhynchus mykiss* [32]. *P. fluorescens* has repeatedly been used as model pathogen in experimental studies using embryos of *C. palaea* [8–10] or *Salmo trutta* [33,34] as hosts. Incubation provokes strain-specific phenotypic changes, such as a direct increase of embryonic mortality [10], and/or delayed hatching and reduced growth [8,9]. The potential of an embryo to mount an effective immune response after pathogen incubation has been demonstrated to depend on the time of exposure [8] and on host embryo genotype [9,12,17,35].

In the present study, gene expression was characterized at the late-eyed developmental stage when paternal genetic effects have shown to become significant [8–11,24,35], and when pathogen-induced changes in gene expression have been observed [9]. Our whitefish system allowed us to control for host genotypes and contrast environment-induced changes in gene expression. To this end, we first assembled expressed transcripts of 24 embryos, providing the first example of the *C. palaea* transcriptome. Then, we examined expression differences in pathogen-infected embryos relative to controls in order to investigate the transcriptomic response against *P. fluorescens*. Additionally, we focused on the possibility of an adaptive immune response by specifically analyzing a set of candidate host immune genes that have been documented to be differentially expressed following pathogen challenge in other salmonids [*e.g.*, [36–38]]. We expected to identify a subset of candidate genes putatively acting as key players during infection with *P. fluoresecens*.

2. Methods

2.1. Experimental protocol of whitefish embryos

We collected large type adult whitefish (*Coregnous palaea*) from their spawning grounds in Lake Geneva using gillnets in December 2010. Ripe fish were immediately stripped of their gametes and these gametes were subsequently used for full-factorial *in vitro* fertilizations following the methods described in von Siebenthal et al. [10]. Sixteen sibships (resulting from a 4 × 4 cross) were haphazardly selected for subsequent work. After being distributed individually to 24-well cell culture plates (Falcon, Becton Dickinson) in a block-wise design, fertilized eggs were raised in a 6.5 °C climate chamber.

Once embryos had reached the late-eyed stage (208 degree days), they were divided into treatment groups, with thirteen replicates of every parental combination receiving *P. fluorescens* ("PF"). The bacterial culture was prepared and enumerated as described in Clark et al. [9], with the suspension being diluted such that inoculation with 100 μl would achieve a concentration of 10^8 bacterial cells/ml in the wells. Nutrient broth ("NB": 3 g meat extract, 5 g bactopeptone, 1 L distilled H_2O; at a dilution of 1:000 in the wells), which is an agent that promotes bacterial growth, was added along with *P. fluorescens*. Consequently, as a control, thirteen replicates per sibship were inoculated with nutrient broth alone. Following treatment, embryonic mortality and days until hatching were recorded.

For statistical analysis of embryonic mortality and time until hatching, 10 replicates per treatment and family were included (the same families that were used for the profiling of their transcriptome). The remaining three eggs per treatment and family were used for RNA extractions. To test for effects of treatment and sire identity on time until hatching, a two-way ANOVA was performed. Average time until hatching per family (by treatment) was entered as the dependent variable. To examine the effects of treatment and sire on embryonic mortality, a logistic regression model was applied with the proportion of dead embryos (out of the 10 initial replicates) per family entered as the response variable. All analyses were done in R v. 3.0.1 [39].

2.2. RNA extraction and preparation

Eggs for RNA extraction were sampled 48 h after infection. RNA was extracted and stored according to Clark et al. [9]. RNA integrity was verified on an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Four sibships, originating from a cross of one dam with four different sires (Fig. 1) were chosen for subsequent analyses since they met the quality criterion of a RIN >8 [40]. In each sample, equal amounts of RNA from three embryos per sibship and per treatment were pooled in order to increase RNA yield for whole transcriptome sequencing (n = 8; Table S1; Illumina, Roche, Basel). cDNA libraries were prepared with 2.1–3.5 μg of starting total RNA using the Illumina TruSeq RNA Sample Preparation kit following the manufacturer's instructions. Libraries were amplified with 15 cycles of PCR and contained TruSeq indexes within the adapters. After KAPA quantification and dilution, libraries were sequenced. RNA-sequencing (RNA-seq) library preparation and sequencing was carried out at Fasteris (Geneva, Switzerland) on an Illumina HiSeq 2000 instrument with 100 bp paired-end (PE) reads. Fasteris developed their own dir-mRNA-dUTP sequencing protocol [41]. After purification and fragmentation of total RNA, dUTPs are added during the first strand cDNA synthesis. This procedure enables the removal of ribosomal RNA and first strand cDNA before PCR amplification, therefore reads are in the original RNA orientation. Eight libraries were pooled per lane and two lanes were sequenced (*i.e.*, each sample was extracted once and then sequenced twice independently as technical replicate and internal validation of the sequencing method).

2.3. De novo assembly of sequencing reads

Before assembly, raw reads were trimmed by removing adapter sequences and ambiguous nucleotides. All reads with quality scores less than 20 and length below 30 bp were removed. A *de novo* assembly was performed with Velvet v 1.2.07 [42,43]. To adapt the assembling process to transcriptome data OASES v. 0.2.08 [44] was used. VELVET builds a hash table of all possible kmers in the dataset and then contigs are designed based on de Brujin graph construction and simplification [43]. Several values of kmer (only odd numbers from 55 to 97) were applied to obtain the optimal assembly. Default parameters were used if not otherwise noted. Paired-end insert average size was set to 200 bp with a standard deviation of 10%. Neither a k-mer average coverage nor a coverage

