Received: 3 June 2021 | Revised: 3 July 2021 | Accepted: 7 July 2021

DOI: 10.1111/jfd.13496

RESEARCH ARTICLE

Journal of Fish Diseases WILEY

Eye fluke effects on Danish freshwater fish: Field and experimental investigations

Yajiao Duan | Louise von Gersdorff Jørgensen | Per Walter Kania | Asma M. Karami | Azmi Al-Jubury | Kurt Buchmann

Laboratory of Aquatic Pathobiology, Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg C, Denmark

Correspondence
Yajiao Duan, Laboratory of Aquatic Pathobiology, Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Stigbøjlen 7, 1870 Frederiksberg C, Denmark.
Email: duanyajiao@sund.ku.dk

Funding information
This specific study did not receive any funding from agencies within the public, commercial or not-for-profit sectors, but Yajiao Duan was supported by a stipend from the Chinese Scholarship Council (No. CSC201706910086)

Abstract

Eye flukes in fish are common in freshwater lakes. Fish become infected by the penetration of cercariae released from freshwater snails, and high infection pressures may be associated with mortalities in a Danish lake. Examination of two other freshwater lakes, combined with laboratory study, supported the notion. We investigated 77 freshwater fish from two lakes and the infection level suggested the occurrence of a high cercarial infection pressure in the Danish lakes. Dominant genera were *Tylodelphys* and *Diplostomum* covering a range of species identified by PCR and sequencing of the *18S* (partial)-*ITS1-5.8S-ITS2-28S* (partial) of the rDNA. Cercariae of the prevalent species *Diplostomum pseudospathaceum* were used to infect zebrafish *Danio rerio* for the elucidation of short-term effects on the fish host. Zebrafish did not display abnormal behaviour when exposed to 200–400 cercariae, but a dosage of 600 and 1,000 cercariae/fish proved lethal. When fish were exposed to sublethal dosages, 19 out of 27 immune genes were significantly regulated and three genes encoding cytokine (IL 4/13B, IL-6 and IL-8) were upregulated at 3 hr post-infection (hpi), whereas others were downregulated especially at a later time point. We suggest that direct massive cercarial penetration of fish surfaces may be detrimental and may represent a threat to fish populations.

KEYWORDS

Diplostomidae, eye fluke, freshwater fish, immune response, pathogenicity, zebrafish

1 | INTRODUCTION

Eye flukes, metacercariae of digenean trematodes within the family Diplostomidae, are prevalent in freshwater fish populations. The final host, carrying the adult trematode in the gut, is a piscivorous bird; freshwater snails serve as first intermediate hosts, releasing infective cercariae; and fish act as second intermediate hosts. Species within the genus *Diplostomum* prefer various sites in the fish eye, such as the lens, and may reduce vision and elicit cataract in the host. Heavy infestations are associated with weight loss as the infections reduce the visual abilities of the host and thereby its food search capacity (Buchmann & Uldal, 1994; Karvonen et al., 2004; Marcogliese et al., 2001; McCloughlin, 2016; Valtonen & Gibson, 1997). Representatives of another genus, *Tylodelphys*, within the same family, colonize the vitreous humour, impair vision and may thereby hamper foraging success (Muñoz et al., 2019).

Other indirect infection effects comprise the parasite-induced alteration of host behaviour, skin pigmentation and thereby susceptibility to predation (Désilets et al., 2013; Flink et al., 2017; Gopko et al., 2015, 2017; Marcogliese et al., 2001; Seppälä et al., 2004, 2005, 2006; Valtonen & Gibson, 1997). These chronical effects were indicated in a winter study where the eye fluke *Diplostomum pseudospathaceum* was associated with a high mortality (57.3%) of juvenile *Rhodeus amarus* (Michálková & Ondračková, 2014). Freshwater fish may harbour the trematodes for years if the infection level is low, but we here present evidence, suggesting that massive invasion is potentially lethal. A mass mortality event of common bream in a freshwater lake (Utterslev Mose) near Copenhagen, Denmark, occurred in the summer 2015. Also, the roach population in the lake was reduced, but no viral or bacterial pathogens were isolated from the surviving fish, whereas a significant load of eye flukes (100% prevalence) was noted (Jensen, 2015). Although a relatively high nitrogen load was recorded in the preceding year (2014) and could play a role for fish performance in 2015, it was suggested to search for additional causative factors. Previous studies had indicated that fish may be severely affected by cercarial penetration and the finding urged us to focus on eye fluke infection levels and their possible impact on fish populations in freshwater lakes. Thus, a controlled infection study in the laboratory had shown that direct penetration of cercariae (1,000 cercariae of *Diplostomum pseudospathaceum* per fish) elicited 100% mortality of rainbow trout fry (5–6 cm body length) within 24 hr (Larsen et al., 2005). This supported results of Wesenberg-Lund (1932) who demonstrated the immediate lethal effects of massive invasion by diplostomid cercariae (released from *Lymnaea stagnalis* and *Radix auricularia*) of crucian carp (*Carassius carassius* Linnaeus, 1758). Following cercarial penetration of the fish surface, the diplostomule migrates towards and penetrates the lens to attain the metacercarial stage, protected from the immune system, because neither immune cells nor effector molecules can access the lens (Niewiadomska, 1986; Pike & Lewis, 1994). It is generally agreed that the time between penetration and ocular invasion is less than 24 hr but dependent on temperature (Erasmus, 1959; Lyholt & Buchmann, 1996; Whyte et al., 1991) and the stimulation of the host immune system is thereby of limited duration (Sitjà-Bobadilla, 2008). *D. pseudospathaceum* may over weeks activate innate and adaptive immune responses in the fish (Haase et al., 2014, 2016a, 2016b; Kalbe & Kurtz, 2006; Scharsack & Kalbe, 2014), but the immune gene expression during the early infection stage (diplostomule migration) is largely unknown. It cannot be excluded that cercarial penetration, especially early infection points, may initiate inflammatory reactions and affect the fish adversely. We have conducted field studies on eye fluke effects in wild freshwater fishes and supplemented them by experimental verification of effects on zebrafish *Danio rerio*. Four species of freshwater fish (*Alburnus alburnus*, *Abramis brama*, *Rutilus rutilus and Perca fluviatilis*) were collected from two different lakes (Lyngby Sø and Bromme Lillesø), and their parasitic metacercariae were identified by PCR and sequencing. From the same lakes, we sampled snails, identified cercariae released from these intermediate hosts and elucidated if the eye fluke infections were reflected by infections in the snails. All parasite specimens were identified with molecular method by amplifying nuclear ribosomal DNA regions comprising *18S* (partial)-*ITS1-5.8S-ITS-28S* (partial), sequencing the PCR products and subsequently performing phylogenetic and BLAST analysis in GenBank. In the laboratory, we infected zebrafish by *D. pseudospathaceum* cercariae and followed the fish reaction including expression of 27 immune genes. Based on the observations, we discuss the potential impact on fish populations of cercariae released from snails when they perform massive penetration of freshwater fish surfaces.

