



Comparative transcriptomics of stickleback immune gene responses upon infection by two helminth parasites, *Diplostomum pseudospathaceum* and *Schistocephalus solidus*[☆]

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ABSTRACT

Immune systems of vertebrates are much more diverse than previously thought, in particular at the base of the vertebrate clade. RNA-seq was used to describe in detail the transcriptomic response of stickleback hosts to infection by two helminth parasites, the trematode *Diplostomum pseudospathaceum* (2 genotypes plus a genotype mix) and the cestode *Schistocephalus solidus*. Based on a global transcription profiling, we present immune genes that are active during chronic or multiple repeated infection. We found that the transcription profiles of *D. pseudospathaceum* genotypes were as divergent as those of the two parasite species. When comparing the host immune response, only 5 immune genes were consistently upregulated upon infection by both species. These genes indicated a role for enhanced toll like receptor (TLR) activity (CTSK, CYP27B1) and an associated positive regulation of macrophages (CYP27B1, THBS1) for general helminth defense. We interpret the largely differentiated gene expression response among parasite species as general redundancy of the vertebrate immune system, which was also visible in genotype-specific responses among the different *D. pseudospathaceum* infections. The present study provides the first evidence that IL4-mediated activation of T-helper lymphocyte cells is also important in anti-helminthic immune responses of teleost fish.

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1. Introduction

In order to defend against infection by parasites and pathogens, animal species have evolved immune systems. In vertebrates, these consist of a more general innate immune response and an adaptive, more specific defense line that also entails immune memory. In recent years, evidence is accumulating that vertebrate immunity is much more diverse than previously thought (Star and Jentoft, 2012). In particular at the base of the vertebrate clade, some fishes seem to have modified important pathways of the adaptive immunity (Star et al., 2011; Haase et al., 2013). Even within species of teleost fishes with classical “textbook” immune systems, immune

responses are complex and show an intricate temporal pattern from first to later infection, with innate immunity being replaced progressively by adaptive immune pathways (Haase et al., 2016). Moreover, different parasite or pathogen species elicit different immune defense pathways (Rauta et al., 2012) while any immune system response is modulated by other environmental conditions (Maizels and Nussey, 2013).

Fishes, including our model species, the three-spined stickleback (*Gasterosteus aculeatus*), are infected by a huge diversity of micro- and macroparasites. In northern European waters, helminths of the classes Cestoda and Trematoda that challenge the immune system upon infection (Scharsack et al., 2007) are particularly prevalent (Kalbe et al., 2002). In helminth infections of mammals, it is well established that those parasites interfere with the balance of T-helper (Th) lymphocyte subsets. At first a Th1 response is induced, which activates cytotoxic cells and phagocytes that eventually eliminate invading parasite larvae. When the parasite establishes, a Th2-induced adaptive immune response is

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upregulated, which mediates the production of parasite-specific antibodies (Muraille et al., 2014; Bashi et al., 2015). These processes are further mediated by coating the parasitic worm with antibodies. After binding, cells can externalize internal vesicles with toxic substances onto the parasite (Janeway et al., 2008). How pattern recognition of multicellular parasites works is not yet fully understood. The response strategy to parasitic worms probably involves toxic products of eosinophils, e.g. reactive oxygen species (Nish and Medzhitov, 2011). The host response likely depends on the size or life stage of the intruding parasite. Other countermeasures might involve an increased creation of barriers, coagulation and even muscle cell contractions (Nish and Medzhitov, 2011). On the other hand, helminths are well known for their ability to manipulate and evade the host's immune system (Allen and Maizels, 1996; Hewitson et al., 2009; Wang et al., 2015). In recent years, helminth-mediated immunity effects have received increasing attention, since positive effects of helminths during autoimmune diseases and allergies are discussed (Danilowicz-Luebert et al., 2011; Graepel et al., 2013; Hernandez et al., 2013).