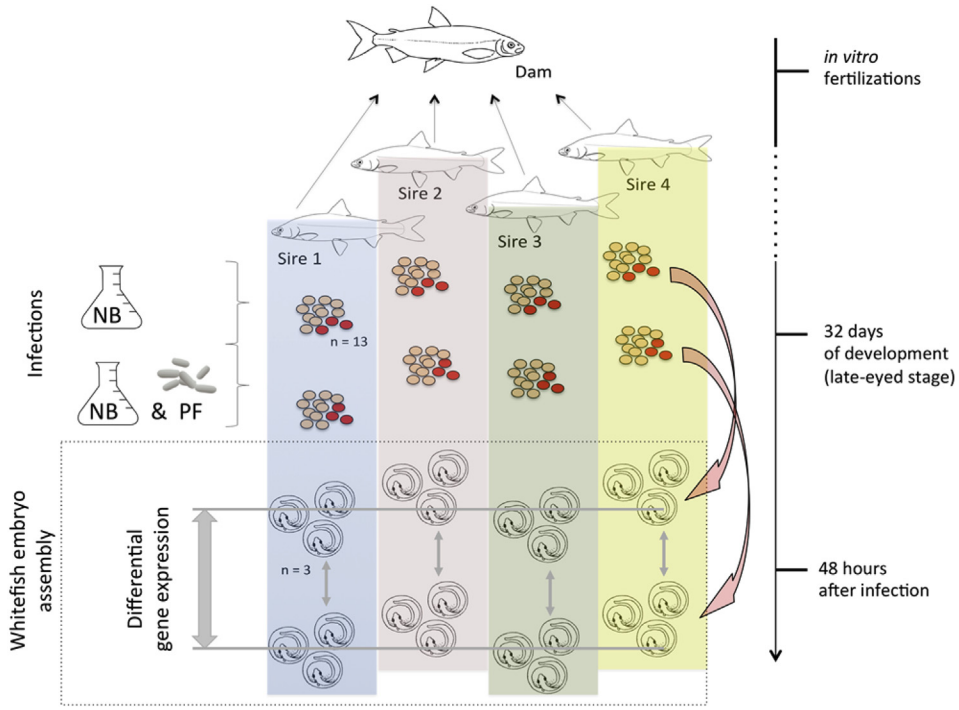

Fig. 1. Experimental design. Four different whitefish sires (Sire 1–4) were crossed *in vitro* with one dam in order to generate four different half-sib families of embryos. Treatment consisted of adding nutrient broth at a concentration of 1:1000 in the wells (NB) or adding the same concentration of nutrient broth and *Pseudomonas fluorescens*, an opportunistic fish pathogen (NB + PF), 208 degree days (32 days at 6.5 °C.) after fertilization. Three embryos per family and treatment (out of a total of 13 embryos each) were collected 48 h after infection for the whitefish embryo assembly and differential gene expression between the two treatments. The rest of the embryos (n = 10) was monitored for survival until hatching.

threshold for nodes was used due to the high dynamic range of transcript expression. Ten assemblies were produced initially. In order to validate *de novo* assemblies and find the best one for further analyses, BWA v. 0.5.9 mapping [45] was carried out on *de novo* contigs using 1 million reads from each library. Reads were mapped with a maximum of 2 mismatches in the first 32 bases of the sequences and a maximum of n mismatches in total. Table S2 summarizes the number of mismatches allowed according to the length of the reads. The assembly with the highest representation of reads from each library was chosen for downstream analysis of differential expression. Reads mapping to several positions of the reference with the same mapping quality were attributed at random to one of the positions with a 0 mapping quality. SAMtools v. 0.1.17 [46,47] was used for BAM/SAM files handling and conversion.

2.4. Differential gene expression

Differential gene expression analysis between the control group of embryos (NB) and treatment with *P. fluorescens* (PF) was carried out before resolving the identity of the specific transcripts; *i.e.*, before annotation. This "blind" analysis uses individual transcripts in the assembly as the features of interest (as opposed to gene or exon) [48]. In order to quantify expression, the coverage of each transcript was computed by applying BEDtools v. 2.9.0 [49]. Coverages from all libraries were then combined into a single file. This file was analyzed in R v. 3.0.1 [39] with the bioconductor package 'DEGseq' v. 1.2 [50], following [51]. Normalized gene expression values were used for statistical analysis as fragments per kilobase of exon model per million mapped reads (FPKM), modified for paired-end data after RPKM [52]. All transcripts were grouped into treatment (PF) and control (NB). Expression was defined as a transcript that is found in all samples of one group and that maps unambiguously against the best assembly. For each normalized count a probability was calculated to determine whether the expression of one group is significantly different from the expression of the other group [53,54]: Briefly, if it is assumed that a read has the same probability to be observed in both groups, the number of reads in each group that mapped to a reference follows a generalized Poisson distribution. Following this distribution it is possible to estimate a probability to observe counts that are more or less frequent between groups. All transcripts that were differentially expressed between the two groups qualified for annotation. Upper quartile normalization was included to improve robustness of differential expression for less abundant transcripts. *P*-values indicate significance of estimated log2 fold changes with a correction for multiple testing (false discovery rate, FDR, at 0.05) [55].

2.5. Gene annotation and ontology

Differentially expressed transcripts were used as queries against the zebrafish model organism database (ZFIN), the UniProtKB/SwissProt database (UniProtKB), the mouse genome informatics database (MGI), and the rat genome database (RGD) using the BLASTX program in Blast2GO v. 3.0 [56,57]. The cutoff E-value was set to 10^{-5}, and only the top gene ID (unigene) was assigned to each transcript. Gene ontology (GO) annotation analysis was performed in Blast2GO. The annotation results were categorized with respect to biological processes, molecular function and cellular component. We tested all GO terms if they are enriched between two distinct gene sets. Again, Blast2GO v. 3.0 was used and GO term enrichment was done using the integrated enrichment analysis function. We tested for differences between treatment (PF2) and control (NB) group using Fisher's Exact Test with a Benjamini – Hochberg multiple testing correction of FDR [58]. The threshold was set as

FDR value < 0.05. Functional groups and pathways encompassing the enriched gene sets were inferred based on the Kyoto Encyclopedia of Genes and Genomes [KEGG; [59]] database using *Danio rerio* as the reference. In the end, transcripts were ranked according to their discriminative power as follows: First, RPKM normalized values were used to calculate minimum, maximum, and median expression values of each group. Then, a centroid was calculated by taking the distance between the two medians of the two groups (PF *vs*. NB). The larger the centroid, the more disjoint are the groups. The ratio of expression from the pathogen-infected group relative to the nutrient broth control group was calculated using centroid distances.

In order to find expressed immune genes at this early developmental stage in whitefish, we also searched their transcriptome for transcripts that are specifically involved in immune functions. GO annotation analysis was performed on all expressed reads in Blast2GO using the search term 'immune system process' with respect to biological process.

2.6. Expression of candidate immune genes

Sixty sequences of immune genes of different species in the salmonid family (*Coregonus* spp., *O. mykiss*, and *Salmo salar*) were retrieved from the NCBI nucleotide databank. These candidate salmonid immune genes were added and aligned to our whitefish embryo assembly in order to improve this assembly. To quantify differential expression of candidate immune genes, all libraries were mapped against this new assembly a second time applying the same procedures as for the differential gene expression analysis above. As sequence information was available for a region of the MHC class I beta chain exon 1 in all whitefish parents of this study [9,60], we also tested for sire-specific expression of this locus.