FIGURE 1 Fish sampling locations (Zealand in Denmark)

2 | MATERIALS AND METHODS

2.1 | Field research

2.1.1 | Fish from a lake experiencing mass mortality

A total of 11 specimens of bream (*Abramis brama*) were obtained from Utterslev Mose (55°43'5.3826", 12°30'28.0902") (16 September 2015) (Figure 1) at 12:00 following a mass mortality event recorded from July 2015. The fish were caught by the use of gill nets by the staff at the Fish Ecology Laboratory (www.fol.dk), whereafter the fish were transferred immediately for a parasitological examination at University of Copenhagen. Abiotic factors in the lake water were also measured on the sampling day showing a pH of 7.8, a water temperature of 9.5°C and an ammonia concentration of 0.4 mg/L. However, in the preceding year, 2014, periods occurred with highly increased total nitrogen concentrations in the lake water (Jensen, 2015).

2.1.2 | Follow-up study on fish from two other lakes

Fish from Lyngby Sø (55°46'27.4866", 12°29'10.2726") and Bromme Lillesø (55°28'52.2402", 11°30'51.5736") (Figure 1) were collected by net traps and transferred alive in aerated water tanks to the University of Copenhagen. The sample included a total of 77 freshwater fish collected between February and November of 2019 (Table 1) comprising bleak, bream, roach and perch. Fish were then placed in aquaria equipped with biofilters and aeration (water temperature 15 ± 2°C) for subsequent examination.

2.1.3 | Laboratory examination

Following euthanization using an overdose of MS-222 (Cat. no. A5040, Sigma-Aldrich) (300 mg/L), fish were weighed (total body weight, *g*), length measured (total body length, cm) and examined externally for the presence of encysted metacercariae beneath the surface of the skin. Metacercariae were excised from the eyes, musculature and fins. Gill filaments, fins and both eyes were removed, placed in a Petri dish and examined under the dissecting stereomicroscope (magnification X 7-40, Leica, Germany). Fish were autopsied by an incision along the midline to recover internal organs for corresponding examination. Predilection sites of the metacercariae were recorded. Metacercariae were counted, collected using a pipette and preserved in 96% ethanol for subsequent molecular analysis.

2.1.4 | Molecular analysis

Individual metacercariae (96% ethanol-fixed) were transferred to Eppendorf tubes (Axygen®, USA). Ethanol was evaporated in the Eppendorf ThermoMixer® Comfort (Hamburg, Germany). We extracted DNA from single specimens with guanidine thiocyanate lysis buffer (Tkach & Pawlowski, 1999) and performed a PCR. The *18S* (partial)-*ITS1-5.8S-ITS2-28S* (partial) of the rDNA was amplified using forward primer-BD1 (5′-GTC GTAACA AGG TTT CCG TA-3′) and reverse primer-BD2 (5′-TAT GCT TAA ATT CAG CGG GT-3′) (Galazzo et al., 2002). PCR conditions used were previously described (Christiansen et al., 2016). DNA was purified from the PCR mixture using Illustra™ GFX™PCR and Gel band purification kit (VWR, Denmark). DNA concentration and purity were measured using a Nanodrop 2000 spectrophotometer (Saveen & Werner ApS, Denmark). Representative sequences (a total of 150) were submitted to the GenBank database under the accession numbers MW135040-MW135189. However, only the complete *ITS1-5.8S-ITS2* sequences (omitting the 18S and 28S parts) were BLAST analysed against sequences at GenBank (NCBI) and used in subsequent phylogenetic analysis.

2.1.5 | Phylogenetic analysis

Using the software MEGA X (Kumar et al., 2018), evolutionary history was inferred by using the maximum likelihood method and general time-reversible model whereafter a bootstrap consensus tree, inferred from 1,000 replicates, was chosen to represent the evolutionary history of the taxa analysed. The percentage of replicate trees in which the associated taxa clustered together in the

TABLE 1 Numbers of fish sampled in the two lakes Lyngby Sø and Bromme Lillesø from February to November in 2019

Location	Fish species	Numbers of fish	Overall prevalence (%)	Month in 2019
Lyngby Sø	Common bleak (*Alburnus alburnus*)	11	90.9	February
	Common bream (*Abramis brama*)	12	83.3	February
	Common roach (*Rutilus rutilus*)	9	100	September
Bromme Lillesø	Perch (*Perca fluviatilis*)	14	100	April
	Common roach (*Rutilus rutilus*)	12	100	April
	Common bream (*Abramis brama*)	2	100	April
	Common roach (*Rutilus rutilus*)	4	100	November
	Perch (*Perca fluviatilis*)	13	100	November

bootstrap test is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories [+G, parameter = 1.9658]). The rate variation model allowed some sites to be evolutionarily invariable ([+I], 13.61% sites). This analysis involved 68 nucleotide sequences. A total of 1,752 positions were seen in the final data set. The two species *Aspidogaster conchicola* and *Aspidogaster ijimai* of Aspidogastrea served as an outgroup.