In the present study, we investigated two helminth species, both of which appear to be excellent immune evaders and manipulators (see also the review by Scharsack et al., 2016), infecting three-spined sticklebacks. The eye fluke *Diplostomum pseudospathaceum* is a digenetic trematode with a complex tri-phasic life cycle, involving piscivorous birds as definitive host, and lymnaeid snails as first and freshwater fish species as second intermediate hosts (Chappell et al., 1994). In the fish, it evades the fish immune system by invading the eye lens (Locke et al., 2015), an immunologically privileged site (Stables and Chappell, 1986), which the migrating parasite stages reach within <24 h after penetration of the fish skin (Whyte et al., 1991). Accordingly, invading diplostomules are exposed to the host's immune system only for a very short time, which in sticklebacks triggers a fast, and partly genotype-specific, innate immune response (Rauch et al., 2006). However, repeated exposure of sticklebacks to the eye fluke induces (incomplete) adaptive immunity pathways eventually (Scharsack and Kalbe, 2014).

Our second model parasite, the cestode *Schistocephalus solidus*, is trophically transmitted and has cyclopid copepods as first, three-spined stickleback as obligatory and specific second intermediate and fish-eating birds as final hosts (Bråten, 1966; Orr et al., 1969). This parasite can modify its surface structure in order to "hide" from the host's immune response (Hammerschmidt and Kurtz, 2005; Franke et al., 2014) and is capable of downregulating the stickleback's granulocyte responses (Scharsack et al., 2004), for which it presumably uses secretory/excretory products (Scharsack et al., 2013) and potentially prostaglandins (Kutyrev et al., 2014).

In teleost fishes, mobilization and activation of granulocytes is considered a significant part of the immune response, e.g. in cyprinids (Hoole and Arme, 1983; Richards et al., 1994; Taylor and Hoole, 1995) and rainbow trout (Sharp et al., 1991) reacting to helminth parasites. Increased production of oxygen intermediates like nitric oxide (NO) and reactive oxygen species (ROS) occurs (Whyte et al., 1989; Secombes and Chappell, 1996; Scharsack et al., 2004), indicating the functional importance of granulocyte and macrophage responses to helminths. Indeed, it has been demonstrated in vitro that rainbow trout macrophages kill larval stages of the eye fluke *Diplostomum spathaceum* (Whyte et al., 1989). However, large helminth parasites of fish do not seem to be killed by cellular responses in vivo (Secombes and Chappell, 1996). Accumulating evidence suggests that the anti-helminthic immune response of many fish species, similarly to mammals, is characterized by a shift from a Th1 response towards Th2-mediated humoral responses (Buchmann, 2012). Molecular studies have shown that the Th1/Th2 system is also present in fish (Takizawa et al., 2008, 2011, 2013).

For one of the model systems used in the present study, the infection of sticklebacks (*G. aculeatus*) with the tapeworm *S. solidus*, it has been suggested that the tapeworm interferes with the Th1/Th2 balance (Barber and Scharsack, 2010). Indeed, adaptive Th2-mediated immunity seems to be detrimental to the parasite. Host genotypes with optimal allelic subsets of a key adaptive immune gene, the major histocompatibility complex class II (MHC II), suppress growth of the parasite once infected (Kurtz et al., 2004).

Here, we combined transcriptome datasets obtained by RNA-seq from two gene expression studies on the long-term effects of infections with multicellular parasites on three-spined sticklebacks. Sticklebacks are established model organisms which can be bred easily in the laboratory. Moreover, various genomic resources are available for this species, such as a well-annotated genome and several transcriptomes (Gibson, 2005).

In both parasites, the stickleback host's gene expression responses were analyzed after several weeks, during a chronic phase of the infection, which in the case of *D. pseudospathaceum* was triggered by repeated exposure to infective parasite stages while in the case of *S. solidus* the ongoing development of the worm in the host's body cavity constituted a chronic disease. Hence, for both parasites we expected to find signatures of an upregulation of adaptive immunity, such as MHC II, immunoglobulins and Th2 cells. In teleosts, it is an open question whether or not helminths interfere with the Th1/Th2 balance. One goal was therefore to assess indications for Th1/Th2 regulation, at least in the case of the *S. solidus* infection.

Given our previous findings on the effects of single exposure to parasite genotypes in sticklebacks (Haase et al., 2014), we expected parasite species-specific host immune responses and generalized host immune responses against parasitic worms (Haase et al., 2014). Hence, we wanted to assess differences and similarities in gene expression of the stickleback host in the face of *Diplostomum* and *Schistocephalus* infections over several weeks, using exactly the same RNA-seq methodology. In a second step, the hypothesized between-species effect was compared with the effect of specific genetic lineages of *Diplostomum pseudospathaceum* on host immune gene expression. This treatment included two single clone lineages and a mix of clonal parasite lineages.