3. Results

3.1. Experimental protocol of whitefish embryos

Incubation of the embryos with nutrient broth alone did not affect mortality (0% mortality), whereas adding *P. fluorescens* caused an average mortality rate of 60% ($SE = 7.84$, $z = 2.39$, d.f. $= 1$, $p = 0.02$). Within the pseudomonad treatment, mortality was not significantly different among sires ($z = 0.16$, d.f. $= 3$, $p = 0.8$). Treatment of embryos with *P. fluorescens* was also associated with significantly delayed hatching (546 ± 6.64 *vs*. 486 ± 5.75, mean $\pm$ SE degree days in the pathogen-infected and the nutrient broth control treatment, respectively; $F = 51.2$, d.f. $= 1$, $p < 0.0001$). A significant sire effect on hatching time was found in the pseudomonad treatment ($F = 7.2$, d.f. $= 3$ $p = 0.007$) but not in the nutrient broth control ($F = 0.39$, d.f. $= 3$ $p = 0.54$).

3.2. Sequencing and assembling of RNA-seq reads

Illumina-based RNA-seq was carried out on whole whitefish embryos at the late-eyed stage (221° days after fertilization). Mean concentration of RNA in these samples was 325 ng/μl (+/− SD of 122 ng/μl; Table S1) and all samples had a RIN of 8 or higher. A total of ~237 Mbp could be assembled after removing ambiguous nucleotides and low quality sequences resulting in 401,137 contigs and 235,807 transcripts. Summary statistics of the sequencing results are shown in Table S3. Ten different whole transcriptome assemblies of whitefish embryos were generated in Velvet. The assembly with the highest representation of reads from each library was chosen for downstream analysis of differential expression. On average there were 79.25% of all reads in each library mapping to this assembly. Contigs had an average length of 324 bp and *N50* of 383 bp. Raw read data are archived at the NCBI Sequence Read Archive (SRA) under Accession BioProject number PRJNA293369.

3.3. Whole transcriptome response

Differential gene expression between the two treatment groups at the 0.05 alpha significance level (FDR-corrected) was confirmed in 1096 transcripts out of 235,807. Expression profiles were congruent between the two technical replicates of each sample (results not shown). Among these 1096 reads, 700 unigenes could be annotated using an open blast search. Mean blast and annotation results are shown in Fig. 2. GO terms (n = 700) were further subdivided into cellular components, biological processes, and molecular function (Fig. S1). Within the category of cellular

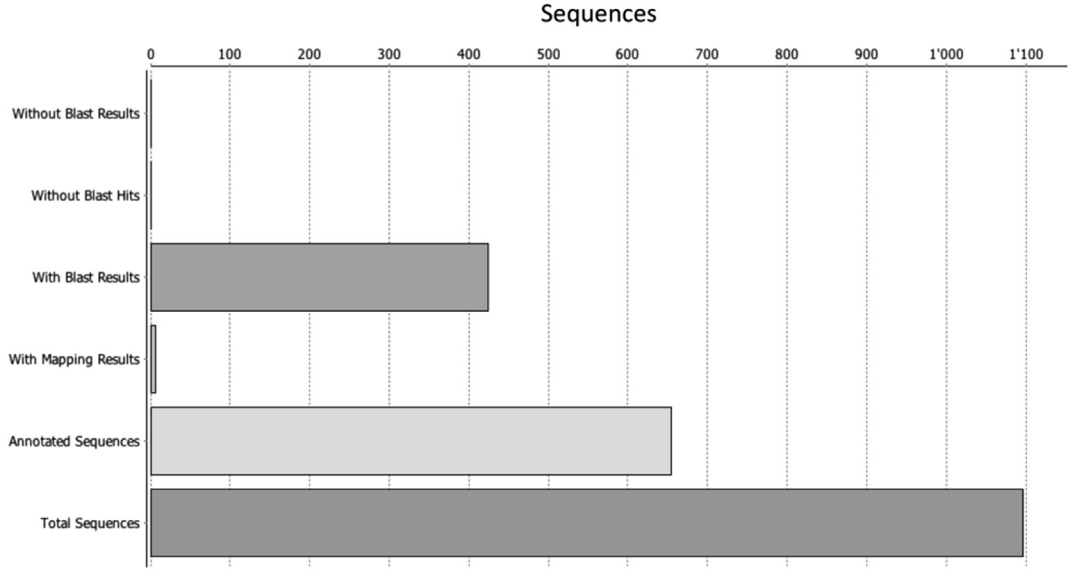

Fig. 2. BLAST and annotation results. A total of 1096 RNA-seq reads from whitefish embryos were blasted against public databases in order to infer their identity. Shown are the numbers of reads that resulted in BLAST and annotation results.

components, 102 genes of protein complexes were differentially expressed between nutrient broth controls and pseudomonad treatment, as well as 45 genes of the cytoskeleton, and 14 genes of the plasma membrane. With regard to biological processes, the majority of genes were involved in signal transduction (n = 57), cellular protein modification processes (n = 43), DNA metabolic processes (n = 28), translation (n = 25), and cytoskeleton organization (n = 24). About a third of all reads could be assigned to the molecular function of ion binding (n = 235), while DNA binding (n = 73), nucleic acid binding transcription factor activity (n = 48), and ATPase activity (n = 37) also present major groups in this category. Enrichment analysis resulted in a selection of 60 different GO categories. They mainly represented genes involved in embryo growth and development. Significantly enriched GO terms are shown in Table S4. The distribution of reads found in different online databases is shown in Fig. S2 and the origin of different species where these genes could be found is illustrated in Fig. 3. Of the 1096 differentially expressed transcripts in this study, 396 could not be identified using public databases (Fig. 4).

KEGG automated pathway analysis of significantly enriched GO terms resulted in 16 differentially expressed functional pathways (Table 1). The most extreme differences were seen in the following three pathways: aminoacyl-tRNA biosynthesis, N-glycan biosynthesis, and purine metabolism. These functional pathways are described in more detail in the Supplementary material including differentially expressed enzymes (Fig. S3).