2.1.6 | Statistical analysis of field data

Raw data were entered into a Microsoft Excel spreadsheet, and descriptive statistics were used to summarize the data. Ecological terms of prevalence, abundance and mean intensity were used according to Bush et al. (1997). The data were analysed statistically using the software SPSS. The non-parametric Friedman test with Dunn's multiple comparisons test was applied to reveal any significant difference of trematode numbers within three host compartments as different species may show preferences due to host tissue tropism. The non-parametric Mann–Whitney test was used for assessing differences between the average number of trematodes at April and November. Statistical significance was considered if $p < .05$ for all statistical tests.

2.2 | Experimental infections on zebrafish

2.2.1 | Ethics

The infection experiment was conducted at the Laboratory of Aquatic Pathobiology fish infection facilities at the University of Copenhagen (Frederiksberg C, Denmark). Animal care and investigations were performed according to license 2020-15-0201-00724 (The Experimental Animal Inspectorate under the Ministry of Food, Agriculture and Fisheries). Fish samples used for tolerance study were killed by immersion into an overdose of MS222 when clinical signs occurred (balance disturbances, swimming upside down, lying on the fish tank bottom). These signs have previously been associated with later mortality (unpublished observation by the author).

2.2.2 | Fish

Wild-type zebrafish were reared in a recirculated system at 27°C with a pH of 7.4 and conductivity at 550 μs. Ten per cent of the water was changed every day, and the fish were fed with live *Artemia* and pelleted dry feed (ZM Fish Food, England) one to three times per day. Adults with the age of 5 months were used for this study. They were acclimatized to room temperature for two days before experimental start.

2.2.3 | Parasites for experimental infection of zebrafish

Infected snails (*L. stagnalis*) were collected in the lake Bagsværd sø (55°46'16.0566", 12°27'39.6864") (Zealand, Eastern part of Denmark). The species identification of snails was confirmed by morphological criteria (Glöer, 2019). All specimens were transferred to the university laboratory (Frederiksberg, Copenhagen area) for subsequent shedding procedures. Individual snails were placed at room temperature in 100-ml beakers with 50 ml filtered (0.45 μm, Filtropur, Darmstadt, Germany) lake water (Buchmann, 2007). The beakers were subsequently examined under a stereomicroscope (magnification X 7-40, Leica, Germany). All snails infected with diplostomid cercariae were offered fresh lettuce in the laboratory. Cercariae to be used for experimental infection were then released by shedding in 100-ml beakers as described above. They were identified as *D. pseudospathaceum* based on the morphology (Niewiadomska et al., 1996), and by PCR with subsequent sequencing of the ITS region and BLAST analysis (NCBI), confirming that the parasite used was identical with *Diplostomum pseudospathaceum* (reference sequence KR269766). The Table S1 contains the achieved molecular data in an Excel file.

2.2.4 | Infection dosage and design for zebrafish exposure

The exposure experiments were performed with three parts

Tolerance study: Exposure of zebrafish was initially performed by using 200, 300, 400, 600 and 1,000 cercariae/fish in order to determine the tolerance of fish. In total, 25 fish were used with 5 fish exposed to each dosage. All fish were placed and exposed in individual tanks (25 tanks in total) with different numbers of cercariae in 200 ml of water (volume of fish tank 1,000 ml). Exposure time was recorded and fish were killed when clinical signs occurred.

Diplostomule migration study: In order to determine the migration of parasites in the fish host, and how fast the diplostomules reach the fish lens, fifty fish were exposed to different dosages (5, 10, 20, 40, 70 cercariae/fish) (10 fish at each dosage). Examination for the presence of metacercariae in lenses was conducted at 2, 4, 6, 7 and 8 hr post-infection, respectively (10 fish at each sampling time). Fish were exposed in duplicate with 10 fish being exposed to cercariae in one 1,000-ml tank with 600 ml of water. Thereafter, fish were kept in separate similarly sized tanks until examination for metacercariae in the lenses.

Immune response to infection: Two non-lethal infection dosages (20 and 70 cercariae/fish) were then selected for exploring the immunological reaction in the exposed fish. Two parallel tanks were used in this study (14 tanks in total with 5 fish in each). A total of 70 fish were used and 3 groups (10 for each group/total 30 fish) served as non-infected and time-point controls to be sampled. Following exposure to cercariae, 2 × 5 fish were sampled for each dosage at 3

and 8 hpi (total 40 infected fish). In this study, each tank contained 5 fish in 600 ml of water in a 1,000-ml fish tank (Figure 2).

2.2.5 | Sampling of organs for qPCR

At specific time points post-challenge (3 and 8 hpi), the fish were killed with an overdose of MS222 (500 mg/L). The viscera package (including intestine, liver and spleen) was removed with fine forceps and immediately transferred to RNAlater (Sigma-Aldrich, Denmark) and kept at 4°C for 24 hr, and then frozen at −20°C until processing for gene expression studies. Due to the small size of the zebrafish, used in the study, it was decided to sample the entire organ package. This secured that relevant organs, in which the diplostomules may migrate on their way to the eye, were included in the investigation. Each group consisted of two duplicated tanks, each tank contained 5 fish. When we analysed gene expression data, each individual sample from all 10 fish was processed individually. The expression data in the two duplicate groups were pooled when it was confirmed that no significant difference was found between them.