In the present comparative study we wanted to identify (i) effects of long-term exposure to *Diplostomum* on RNA expression patterns in the stickleback host, and (ii) similarities and differences in global gene expression in response to long-term exposure to *Diplostomum* and *Schistocephalus*.

2. Materials and methods

The present study compares two data sets, one on the global transcriptome response of stickleback hosts to the eye fluke *D. pseudospathaceum*, which has already been published (Haase et al., 2014, 2016), and a second one on exposing sticklebacks to the tapeworm *S. solidus*, which is analyzed here for the first time. Previously unpublished RNA-seq data for sticklebacks infected with *S. solidus* were deposited into the GenBank trace file archive (BioProject PRJNA308353). In case of the *D. pseudospathaceum*–stickleback data set (BioProject PRJNA253091 and BioProject PRJNA276419), published RNA-seq data (Illumina, San Diego, CA, USA) of two transcription-profiling projects was reanalyzed to enhance the comparability of gene expression differences within both experiments.

In the first sub-experiment, sticklebacks were exposed to *D. pseudospathaceum* according to one of three treatments, either one of two single clones (*Diplostomum* clone I, *Diplostomum* clone XII) or a mix of clones (*Diplostomum* clone mix). The *Diplostomum* clone mix contained clones I and XII plus six additional clones (Rieger

Table 1

Treatment overview of analyzed RNA-seq data. The replication level in each treatment was $N=4$ for *Diplostomum* and $N=14$ – 17 for *Schistocephalus* infection. Given is the infecting parasite (*Diplostomum pseudospathaceum* or *Schistocephalus solidus*); “genotype” gives the genetic diversity of the parasites used for infection of sticklebacks; “habitat” shows the host and parasite habitat; “inf.temp” is the temperature at which sticklebacks were exposed to the parasite; “const.temp” is the temperature at which infected sticklebacks were kept; “sampling.dpi” is the number of days fish were sampled post infection; n/a = not applicable.

Parasite	Genotype	Habitat	inf.temp	const.temp	sampling.dpi
sham infection	n/a	Lake	18 °C	18 °C	49
<i>Diplostomum</i>	Clone I	Lake	18 °C	18 °C	49
<i>Diplostomum</i>	Clone XII	Lake	18 °C	18 °C	49
<i>Diplostomum</i>	Clone Mix	Lake	18 °C	18 °C	49
sham infection	n/a	River	18 °C	13/24 °C	50
<i>Schistocephalus</i>	Genotype Mix	River	18 °C	13 °C	50
<i>Schistocephalus</i>	Genotype Mix	River	18 °C	24 °C	50

et al., 2013). Fish were kept in tanks in groups of 10 fish per tank, and for 5 weeks, every week 20 cercariae per fish were added to the tank. After two more weeks, fish were placed in small tanks and individually exposed to 100 cercariae of either *Diplostomum* clone I, XII or the clone mix. Fish were sacrificed by an incision into the head, 4 h after the final infection, and immediately placed in RNAlater. Non-exposed fish were used as a control. Head kidney tissue of sticklebacks was used for RNA-sequencing (Illumina). See Haase et al. (2014, 2016) for complete details of the experimental procedure.

In the second sub-experiment, sticklebacks were infected with *S. solidus*. Sticklebacks were laboratory-raised F1 offspring from 8 parental pairs (families) originating from a brook in north-west Germany (Ibbenbürener Aa; 52°17'31.76" N, 7°36'46.49" E). At the start of the experiment, sticklebacks were 5–6 months old and sexually immature. Experimental groups were gender-matched, resulting in even sex ratios across treatments. Stickleback families were assigned to the treatments in a balanced design. In order to achieve a homogeneous load, single host fish were exposed to two infected 1st intermediate hosts (copepods) containing a total of 3 proceroids of different genotypes. After long-term (50 days) parasite exposure the experiment resulted in sticklebacks being infected with 1–3 parasites. As one of the organs with major immunological activity during chronic infection, extracts of liver were used for RNA-sequencing (Illumina platform).

In both sub-experiments, sticklebacks were sympatric with their respective parasite genotypes, and infection was ongoing for about 7 weeks (i.e. chronic); in case of the *Diplostomum* experiment, infection rounds were conducted weekly (see Table 1 and Table S1 in the supplementary online Appendix for an overview). To ensure comparability between experiments, we selected fish with similar experimental parameters in terms of fish age and feeding regime, while there were differences in water temperature (Table 1). In the *Schistocephalus* sub-experiment, experimental fish from both water temperatures were analyzed jointly to achieve a similar temperature effect as in the *Diplostomum* sub-experiment with an intermediate temperature.