3.4. Immune gene expression

We could assign 45 different transcripts to putative GO term immune functions of which 13 were differentially expressed between the two treatment groups at the 0.05 alpha significance level (FDR-corrected; Table 2). While pro-interleukin-16 was significantly upregulated in pathogen-infected embryos relative to the nutrient broth controls, all remaining transcripts were significantly downregulated in the pseudomonad treatment. The majority of these transcripts mapped to b-cell leukemia transcription factors (n = 9 different isoforms). Two more transcripts mapped to interleukin enhancer binding factor, and one transcript mapped to t-cell leukemia homeobox protein. All of these results are based on unigenes of the zebrafish model organism database (ZFIN). The remaining transcripts in association with immune functions included complement factors c, h and i, leukocyte surface antigen cd53, MHC class I antigens, cd 151 antigens, cra b, and lymphoid enhancer binding factors. These latter genes were expressed, but not significantly different between pathogen-infected embryos and nutrient broth controls.

Sixty additional sequences of salmonid immune genes were retrieved from GenBank and included in the whitefish transcriptome assembly. Mapping all libraries against this new assembly revealed four more differentially expressed immune genes: MHC class I (downregulated), MHC class II (upregulated), TNF-alpha 2 and T-cell differentiation protein (upregulated; Table 3). Eight more genes that have previously shown to be involved in immune functions were expressed, although not significantly different between nutrient broth control and pathogen treatment (IgH locus B, IgH locus D, CD4 protein, four transcripts of MHC class I and one transcript of MHC class II). Mapping RNA-seq reads against MHC class I alleles in the same species (*C. palaea*) revealed sire-specific expression of alleles (Genes Nr. 10, 11, 12, 21, and 36 in Table 3; other than this specific MHC class I gene, we did not test for sire-specific expression).

4. Discussion

Our study provides the characterization of differential gene expression in non-model fish embryos following infection. We worked with samples of a wild population. In order to control for potentially confounding genetic and environmental effects, our host embryos were produced *in vitro* and comparisons were made within families.

4.1. Whitefish embryonic performance

P. fluorescens increased embryonic mortality markedly in our experiment, causing on average more than 60% mortality in all families. Besides mortality, the pseudomonad also delayed hatching. However, a pattern of delayed hatching could also have been

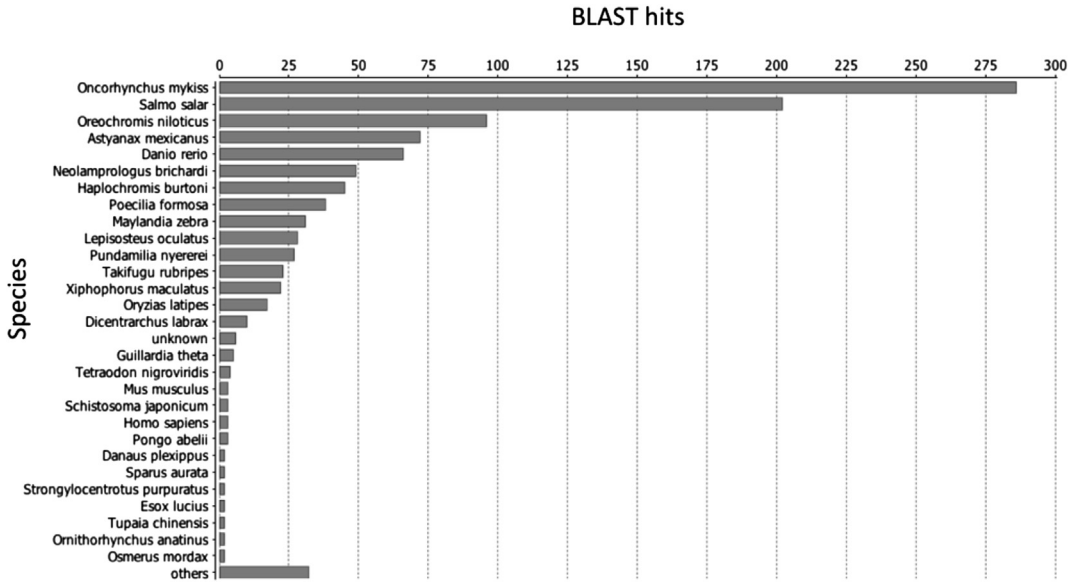

Fig. 3. Distribution of reference species used for the identification of gene ontologies in whitefish embryos. Transcripts were blasted against public databases in order to infer their gene identity. Shown are the numbers of differentially expressed whitefish embryo transcripts that matched a specific species in the protein sequence database (UniProtKB).