2.2.6 | RNA extraction, cDNA synthesis and real-time PCR

Total RNA was extracted from tissue samples using GenElute™ kit (Cat. no. RTN350-1KT, Sigma-Aldrich, Denmark), according to the manufacturer's instructions and subsequently DNase treated with DNase I (Cat. no. ENO521, Thermo Scientific, Denmark). The quantity and purity of RNA were measured at 260/280 nm (NanoDrop 2000 Spectrophotometer, Saveen & Werner ApS, Denmark), and DNAse efficacy and RNA integrity were evaluated by electrophoresis on 1% agarose gels with ethidium bromide (EtBr) staining. The first-strand cDNAs were synthesized using 1,000 ng of total RNA, MultiScribe Reverse Transcription reagent (Thermo Fisher Scientific, Denmark), and Oligo dT primers in a 20-µl set-up. The reaction was placed at 25°C for 10 min and 37°C for 60 min in a Thermal Cycler (T100™ Thermal Cycler, Bio-Rad, Denmark). Subsequently, the synthesized cDNA was stored at −20°C until further use. The expression of immune-associated genes was evaluated by qPCR assays using the synthesized cDNA with specific primers and corresponding probes listed in Table S2. The endogenous reference genes (ELF1-α, β-actin and RP13a) were used to normalize the relative expression of the target genes. The qPCRs were carried out in a 96-well plate with volume of 12.5 µl (2.5 µl cDNA, 6.25 µl Brilliant III Ultra-Fast QPCR Master Mix (AH diagnostics as, Denmark), 1.0 µl primer-probe mixture (forward primer [10 µM], reverse primer [10 µM] and TaqMan probe [5 µM]) and 2.75 µl RNase-free water. The reactions were performed on an AriaMx Real-Time PCR system (AH diagnostics as, Denmark) under the following conditions: 95°C for 3 min followed by 40 cycles denaturation at of 95°C for 5 s and 60°C for 10 s. In order to exclude any reaction inhibition by gut material, we tested for this possibility by calculation the amplification efficiency of the individual samples using the freeware LinRegPCR (Ruijter et al., 2009; Tuomi et al., 2010). Using all samples, we found an average of efficiencies of 100.4% ± 10.7% and an average of r^2 of 0.987 ± 0.005. These levels indicated that no inhibitory effect was evident.

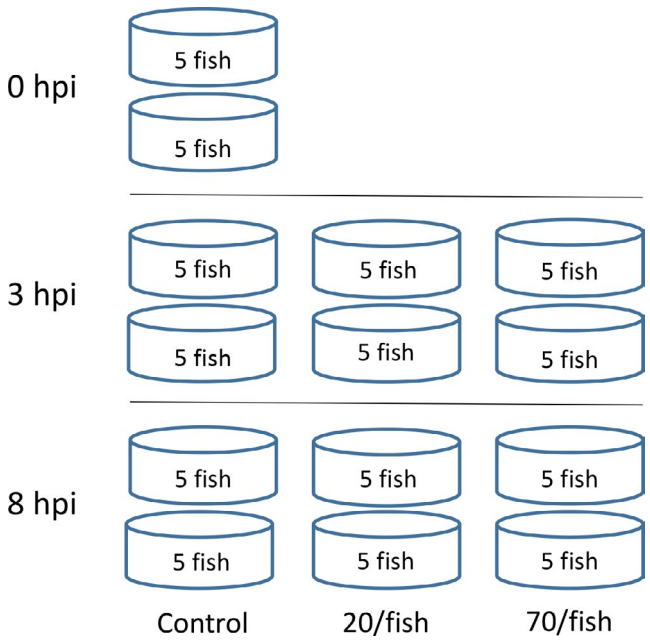

FIGURE 2 Study design showing the challenge model. Each section included 10 fish divided into two parallel tanks. hpi: hours post infection

2.2.7 | qPCR data analysis

The $2^{-\Delta\Delta Ct}$ method was applied to determine the relative gene expression presented as the fold increase or decrease of the infected group relative to the time point control groups (mean expression level adjusted to 1). Duplicate tanks were utilized for the elimination of tank effects. Before pooling data from duplicate samplings, it was confirmed (Student's *t* test) that gene expression data did not differ significantly. To account for biological variation, only gene regulations with at least 2-fold change were considered significant. The statistical difference between groups was determined using a Student's *t* test applying a probability level of 5% ($p < .05$).

3 | RESULTS

3.1 | Field study

3.1.1 | Lake Utterslev Mose in 2015

All the 11 bream specimens were infected with parasites (in this initial field study, they were merely identified to genus level). Fish eyes were infected with metacercariae (genera *Diplostomum* and *Tylodelphys*) with a 100% prevalence (mean intensity 100 parasites

per fish). Prevalences of other parasite types were for metacercariae of *Apatemon* in the pericardium (72%), metacercariae of *Ichthyocotylurus* in the peritoneum (18%), monogenean parasites on gills (*Dactylogyrus*) 9%, myxosporidia (*Myxobolus*) in gills 54% and intestinal tapeworms (*Khawia*) 18%. The intensities of these other parasite types were low (1–5 parasite stages per infected host).

3.1.2 | Lakes Lyngby Sø and Bromme Lillesø in 2019

A total of 77 fish in both lakes (32 from Lyngby Sø and 45 from Bromme Lillesø) comprising common bleak (*Alburnus alburnus*), common bream (*Abramis brama*) and common roach (*Rutilus rutilus*) and perch (*Perca fluviatilis*) was examined. Except for samples in February all fish were infected with one or more species (Table 1).