Both sub-experiments were biologically replicated, albeit with different numbers of replicates. In the *Diplostomum* sub-experiment we had four replicates for *Diplostomum* clone I, clone XII and clone mix and three replicates for *Diplostomum* control (i.e. unexposed fish from the *Diplostomum* sub-experiment); in the *Schistocephalus* sub-experiment there were 14 replicates for infected sticklebacks and 17 replicates for non-infected control fish (i.e. sham exposed fish from the *Schistocephalus* experiment). This resulted in 76,177,176 reads for infection treatment with *Diplostomum* clone I, 94,939,082 reads for *Diplostomum* clone XII, 81,766,064 reads for the *Diplostomum* clone mix and 46,487,618 reads for the *Diplostomum* control, as well as 170,117,944 reads for *Schistocephalus* infection treatment and 187,112,978 reads for the *Schistocephalus* control (see Table S1). Sequence reads were aligned to the genomic backbone via TopHat v2.0.13, using Bowtie

2 version 2.2.4, with standard parameters. Reads were aligned to the ensembl *Gasterosteus aculeatus* genome, version 79 (www.ensembl.org/Gasterosteus_aculeatus/Info/Index). Differential gene expression was calculated via the Cufflinks package v2.2.1. We used a false discovery rate (FDR) of $\alpha=0.05$ for correction of multiple testing. To enhance comparability of the data, datasets of both sub-experiments were combined in a single Cuffdiff analysis. For further analyses, custom-made Python 3.4.1 scripts were used. Only differential expression in comparison with the respective treatment control was taken into account.

To analyze differentially expressed genes for enrichment of Gene Ontology (GO) categories, corresponding stickleback GO terms were extracted via the ensembl biomart filter, *Gasterosteus aculeatus* genome version 79. Tests for GO term enrichment were performed via the Gene Ontology Enrichment Analysis Software Toolkit (GOEAST, <http://omicslab.genetics.ac.cn/GOEAST/>) with standard parameters (FDR = 0.1). We tested for enrichment of GO terms in upregulated genes for all four infection treatments (*Diplostomum* clone I, *Diplostomum* clone XII, *Diplostomum* clone mix and *Schistocephalus*) and with a combined set of all genes upregulated in all *Diplostomum* treatments to account for species-specific differences.

To estimate the differential gene expression of putative immune genes, we used a reference list based on earlier studies (Star et al., 2011). To account for changes in genome annotations the list was updated. For this purpose we extracted human gene names associated with the GO category “immune system process” (GO:0002376) from the ensembl biomart filter and matched those to the most recent stickleback genome annotation. The list was complemented with immune genes published in Star et al. (2011). When necessary, missing gene names were obtained via BLASTx 2.2.29+ of stickleback coding sequence data from ensembl, genome version 79. Overall, 1123 putative immune genes, including splice variants, were identified (see Table S2 in the supplementary online Appendix). The immune gene reference list was used to check for the presence of differentially expressed immune genes.

We also visualized global immune gene expression patterns among *S. solidus* and *D. pseudospathaceum* infections using a heat map. This was based on all differentially expressed genes and their $\log_{10} + 1$ -transformed fold changes of expression values and visualized based on a Euclidean distance matrix implemented into the function heat map.2 of the R-package “gplots” (Warnes et al., 2015).

3. Results

The immune gene expression patterns varied markedly among the four different infection treatments (Fig. 1). Qualitatively, the divergence elicited by three genetically different infection treatments of the trematode *D. pseudospathaceum* were as large as some of the inter-species divergences among *S. solidus* and *D. pseudospathaceum*.