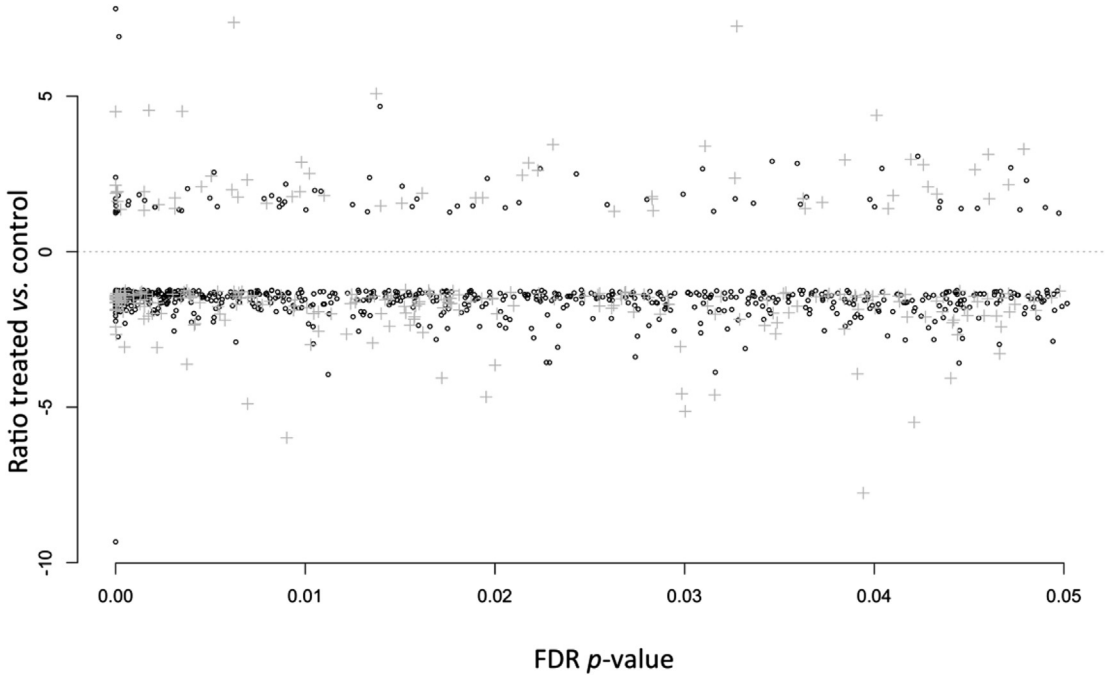

Fig. 4. Differential expression of 1096 transcripts assembled for the whitefish embryo transcriptome. Results are shown as the log2 fold change in expression versus *p*-values that had been corrected for multiple testing (FDR). Only differentially expressed transcripts between pathogen-infected embryos and nutrient broth controls are displayed. More than one third of these transcripts could not be identified (depicted as grey crosses) due to incomplete reference data for whitefish, leaving a set of exciting candidate genes to be identified in the near future (see discussion).

Table 1
Differentially expressed KEGG pathways in whitefish embryos.

Pathway (KEGG)[a]	Enzyme	Enzyme ID[b]	Length (bp)	*p*-value	Centroid ratio[c]
Aminoacyl-tRNA biosynthesis	Ligase	ec:6.1.1.1	3555	0.011	1.5
Aminoacyl-tRNA biosynthesis	Ligase	ec:6.1.1.11	1252	0.004	1.4
Aminoacyl-tRNA biosynthesis	Ligase	ec:6.1.1.12	1962	0.002	1.6
Aminoacyl-tRNA biosynthesis	Ligase	ec:6.1.1.19	1161	0.013	1.7
Aminoacyl-tRNA biosynthesis	Ligase	ec:6.1.1.20	1231	0.007	6.3
Aminoacyl-tRNA biosynthesis	Ligase	ec:6.1.1.21	3293	<0.001	1.5
Aminoacyl-tRNA biosynthesis	Ligase	ec:6.1.1.22	688	0.022	1.4
Arginine and proline metabolism	Dioxygenase	ec:1.14.11.2	4490	<0.001	2.5
Biosynthesis of unsaturated fatty acids	Reductase	ec:1.1.1.100	276	<0.001	0.43
Citrate cycle (TCA cycle)	Dehydrogenase (succinyl-transferring)	ec:1.2.4.2	1975	0.01	0.44
Cysteine metabolism	Isomerase	ec:5.3.1.23	3001	0.01	0.29
Fatty acid biosynthesis	Synthase I	ec:3.1.2.14	277	0.001	0.66
Glycerolipid metabolism	Lipase	ec:3.1.1.3	2057	0.049	0.41
Glycerophospholipid metabolism	Dehydrogenase (NAD+)	ec:1.1.1.8	4306	0.004	2.1
Inositol phosphate metabolism	3-kinase	ec:2.7.1.127	3269	0.024	0.83
Inositol phosphate metabolism	Phospholipase C	ec:3.1.4.11.1	1730	0.001	0.58
Lysine degradation	N-methyltransferase	ec:2.1.1.43	2625	0.002	0.45
mTOR signaling pathway	Protein kinase	ec:2.7.11.24	3080	0.038	0.21
N-Glycan biosynthesis	6-beta-N-acetylglucosaminyl-transferase	ec:2.4.1.155	1437	0.002	0.17
One carbon pool by folate	Reductase	ec:1.5.1.3	4327	0.008	0.62
Purine metabolism	RNA polymerase	ec:2.7.7.6	3201	<0.001	0.17
Purine metabolism	Adenylpyrophosphat-ase	ec:3.6.1.3	2688	<0.001	0.21
Pyruvate metabolism	Synthase	ec:2.3.3.9	2387	0.027	0.71
T cell receptor signaling pathway	Phosphatase	ec:3.1.3.16	1514	0.047	0.47

[a] Functional pathways of significantly enriched GO terms were retrieved using Blast2GO [56,57] and zebrafish information in the KEGG database.
[b] Full pathways for aminoacyl-t-RNA biosynthesis, N-glycan biosynthesis, and purine metabolism including enzymes of interest are shown in the Supplementary material.
[c] Centroid ratio gives the ratio of expression in treated:control (control = nutrient broth at a concentration of 1:1000 in each well, and treated = nutrient broth and *Pseudomonas fluorescens*). Positions of every enzyme in the whitefish embryo transcriptome assembly are listed in the Supplementary material Table S5.

caused by higher mortality of individuals in the pseudomonad treatment that would have hatched earlier. Our data does not allow the discrimination between the two scenarios. Increased mortality and delayed hatching confirm previous findings using the same host-pathogen system [8–10]. Moreover, a previous investigation on other families of the same whitefish population showed a link between the expression of an MHC class I gene and the time until hatching: the less the gene was expressed the earlier the embryos hatched, suggesting a trade-off between immune function and development in whitefish embryos [9].

Table 2
Expressed immune genes in the whitefish embryo transcriptome.