3.2 | Parasite species from fish in Lyngby Sø and Bromme Lillesø

In Lyngby sø, six species of trematodes in fish were identified (*D. pseudospathaceum*, *D. mergi*, *D. paracaudum*, *Diplostomum* sp., *P. cuticola* and *T. clavata*). *P. cuticola* dominated in bleak and bream with 81.8% and 50% prevalence, respectively. *T. clavata* showed the highest prevalence in roach (100%). The highest abundance/mean intensities of individual parasite species were for *P. cuticola* (3.73/4.56), *Diplostomum paracaudum* (13.60/32.60) and *T. clavata* (15.89/15.89). Besides, monogeneans (*Paradiplozoon* sp. and *Dactylogyrus* sp.) also myxosporeans (*Myxobolus* sp.) were found in bleak and bream (Figure 3).

In Bromme Lillesø, eight trematode species were identified from fish including *D. baeri*, *D. pseudospathaceum*, *P. brevicaudatum*, *T. clavata*, *D. mergi*, *D. paracaudum*, *P. cuticola* and *Hysteromorpha triloba*. In perch, *T. clavata* showed the highest prevalence both in April (100%) and September (84.6%). In roach, *T. clavata* exhibited a peak prevalence in April (83.3%) and *P. cuticola* in September (100%). *T. clavata* showed the highest intensities in April and November. In bream, only two species of trematodes were discovered, *D. pseudospathaceum* with 100% prevalence and *T. clavata* with 50% prevalence. In addition to trematodes, cestodes (*Triaenophorus nodulosus*), myxosporeans (*Myxobolus* sp.), nematodes *(Camallanus lacustris)*, monogeneans (*Ancyrocephalus percae*), crustaceans (*Ergasilus sieboldi*), acanthocephalans (*Acanthocephalus lucii*) were also recorded. All these parasites were present at relatively low prevalences and abundances (Figure 3). The Table S3 contains the achieved molecular data in an Excel file. The result of the phylogenetic analysis is presented as a phylogram in the Figure S1.

3.3 | Host tissue tropism

We compared the occurrence of the different metacercariae with the preferred site in the fish host to indicate host tissue tropism. Metacercariae dominated in the skin of bleak with a lower infection in the vitreous humour ($p < .05$). The number of metacercariae in the lens of bream in Lyngby Sø was significantly lower than other infection sites (Figure 4). *T. clavata* showed a high preference for the vitreous humour in other fish species, such as roach (Lyngby Sø and Bromme Lillesø) and perch in Bromme Lillesø ($p < .05$). Besides, in Bromme Lillesø, the number of trematodes in the lens of perch and in the vitreous humour of roach differed significantly ($p < .05$) (Figure 4).

3.4 | Tolerance of zebrafish to experimental cercarial exposure

Exposures of adult zebrafish to cercarial dosages of 600 and 1,000 cercariae/fish were detrimental and considered fatal. All fish exposed to the high number of cercariae (600 or 1,000 per fish) showed abnormal behaviour (swimming erratically, balance disturbances including tilting, swimming upside down, lying on the fish tank bottom) and had to be killed within 30 and 10 min, respectively.

3.5 | Parasite migration in the fish

The number of metacercariae reaching the lens was positively correlated with the infection dosage (Figure 5), but approximately 50% of the cercariae reached the lens within 8 hpi, independently of the dosage.

3.6 | Expression of immune-relevant genes

Genes encoding immune relevant molecules were expressed at different levels, and only three genes were upregulated, mainly at 3 hpi, whereas 16 genes were downregulated at 3 hpi and/or 8 hpi when compared to non-exposed control fish (Figure 6).

3.6.1 | Transcription factors

All the three genes encoding transcription factors Fox P3 (Figure 6a), GATA-3 (Figure 6b) and T-bet (Figure 6c) were not regulated at 3 hpi but were downregulated at 8 hpi (both low and high dose) (Figure 6a–c).

3.6.2 | Cytokines

Fifteen genes associated with inflammation were analysed in this study and twelve genes were significantly regulated, when compared to the time point control groups (Figure 6d–o). The expression of IL-1β was downregulation at hour 8 pi (Figure 6d), which also applies to IL-17 A/F1 (Figure 6j), IL-17A/F3 (Figure 6l) and TNFα (Figure 6o). The expression of genes encoding IL-10 (Figure 6h),