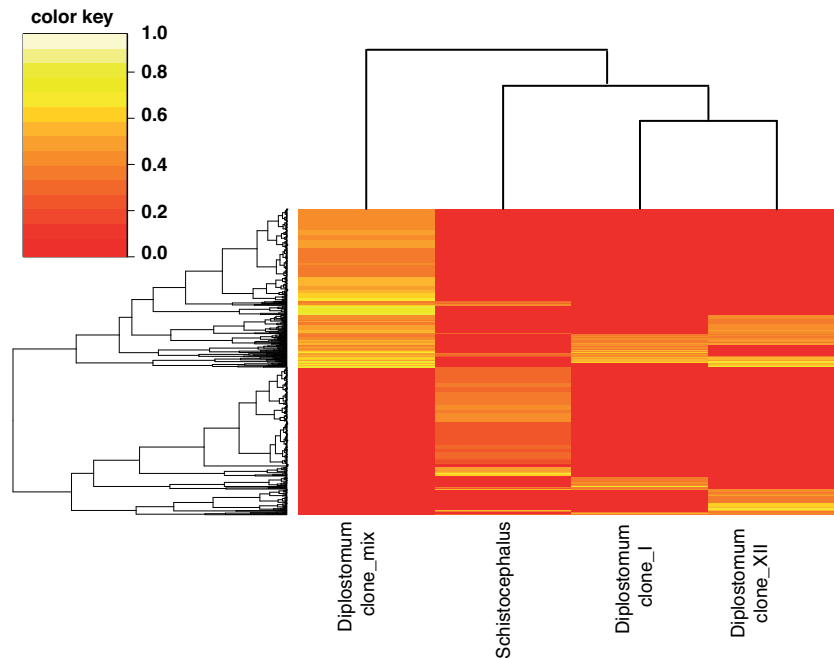


Fig. 1. Heat map of all differentially expressed immune genes in sticklebacks exposed to *Schistocephalus solidus* and *Diplostomum pseudospathaceum*. Displayed according to a color code are $\log_{(10+1)}$ -transformed \log_2 fold changes of genes which are significantly upregulated in one or more treatments. Fold change values of genes which showed no significant upregulation were set to zero (see supplementary Table S3 for differentially expressed genes with unmodified values).

In sticklebacks exposed to *D. pseudospathaceum*, 107 (0.5%) genes were differentially expressed due to clone I treatment, 261 (1.2%) due to clone XII and 381 (1.7%) in fish exposed to a clone mix (see Table S3 in the supplementary online Appendix). Sticklebacks infected with *Schistocephalus* showed differential gene expression of 423 genes (1.9%; Table S3). Of those genes, 32 showed shared upregulation in the *Diplostomum* and *Schistocephalus* treatment groups. 427 and 245 genes were unique to the *Diplostomum* and *Schistocephalus* treatment, respectively. Within the *Diplostomum* treatment group, 30 genes showed unique upregulation for *Diplostomum* clone I, 51 for clone XII and 247 for the clone mix treatment. 25 genes were shared between clone I and the clone mix, whereas clone XII shared 57 genes with the clone mix. 41 genes showed shared upregulation between all three *Diplostomum* treatments, while no genes were shared between clone I and clone XII (Table S3). Since the experiments were conducted separately, expression values cannot be directly compared. However, gene expression for each single data set should still be valid and thus genes differentially expressed should be representative of the respective dataset.

In the GO enrichment analysis of upregulated genes, fish infected with *D. pseudospathaceum* showed 19 enriched GO terms for *Diplostomum* clone XII. Of those, one was associated with a biological process (GO:0007017, microtubule-based process). No GO terms were enriched in the *Diplostomum* clone I and clone mix treatments. Sticklebacks exposed to *S. solidus* showed 20 enriched GO terms (Table S4). Three of those were associated with a biological process (GO:0002573, myeloid leukocyte differentiation; GO:0002682, regulation of immune system process; GO:0018149, peptide cross-linking). The genes associated with an immune response (GO:0002682) were SPI1B, IRF8, INPP5D and C1QC.

Comparison of the differentially expressed, upregulated genes to our list of putative immune genes revealed 66 upregulated genes (Fig. 2). Of those, two were shared between all treatments (ENS-GACG00000000141, THBS1 (1 of 2)), and six were shared between the three *Diplostomum* treatments (DDIT4, CALR, MELK, CDK1, IL4R, SOCS3). The *Schistocephalus* and *Diplostomum* clone mix shared