Link to assembly[a]	Length (bp)	*p*-value	Centroid ratio[b]	Description[c]
44	674	0.002◆[d]	2.5	Pro-interleukin-16-like
35	276	0.12	2	Hypothetical protein BRAFLDRAFT_66570
31	2028	0.16	2.5	Complement factor i
1	3131	0.16	2	Complement factor h precursor
9	1354	0.22	2	cd99 antigen precursor
5	338	0.23	2.5	Leukocyte surface antigen cd53
23	1150	0.24	1.67	Mannan-binding protein-associated serine protease 3 precursor
10	770	0.24	2.5	Complement component c8 alpha chain precursor
43	1422	0.27	1.67	mhc class i antigene
38	1228	0.28	1.43	b-cell receptor-associated protein 29-like
34	2563	0.29	2.5	Complement receptor-like precursor
11	1277	0.3	1.67	cd151 antigen-like isoform x1
20	2514	0.3	0.63	b-cell cll lymphoma 11ba (zinc finger protein) isoform x1
26	1286	0.3	0.63	Pre-b-cell leukemia transcription factor 3b isoform 2
8	1481	28	0.67	Myeloid ecotropic viral integration site-related gene isoform cra_b
33	2477	0.27	0.56	Complement receptor-like precursor
36	594	0.27	0.48	Lymphoid enhancer-binding factor 1
28	934	2.6	0.53	t-cell leukemia homeobox protein 3-like isoform x2
22	4869	0.25	0.71	Squamous cell carcinoma antigen recognized by t-cells 3-like
7	1481	0.23	0.77	Myeloid ecotropic viral integration site-related gene isoform cra_b
2	733	0.22	0.63	Mucin-1 precursor (muc-1)
42	943	0.21	0.67	b-cell cll lymphoma 9-like protein
30	452	0.21	0.56	Uncharacterized protein LOC101174103
15	911	0.19	0.71	b-cell translocation gene 3
17	1901	0.19	0.56	b-cell lymphoma leukemia 11b 3 prime
40	2457	0.17	0.59	b-cell cll lymphoma 11ba (zinc finger protein) isoform x2
16	1901	0.17	0.59	b-cell lymphoma leukemia 11b 3 prime
13	2548	0.16	0.77	pre-b-cell leukemia transcription factor 1-like
39	2457	0.16	0.53	b-cell cll lymphoma 11ba (zinc finger protein) isoform x1
3	2656	0.12	0.63	cdk8 protein
37	436	0.12	0.67	Interleukin enhancer-binding factor 2 homolog
41	3351	0.11	0.53	b-cell cll lymphoma 11ba (zinc finger protein) isoform x2
4	2656	0.1	0.71	cdk8 protein
29	2929	0.04◆	0.77	Pre-b-cell leukemia transcription factor 3-like isoform x2
24	821	0.04◆	0.36	b-cell lymphoma leukemia 11a-like
18	1083	0.03◆	0.4	b-cell lymphoma leukemia 11b-like isoform x3
45	437	0.02◆	0.77	interleukin enhancer-binding factor 2 homolog
14	2444	0.02◆	0.77	Pre-b-cell leukemia transcription factor 1-like isoform x5
27	963	0.01◆	0.5	t-cell leukemia homeobox protein 1-like
6	1472	0.01◆	0.63	pre-b-cell leukemia transcription factor 3-like isoform x9
12	1801	0.01◆	0.67	pre-b-cell leukemia transcription factor 1-like isoform x5
25	1776	0.001◆	0.83	Interleukin enhancer-binding factor 2 homolog isoform x3
19	2514	0.001◆	0.53	b-cell cll lymphoma 11ba (zinc finger protein) isoform x1
21	5761	0.0001◆	0.67	b-cell cll lymphoma 11ba (zinc finger protein) isoform x2
32	903	0.0001◆	0.71	b-cell cll lymphoma 7 protein family member a

[a] The position of every immune gene in the whitefish embryo transcriptome assembly is listed in the Supplementary material Table S6.
[b] Centroid ratio gives the ratio of expression in treated:control (control = nutrient broth at a concentration of 1:1000 in each well, and treated = nutrient broth and *Pseudomonas fluorescens*).
[c] The description for every gene is based on gene ontologies (GO terms) as retrieved from Blast2GO [56,57].
[d] Diamonds indicate significant differential expression at the 0.05 alpha significance level (FDR-corrected).

The mechanisms of how the pseudomonad affects and invades the embryo host have not been described yet. In adult *Paralichthys olivaceus*, *P. fluorescens* adheres to the host tissue, followed by its invasion into the host cells, biofilm formation and cytotoxicity [61]. An essential component of host invasion is the autotransporter *PfaI* that interacts with host cells and modulates the host immune response [61]. Many *P. fluorescens* strains are able to secrete an extracellular protease that adheres to host mucus, modulates host immune responses and disseminates into host blood cells affecting host mortality [62]. Below we discuss a compilation of differentially expressed genes in whitefish embryos that were identified in this study and that provide a starting point for further investigation of, for example, bacterial virulence factors and embryo–pathogen interactions.

4.2. Whole transcriptomic response

Differentially expressed gene products between pathogen-infected embryos and nutrient broth controls were summarized using three main categories: cellular components, biological processes and molecular function. Within each category we found major groups of genes that were affected by *P. fluorescens*. In order to characterize the host-pathogen interaction of whitefish embryos and *P. fluorescens* in more detail, we zoomed into the major enriched GO term transcripts and identified differentially expressed functional pathways. Ion binding was highly noticeable. It was linked to almost 30% of all known, differentially expressed gene transcripts. The involvement of zinc ions in a plethora of bacterial virulence pathways makes zinc homeostasis a promising target for antimicrobial strategies [63]. Bacterial pathogens have been shown to produce high-affinity zinc importers in order to grow and multiply in the infected host. Hence, the upregulation of zinc pathways is likely pathogen-mediated.

Another prominently upregulated pathway in pathogen-infected embryos was aminoacyl-tRNA-biosynthesis. Loaded tRNAs are of great value for many pathogens [64,65]. The pathogen might import host products that are valuable for its own metabolism [66]. *Legionella* infections in *Dictyostelium* revealed the

Table 3
Expressed immune genes in whitefish embryos using candidate salmonid genes in the mapping assembly.

Link to GenBank[a]	Gene	Species	C1[b]	T1[c]	C2	T2	C3	T3	C4	T4	*p*-value[d]	Centroid ratio[e]
1	MHC class II exon 1-3	*C. clupeaformis*	+	+	+	+	+	+	+	+	0.001	33.3
2	TNF-alpha 2	*S. salar*	+	+	+	+	+	+	+	+	0.002	19.8
3	MHC class I	*Coregonus* sp.	+	+	+	0	+	+	+	+	0.002	0.32
4	T-cell differentiation protein	*S. salar*	+	+	+	+	+	+	+	+	0.004	1.47
5	IgH locus B	*S. salar*	+	+	+	+	+	+	+	+	0.12	20.1
6	IgH locus D	*O. mykiss*	+	+	+	+	+	+	+	+	0.12	10.2
7	CD4 protein	*S. salar*	+	+	0	0	+	+	+	0	0.26	0.61
8	MHC class I	*S. salar*	+	+	+	+	+	+	+	+	0.31	1.88
9	MHC class II exon 1-3	*C. clupeaformis*	+	+	+	+	+	+	+	+	0.34	50.2
10[f]	MHC class I	*Coregonus* sp.	+	+	0	0	+	+	0	0	0.51	0.64
11[f]	MHC class I	*Coregonus* sp.	+	+	0	0	+	+	0	0	0.64	0.5
12[f]	MHC class I	*Coregonus* sp.	0	0	+	+	+	+	0	0	0.9	0.49
13	TNF-alpha 1	*S. salar*	+	0	0	0	0	0	+	0	–	–
14	Interleukin 1 beta 2	*O. mykiss*	0	0	0	0	0	0	0	0	–	–
15–20	MHC class I	*Coregonus* sp.	0	0	0	0	0	0	0	0	–	–
21[f]	MHC class I	*Coregonus* sp.	+	+	0	0	+	0	0	0	–	–
22–35	MHC class I	*Coregonus* sp.	0	0	0	0	0	0	0	0	–	–
36[f]	MHC class I	*Coregonus* sp.	+	+	0	0	+	0	0	0	–	–
37–53	MHC class II antigen	*Coregonus* sp.	0	0	0	0	0	0	0	0	–	–
54	CD4 protein	*S. salar*	0	0	0	0	0	0	0	0	–	–
55–59	T-cell receptor alpha	*S. salar*	0	0	0	0	0	+	0	0	–	–
60	CD3 epsilon	*S. salar*	0	0	0	0	0	0	0	0	–	–