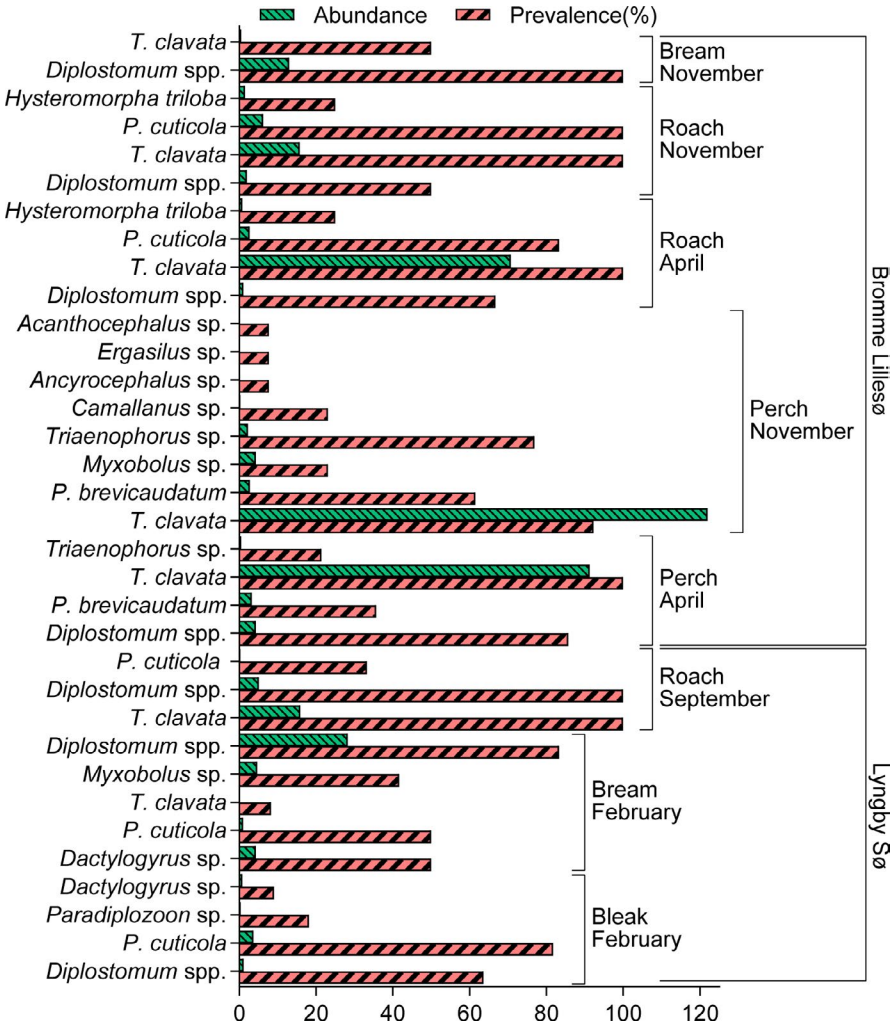

FIGURE 3 Parasites infecting different fish species in two lakes. *Diplostomum* spp comprised different species (more data shown in Table S2). F: February; A: April; S: September; N: November

IL-17A/F2 (Figure 6k), IL-22 (Figure 6m) and IFN-γ (Figure 6n) was not regulated at 3 hpi, but downregulated at 8 hpi but only after low-dosage exposure. Three genes, encoding IL-4/13B (Figure 6e), IL-6 (Figure 6f) and IL-8 (Figure 6g), were significantly upregulated. The IL-12-encoding gene was downregulated at 3 hpi following exposure to a low dosage (Figure 6i).

3.6.3 | Immunoglobulins, acute phase proteins and toll-like receptors

Genes encoding IgM and IgZ (Figure 6p,q) were downregulated at 8 hpi (both low and high exposure dose) for IgZ2 (Figure 6q) and at low dose for IgM (Figure 6p). Fish exposed to a low cercarial dosage showed downregulation of genes encoding SAA (Figure 6r) and TLR2 8 hpi (Figure 6s).

4 | DISCUSSION

We performed a survey of parasite infections in fish in three freshwater lakes in Denmark and recorded a high prevalence and intensity of eye flukes. According to our previous study (Duan et al., 2021), it was seen that the eye flukes have higher host specificity in snails (including miracidial invasion, sporocyst formation, cercarial production and release). In contrast, the metacercarial stage is less specific as the four teleost species investigated carried one or more eye fluke species. It is therefore evident that due to the narrow host specificity of trematodes in snails (Van der Knaap & Loker, 1990), one must collect specific snail species in order to reflect the infection risk of fish in a certain lake habitat. For instance, various species of the genus *Diplostomum* are largely reported from the snail species *Lymnaea stagnalis* (Karvonen et al., 2003, 2004, 2006; Loy & Haas, 2001; Lyholt & Buchmann, 1996; Morley et al., 2005; Riley & Chappell, 1992). A total of 250 and 150 snails from Lyngby Sø and Bromme Lillesø (23.6% and 29.0% total prevalence), respectively, were analysed in this study. The most common eye fluke species in the different fish species is *T. clavata* reaching 100% prevalence in roach, whereas the different species of the genus *Diplostomum* showed a more specific orientation towards the different species of fish. In the present study, Bromme Lillesø hosted two species of eye flukes (*T. clavata* and *D. mergi*), which were overlapping in snail and fish and showed a higher transmission compared to Lyngby sø. Moreover, *T. clavata* was the dominating species in Bromme Lillesø