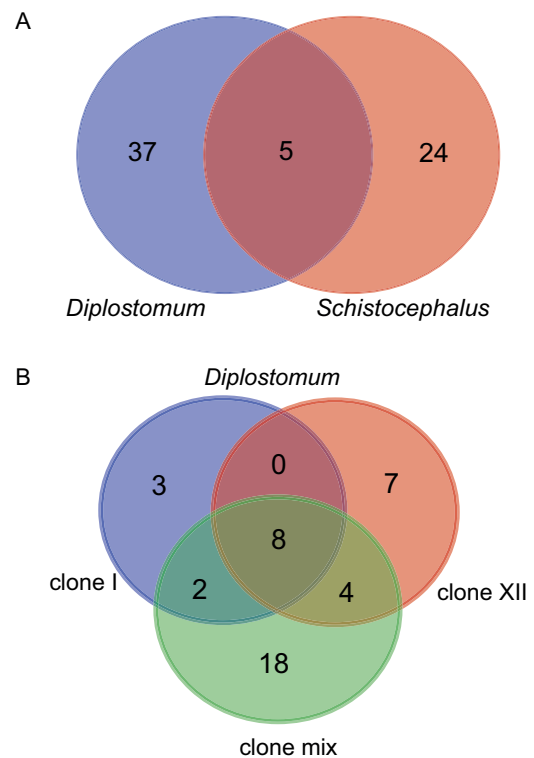


Fig. 2. Venn diagrams of pre-defined differentially expressed, upregulated immune genes. (A) Number of genes shared between sticklebacks infected with *Diplostomum* and *Schistocephalus*, as well as the number of genes unique for each experimental group. (B) Number of genes shared between all combinations of *Diplostomum* treatments, as well as the genes upregulated in a certain *Diplostomum* treatment only (clone I, clone XII or clone mix).

one gene with *Diplostomum* clone I (CYP27B1) and one gene with *Diplostomum* clone XII (C1QC). One gene was also shared between *Schistocephalus* and the *Diplostomum* clone mix alone (CTS). The

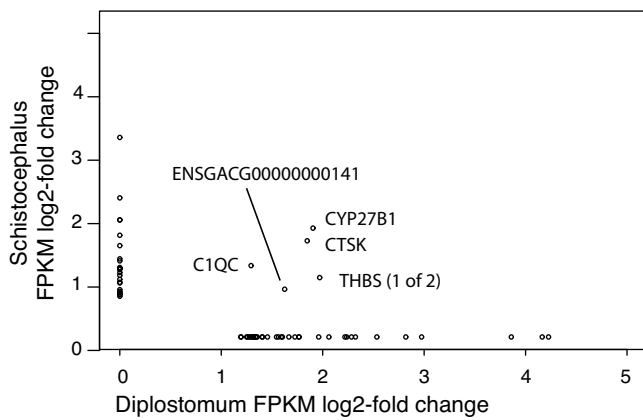


Fig. 3. Directional expression of shared upregulated genes in sticklebacks infected with *Diplostomum pseudospathaceum* and *Schistocephalus solidus*. The plot shows fold changes (\log_2 -scale) relative to the respective uninfected controls based on FPKM (fragments per kilobase of exon per million fragments mapped) for all predefined immune genes differentially expressed in the presence of either parasite species. Where genes are exclusively regulated in one treatment, the FPKM value in the other treatment is set to zero. Five genes show consistent differential gene expression in both treatments.

Diplostomum clone mix shared one gene with clone I (PYCARD (2 of 3)) and three genes with clone XII (SHC1, DUSP6, CEBPB). In total, five genes were shared between the *Diplostomum* treatments and the *Schistocephalus* treatment (ENSGACG00000000141, THBS1 (1 of 2), CYP27B1, C1QC, CTSK). Interestingly, all of them were upregulated in both treatment groups (Fig. 3). Regarding genes active in single treatments only, we found three in *Diplostomum* clone I (CIB1, SEC61B, KIF23), seven in clone XII (IRG1, IRF3, TEC, KIF23 (2 of 2 isoforms), KIF11, ENPP1, KIF4A), 17 in *Diplostomum* clone mix (DUSP7 (2 of 2 isoforms), ITGA5, PDE4D, KDR, SBNO2, FOS, SLC16A3 (1 of 2), SLC16A1, ENSGACG00000019078, HYAL2 (1 of 2 isoforms), SLC7A7, PIK3R1, TGFBR2, TINAGL1, ITGA4, NR4A1, RGCC), and 24 in the *Schistocephalus* treatment (IRF8, AZU1, COL1A2, CYBB, RPS6KA1, C3 (4 of 8 isoforms), EGR1, FGF19, INPP5D, CORO1A, CYBA, HRH1, NCF1, COLEC12, CAV1, TCF21, NCKAP1L, AXL, LCP1, SKAP2, APOA4 (4 of 4 isoforms), CTSS (2 of 2 isoforms), HCK, C1QTNF5).