[a] Candidate sequences related to the major histocompatibility complex (MHC) in different salmonid species were retrieved from GenBank and added to the whitefish assembly in order to detect differentially expressed genes in whitefish embryos. Gene numbers correspond to the following GenBank accession numbers: 1 = HQ287750.1 [86], 2 = DQ787158.1 [37], 3 = AF213288.1 [60], 4 = GO065018.1 [38], 5 = GU129140.1 (direct submission), 6 = AH014877.2 (direct submission), 7 = NM_001123611.1 (direct submission), 8 = AAA49602.1 [36], 9 = HQ287749.1 (direct submission), 10 = AF213289.1 (direct submission), 11 = AF213295.1, 12 = AF213312.1, 13 = DQ787157.1 (direct submission), 14 = AJ245925.2 (direct submission), 15 = AF213287.1, 16 = AF213300.1, 17 = AF213301.1, 18 = AF213292.1, 19 = AF213297.1, 20 = AF213291.1, 21 = AF213294.1, 22 = AF213290.1, 23 = AF213298.1, 24 = AF213296.1, 25 = AF213299.1, 26 = AF213288.1, 27 = AF213293.1, 28 = AF213306.1, 29 = AF213307.1, 30 = AF213309.1, 31 = AF213302.1, 32 = AF213310.1, 33 = AF213308.1, 34 = AF213304.1, 35 = AF213305.1, 36 = AF213311.1, 37 = JN029749.1 (direct submission), 38 = JN029750.1, 39 = JN029751.1, 40 = JN029752.1, 41 = JN029753.1, 42 = JN029754.1, 43 = JN029755.1, 44 = JN029756.1, 45 = JN029757.1, 46 = JN029758.1, 47 = JN029759.1, 48 = JN029760.1, 49 = JN029761.1, 50 = JN029762.1, 51 = JN029763.1, 52 = JN029764.1, 53 = JN029765.1, 54 = DQ867018.1 (direct submission), 55 = EF466709.1 (direct submission), 56 = EF466717.1, 57 = EF466719.1, 58 = EF466721.1, 59 = EF466869.1, 60 = BT060075 (direct submission).

[b] C stands for control group (nutrient broth at a concentration of 1:1000 in each well) and.

[c] T stands for treated group (with nutrient broth and *Pseudomonas fluorescens*).

[d] *P*-values were FDR-corrected and considered significant at ≤0.05.

[e] Centroid ratio gives the ratio of expression in treated:control. A plus indicates that a gene is expressed, whereas a zero shows that we could not detect expression.

[f] Gene numbers 10, 11, 12, 21, and 36 are MHC class I alleles that were expressed in the corresponding sire genotypes only. Gene numbers 20, 22, and 27 were found in the same whitefish dam in a previous study [9].

upregulation of many aminoacyl-tRNA synthetases already early in infection, and most of them remained upregulated throughout the course of infection [67]. Differentially expressed genes of cytoskeleton synthesis and plasma membrane maintenance, as we found them here in whitefish embryos, have shown to play a role in the transport of these metabolites to the pathogen [68].

The metabolism of arginine and proline was also upregulated relative to the control. Arginine in particular has been shown to represent an essential amino acid for a few pathogenic bacteria [69]. It is likely that the increased metabolism of this amino acid is pathogen-mediated, especially if the amino acid is needed for proliferation of *P. fluorescens*. Alternatively, arginine can play the role of a substrate for the host's production of antimicrobial agents [70]. Host-mediated upregulation of arginine synthesis may therefore not be ruled out.

Another pathway that was found to be markedly upregulated in pathogen-infected embryos was the glycerophospholipid metabolism. Many pathogens secrete or contain phospholipase activity that can destabilize plasma membranes of their hosts in order to invade cells or to extract nutrients [*e.g.*, [71,72]]. For *P. fluorescens* a hemolytic activity has been characterized with the purpose of acquiring iron from lysed blood cells [73]. Consequently, the upregulated pathway in whitefish embryos might result from an attempt of the host to stabilize its membranes [74]. Glycerophospholipids have also been shown to be involved in the host's chemokine production during bacterial infections [75], such as interleukin release [76]. This aligns well with our observation of upregulated interleukin-16 production in whitefish embryos that had been exposed to the pathogen.

In contrast to those aforementioned upregulated pathways in pathogen-infected embryos, the majority of pathways were downregulated relative to the control. Downregulated pathways in whitefish embryos included the biosynthesis of unsaturated fatty acids, glycerolipids and N-glycan. Furthermore, the metabolism of cysteine, inositol phosphate, purine, pyruvate, the citrate cycle, and lysine degradation were also downregulated. These are all essential metabolic pathways in vertebrate embryos [7]. They are involved in energy production, growth, and cell-differentiation during embryogenesis. Their down-regulation might stem from a compromise with immune functions [77]. This compromise could potentially lead to reduced growth and later hatching, as it has been observed in previous studies of pathogen-infected whitefish embryos [8,9].

Pathogens sometimes manipulate the host by differentially regulating its metabolism; *i.e.*, upregulating activities that produce suitable nutrients for the pathogen and downregulating the host-specific metabolism [65,67,68]. Whether the observed up- and downregulations are mediated by the pathogens or the hosts remains unclear.