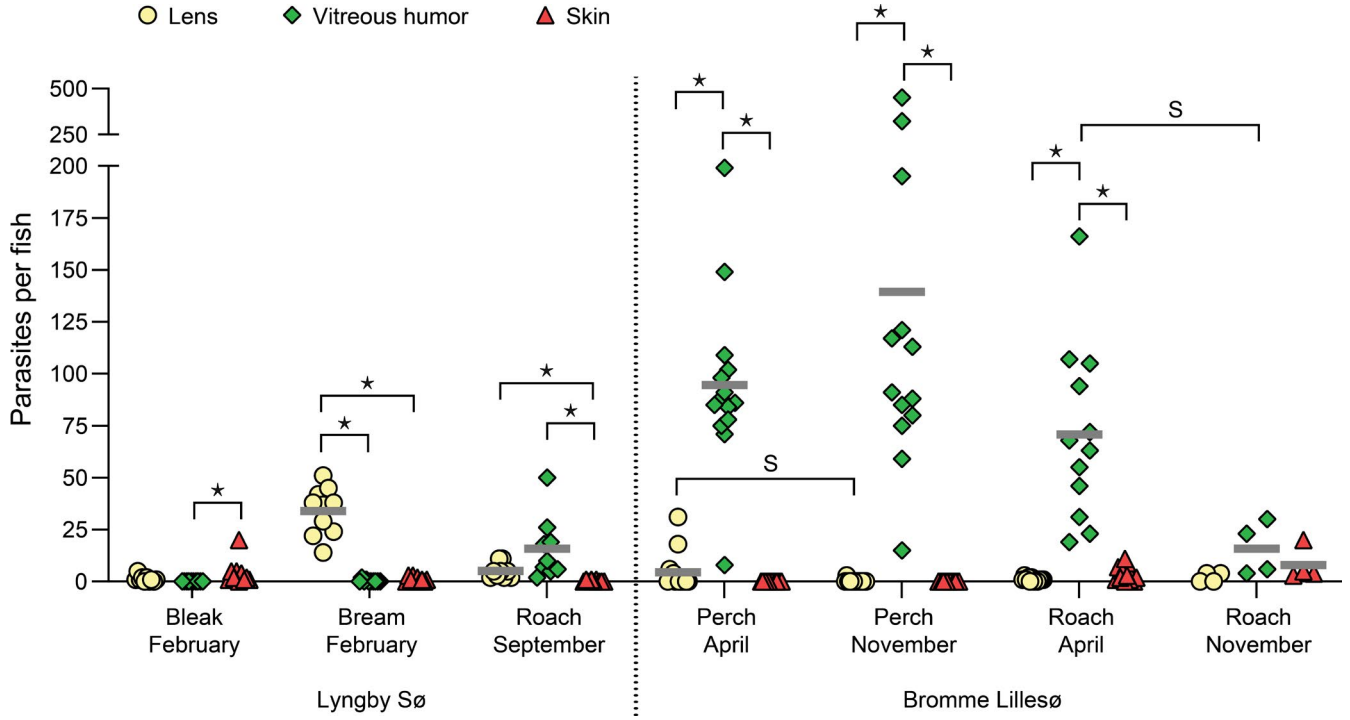

FIGURE 4 Host tissue tropism and seasonal occurrence. Note: Data from infected fish in Lyngby Sø (left to the stipulated vertical line) and Bromme Lillesø (right to the stipulated vertical line). Brackets with an asterisk above indicate significant difference of number of parasite between the indicated locations (the non-parametric Friedman test with Dunn's multiple comparisons test, $p < .05$). Brackets with S above indicate seasonal significant difference of number of parasites for the fish species indicated (the non-parametric Mann–Whitney test, $p < .05$)

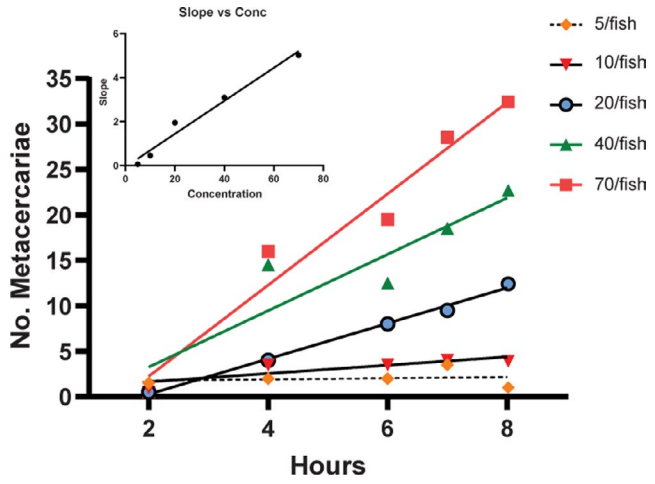

FIGURE 5 Accumulation of metacercariae in lens. Accumulation line of *D. pseudospathaceum* in zebrafish lens. Linear regression of slope and concentration shown in upper right. $R^2 = .9737$

with high prevalence in all fish hosts. Eye flukes *D. pseudospathaceum*, *D. baeri* and *D. brevicaudatum* exhibited a very high prevalence in Bromme Lillesø, whereas *T. clavata* occurred in both lakes and both in roach and perch at a relatively high prevalence. European perch has commonly been reported as host for this species (Morley & Lewis, 2020; Muñoz et al., 2017, 2019; Vivas Muñoz, 2019), but species-specific preference seems to be low. Roach exhibited the highest species richness in both Lyngby Sø (five species) and Bromme Lillesø (six species) consistent with previous studies on freshwater fish in Poland (Dzika et al., 2008). No zoonotic parasites were discovered in roach in our study, but zoonotic opisthorchid metacercariae (*Pseudamphistomum truncatum*) were previously reported from roach in the Danish lake Furesø (Skov et al., 2008) and in the Gulf of Finland (Eriksson-Kallio et al., 2017).

The host tissue tropism of different species of trematodes (metacercariae) was indicated as different species preferred the lens, vitreous humour or the skin (Table S2). Four species of metacercariae were parasitizing the fish lens (*D. mergi*, *D. paracaudum*, *Diplostomum* sp. and *D. pseudospathaceum*); three species (*T. clavata*, *D. baeri* and *Posthodiplostomum brevicaudatum*) were found in the vitreous humour, and two species *Posthodiplostomum cuticola* and *Hysteromorpha triloba* were found in the fish skin. The host tissue tropism has previously been reported with *T. clavata* located in the vitreous humour (Buchmann et al., 1997) and *Hysteromorpha triloba* also located in fish body cavity (Sereno-Uribe et al., 2019). It is worth noting that *Posthodiplostomum brevicaudatum* (Nordmann, 1832), congeneric to *Posthodiplostomum cuticola*, causing black spot skin disease, was found encysted in the fish eye, as reported by previous studies (Stanevičiūtė et al., 1998; Wisniewski, 1958). In our case, *P. brevicaudatum* was only discovered from perch in Bromme Lillesø, aligning with records from Poland (Morozinska-Gogol, 2013).