4. Discussion

In recent years our knowledge on the diverse immune reactions of vertebrates has been greatly expanded, in particular in a basal group of vertebrates, the teleosts. One new focus were responses to genetic diversity within (macro-)parasite species (Rauch et al., 2006; Lenz et al., 2013) and alternative immune system setups (Star et al., 2011; Haase et al., 2013; Buonocore and Gerdol, 2015). The present study dissects immune gene expression of a teleost model fish, the three-spined stickleback, to two helminth species, with the goal to identify commonalities and differences.

This comparative data set contains three types of useful information: (i) the RNA-seq pattern of chronic responses to infection with either of the two macroparasites; (ii) an analysis of the overlap in gene expression responses. Here we focus on the latter part, although comparability is compromised between both sub-experiments owing to different tissues and slightly different conditions. Notwithstanding, the analysis is useful if we restrict ourselves to analyzing the relative expression differences between infected/non-infected individuals within the sub-experiments.

The lack of a consistent immune response involving similar genes and pathways to both infections was one clear result. We found five immune genes that were upregulated in response to both parasite infections. These could be attributed to a gen-

eral anti-helminthic immune reaction, at least towards both investigated species. Among them, we found genes involved in matrix degradation (CTSK, C1QC, THBS1), which point to an ongoing immunological process that targets the parasitic tegument (see www.uniprot.org). This is supported by the mediation of TLR activity (CTSK, CYP27B1) and the resulting positive regulation of macrophages (CYP27B1, THBS1). TLRs as important pathogen recognition receptors have been identified in different fish species, associated with macrophages and granulocytes, the main immune cells of an innate immune response in fish (reviewed in Magnadóttir, 2010). It is evident now that innate and adaptive immunity cannot be seen as separate systems but rather as a defense network (Magnadóttir, 2006). Thus, the presence of C1QC, a complement component which acts as a linker between innate and acquired immunity, further supports our view of an ongoing immune response. C1Q binds to antibodies and activates other C1 subunits to form the complement component C1QC, thus leading to the initiation of the classical complement pathway (see www.uniprot.org). This indeed suggests that adaptive, antibody-mediated immunity is upregulated in both helminth infections. We have already observed in an earlier study that gene expression of the complement system (C3–C9, among others) shows parasite genotype dependent differences during the first stages of *D. pseudospathaceum* infections (Haase et al., 2014). But here, stickleback immune gene expression was analyzed 4h after exposure to *D. pseudospathaceum* and upregulated complement components were attributed to the alternative complement pathway, solely based on the activity of innate immunity (Haase et al., 2014).

In the case of chronic infections by either of the two species *D. pseudospathaceum* and *S. solidus*, the presence of C1 subunits points to a constant reactivation of the complement system, presumably associated with an upregulation of adaptive immunity. This was also supported by the observed upregulation of genes of the major histocompatibility complex (MHC). This involved genes associated with MHC class II antigen presentation (CTSK) and MHC class I alpha antigen presentation (ENSGACG00000000141) possibly activating cytotoxic T cells mediated by MHC I (see www.uniprot.org). In addition, the expression patterns identified also point towards an MHC II and Th2-mediated activation of B cells and antibody production. Due to the nature of the experimental approach, differences in the effects of single versus mixed genotypes in *S. solidus* cannot be controlled. This might influence the comparability of the two experiments involving *D. pseudospathaceum* and *S. solidus*. To circumvent this problem we selected *Diplostomum* fish which had been exposed to single and mixed parasite genotypes. This should guarantee an identification of similarities induced by single and multiple parasite genotypes. Furthermore, due to different organs used in the *D. pseudospathaceum* and *S. solidus* studies, we cannot rule out an effect of organ type on the differences observed between both treatment groups. But this will mostly affect the differences between both groups, not the similarities. We are therefore convinced that inferences on genes that share upregulation in the *S. solidus* and *D. pseudospathaceum* infection treatments are conservative with respect to the research question.