4.3. Immune gene expression

With regard to putative immune functions, there was a markedly upregulated transcript of pro-interleukin-16. Most other

transcripts were substantially downregulated in pathogen-infected embryos, including nine isoforms of b-cell leukemia transcription factors, two transcripts mapping to an interleukin enhancer binding factor, and one transcript mapping to the t-cell leukemia homeobox protein. B-cell leukemia transcription factors play a leading role in lymphoblastic leukemia [78]. The combination of different b-cell leukemia transcription factors has been shown to be involved in blood cell differentiation and renewal [79]. Their conspicuous differential expression in this study provides further evidence for a correlation of *P. fluorescens* infections and host blood parameters. Congruent results have been found in adult carp (*C. carpio*) with a significant decrease of hematocrit, erythrocyte number, total plasma protein albumin and ion levels following *P. fluorescens* infections [80].

We also found a number of expressed transcripts mapping to presumed immune functions, which were not significantly different between pathogen-infected embryos and the control group. In order to sensibilize the mapping of whitefish embryo transcripts and improve the resolution of the assembly, 60 sequences of candidate salmonid immune genes were added. Here, differentially expressed whitefish embryo transcripts during infection mapped additionally to MHC class I genes in different *Coregonus* species, MHC class II genes in *Coregonus clupeaformis*, and TNF-alpha 2 and T-cell differentiation protein in *S. salar*. MHC class I genes were mostly downregulated, which might involve pathogen-mediated suppression of transcription [81,82] or a trade-off by the host with other metabolic functions or immune pathways [83]. The combined upregulation of MHC class II genes and a T-cell differentiation protein provides evidence for the activation of the host's adaptive immune response. However, targeted immune-assays are needed to confirm this result. Nevertheless, this finding corroborates previous results on the importance of MHC class I [9,22] and class II genes [15,17,24] in embryonic survival during experimental pathogen infections. Mapping against specific MHC class I beta exon 1 sequences in the same species even revealed sire-specific (or allele-specific) expression. This high resolution of mapping is lost if only a consensus assembly is used as a reference since the assembling algorithm cannot include all actual alleles at this highly polymorphic locus [84].

4.4. Transcriptomic host responses in non-model fish species

With the paucity of genomic resources available for most non-model organisms, RNA-seq-based gene expression analysis offers a great opportunity for unraveling host gene responses to their pathogens in an ecologically relevant context. Our approach to filter out differentially expressed reads implied: (i) expression of a transcript in all samples within a treatment group, (ii) a stringent similarity threshold to the open reference database, and (iii) upper quartile normalization to improve robustness of differential expression for less abundant transcripts. Accordingly, this conservative approach was chosen in order to make sure that the expression of a gene is essential, to increase confidence in the inferred identity of a gene transcript, and to avoid outliers that are due to the extreme expression in a few individuals. Of the 1096 differentially expressed transcripts in this study, 396 could not be identified using public databases. Twenty-five percent of these reads were among the most abundant reads in challenged whitefish embryos. This calls for more research on their functions in the embryonic host-pathogen interactions. Incomplete reference continues to be a major challenge for studies of ecological and evolutionary genomics in non-model organisms [21]. As an example, the inclusion of 60 candidate salmonid immune genes significantly improved the resolution of our assembly for identifying differentially expressed immune genes. The results from this study may be updated as new sequence information of salmonids is obtained in the future.

Our salmonid embryo model allowed us to keep environmental variation at a minimum and to control for the genetic background of the embryos (*i.e.*, genetic and maternal environmental effects). We identified a suite of presumably immune-relevant genes in whitefish embryos that responded to pathogen infection. This provides a first insight into which processes are likely to be important for whitefish embryos. The extension from between treatment group differences in expression patterns to within group variation will lead to the quantification of the components of phenotypic variation under various ecological conditions [85].

5. Conclusions

When studying the transcriptomic response in pathogen-infected whitefish embryos compared to a control group, we captured a broad representation of whitefish genes at this developmental stage, including previously undescribed transcripts. Transcript levels of genes are reported, applying a “blind” approach whereby reads are first quantified, normalized, and only differentially expressed reads are identified using multi-organisms, public databases. For non-model species in particular, this approach turned out to be time efficient [48]. In parallel, we mapped all expressed reads against a salmonid-specific reference of immune genes to discuss the capabilities of whitefish embryos to mount an adaptive immune response at this early developmental stage. As we looked at the whole transcriptomic response to *P. fluorescens* infection, we were able to shed light on genes that a) the pathogen might manipulate to establish and/or maintain an infection and also b) the host can regulate as response in terms of reducing damage or potentially initiating an immune response; while we experimentally controlled for potential effects of host family. Accordingly, a few promising motives could be identified: (1) Host production of several metabolites was increased, such as nucleotides, loaded tRNAs, arginine, proline, and zinc ion pathways. These metabolites most likely provide additional nutrients to the pathogen [64,65]. (2) Transcripts that contribute to nutrient transporters were also differentially expressed, such as genes involving glycerophospholipids, cytoskeleton formation, cytoplasmic membrane-bound vesicles, transmembrane transporter activity, and ATPase activity. (3) The involvement of several different genes associated with host blood differentiation suggests a central role of this metabolism during the whitefish embryo-*P. fluorescens* interaction [80]. (4) The production of many essential metabolites for the host development was downregulated, most probably due to a trade-off with the activation of other metabolic functions in host embryos. (5) Several transcripts of putative immune genes in whitefish embryos were differentially expressed, including a prominent upregulation of the MHC class II pathway and the corresponding T-cell differentiation protein. In summary, we have demonstrated an extensive transcriptomic response. The elaborate list of differentially expressed pathways in pathogen-infected *C. palaea* embryos expands our knowledge on host-pathogen interactions in fish embryos and encourages more research using this very promising model.

Author contributions

L.Fa., L.Fu., C.W., and L.W. designed the project. L.Fu. and C.W. supervised the project. E.C., C.W. and L.W. sampled the fish, did the *in vitro* fertilizations, and distributed the fertilized eggs to plates. All further manipulations and measurements were done by E.C. and L.W. L.W. and L.Fa. performed the molecular genetic and the statistical analyses. L.W. wrote a first version of the manuscript that

was then critically revised by E.C., L.Fu., and C.W.

Competing financial interests

All authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2015.08.035.

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