The general immune response against *D. pseudospathaceum* shows MHC activity (ENSGACG00000000141) and positive regulation of macrophages (THBS1); both are shared with the *S. solidus* infection treatment. Furthermore, the genes suggest immunoglobulin and chemokine production (IL4R), increasing support for TLR activity (CDK1) and responses to cellular and oxidative stress (MELK, CALR, DDIT4; see www.uniprot.org). Further support for these responses can be found in genes shared between either one of the two single clone treatments and the clone mix. We found responses to oxidative stress (SHC1) and signs of TLR activity (DUSP6, CYP27B1). Cytokine and chemokine processing and activation (PYCARD) are also supported, as well as macrophage

activity and MHC-based immune responses (CEBPB). Particularly interesting is the upregulation of interleukin 4 (IL4R). In mammals (mice/humans) it is well established that IL4 induces a Th2 response during helminth infections (Kopf et al., 1993; Sekiya and Yoshimura, 2016). To the best of our knowledge, the present study provides the first evidence that IL4-mediated activation of Th cells is also important in the anti-helminthic immune responses of teleost fish.

In the response to *Diplostomum* clone I, the gene CIB1 indicates an increase in cell proliferation and migration, probably associated with genes involved in MHC class I (SEC61B) and class II antigen processing and presentation (KIF23). In *Diplostomum* clone XII we also saw MHC class II antigen processing and presentation (KIF23, both isoforms, KIF11, KIF4A). It is interesting to note that different isoforms of KIF23 are active in both monoclonal *Diplostomum* treatments. This underlines that hosts are able to respond to specific parasite genotypes on the level of gene expression. Besides the activity of MHC class II-mediated immunity and T-cell development (TEC), we also found upregulated genes involved in macrophage and TLR activity (IRG1, IRF3). The *Diplostomum* clone mix caused the largest number of differentially expressed genes. We have already observed this pattern in sticklebacks that were exposed to *D. pseudospathaceum* only once (Haase et al., 2014). It is likely that the larger genetic diversity enhances immune stimulation and challenge owing to a larger array of genotype-specific host responses. Thus, we found basically similar responses as in the monoclonal treatments *Diplostomum* clone I and clone XII. We observed TLR activity (DUSP7, CTSK, FOS) and MHC class II antigen presentation (CTSK), combined with macrophage activity (SBNO2). In addition, we found MHC class II related T-cell activity (PDE4D, ENSGACG00000019078 [IL8], PIK3R1, ITGA4) and genes involved in leukocyte migration (SLC16A3, SLC16A1, ITGA5, SLC7A7, PIK3R1).

The small number (8 out of 1123; 0.7%) of upregulated immune genes shared between the *Diplostomum* treatments is likely due to the redundant nature of immune system processes (Nish and Medzhitov, 2011), especially since immunological processes associated with the detected genes seem to overlap (e.g., TLR activity; Haase et al., 2014). However, we cannot rule out the possibility that in the adaptive immune system specificity is accomplished via binding capacities of B-cells and less via differentiation in gene expression.

The GO term analysis did not reveal any increase in immune gene-related activity in any of the three *D. pseudospathaceum* infection treatments. In contrast, infection with *S. solidus* showed an enrichment of genes related to immunity. The failure to detect GO enrichments upon *D. pseudospathaceum* infection is consistent with the view that the stages of this trematode in the second intermediate fish host are immunologically protected when living in the eye lens.

Considering the habitat differences of fish collected in the two sub-experiments, the treatments with *Diplostomum* and *Schistocephalus* were only compared to their respective control groups, which lived in the same habitat. Thus, habitat effects were controlled for by the respective control groups and should not play a major role in the observed gene expression differences.

Vertebrate immune systems possess redundancy at many different levels (Nish and Medzhitov, 2011), in particular at the base of the vertebrate clade, the bony fishes (Haase et al., 2014; Buonocore and Gerdol, 2015). This has also become apparent in the present comparative study, where various elements of immune response can be found in stickleback host responses to both *S. solidus* and *D. pseudospathaceum* infections. Furthermore, parasite genotype-specific responses to the long-term exposure to *D. pseudospathaceum* involve the use of similar mechanisms. Host organisms are likely to encounter several pathogenic species in

their natural environment, including different multicellular parasites. The parasitic species composition and the timing of infections might influence the host immune reactions (Maizels and Nussey, 2013). Thus, a next step would incorporate a simultaneous infection of sticklebacks with *D. pseudospathaceum* and *S. solidus* to increase our understanding of host immune reactions against parasitic worms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.zool.2016.05.005>.

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