In the host tissue tropism analysis, metacercariae parasitizing the lens and the vitreous humour (mainly eye flukes) showed a high

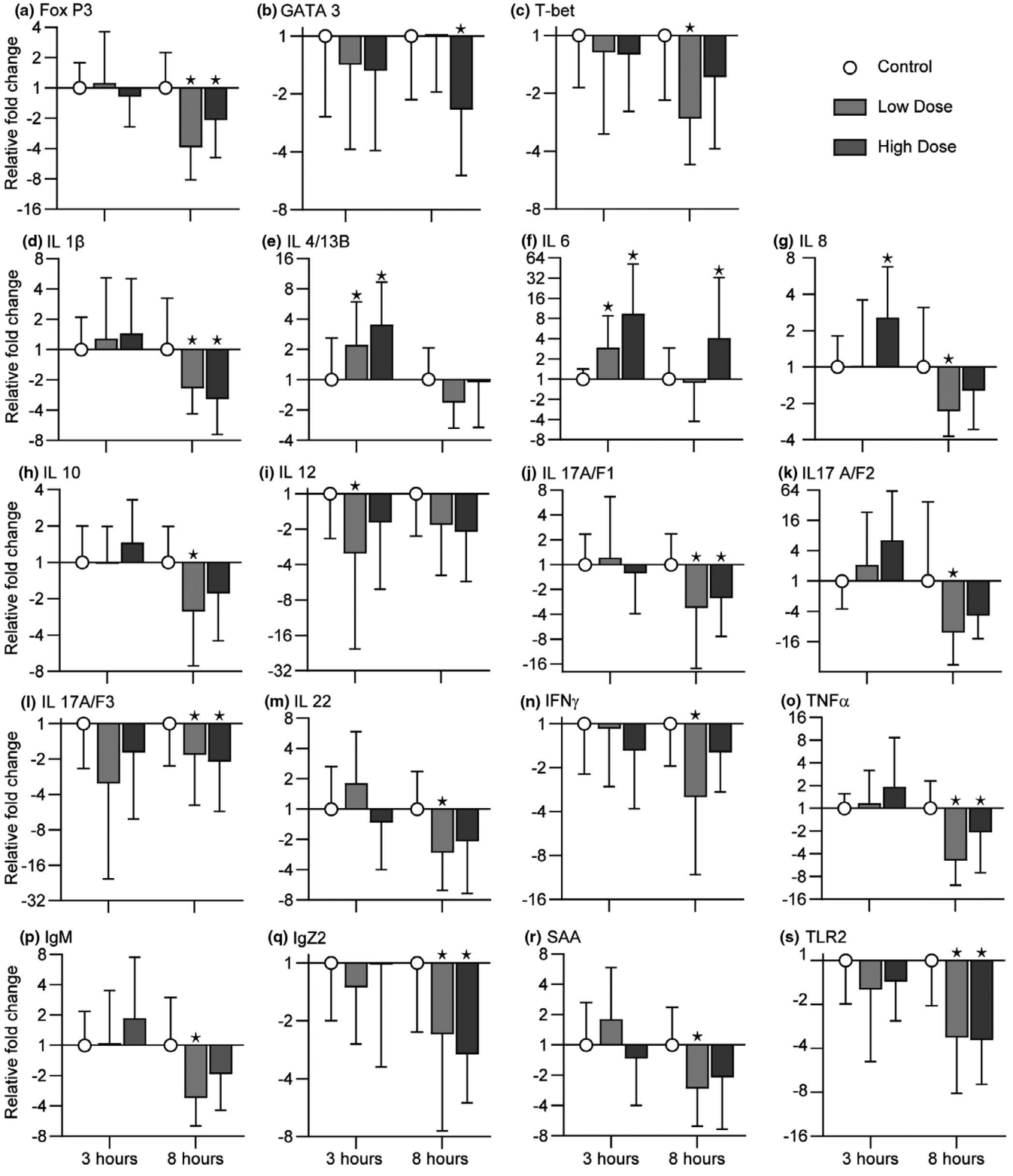

FIGURE 6 qPCR analysis. Relative fold change was calculated as $2^{-\Delta\Delta Cq}$. Due to the exponential nature, the geometric mean and geometric standard deviation were used. Only significant results are shown. "a-s" were shown as short names of genes, full names of genes were given in Table S2. A comprehensive summary of the gene expression study, including all the genes investigated, is present as Table S4. *$p < .05$ (Student's t test), fold change >2

infection level. The current hypothesis is that parasites manipulate the host behaviour and leave them as easier prey for a predator, which may serve as a final parasite host (Gopko et al., 2015; Lafferty & Morris, 1996; Muñoz et al., 2017; Poulin, 2010; Seppälä et al., 2004, 2005) and such a relation is likely to occur also in the investigated Danish lakes.

4.1 | Effects on fish

The prominent occurrence of infected snails and the high eye fluke infection level in fish raises the notion that the direct penetration of fish at peak shedding periods may expose fish to such a high infection pressure that survival is affected. Eye flukes are particularly relevant to discuss in this context due to their high prevalence, whereas other parasite species were present in much lower numbers in the investigated lakes. It is acknowledged that all the recorded parasite types (monogeneans, myxosporeans, cestodes, nematodes, acanthocephalans, crustaceans), and also adverse levels of abiotic factors (pH, Nitrogen, Oxygen), represent a threat to fish health when occurring at a certain intensity. The only obvious abiotic environmental change in the lake Utterslev Mose recorded was a high concentration of Nitrogen in 2014, and it cannot be excluded that other parasite types detected may affect fish population levels. However, in the present study these parasite types were not represented to the same extent that as the digenean metacercariae. We therefore suggest that, under conditions favouring cercarial shedding from snails (such as high summer temperatures), mortalities may be induced by massive cercarial invasion of fish. Temperature is particularly relevant as the shedding is highly temperature dependent and peaks at temperatures above 20°C (Lyholt & Buchmann, 1996), and in this study, temperature may have played a role for the infection pressure during the summer period. It is generally observed that eye flukes accumulate in fish during the season with moderate infection levels (Lyholt & Buchmann, 1996), but in our field studies (in Bromme Lillesø and Lyngby sø), this was not seen, which could be caused by fish mortality, among the most heavily infected fish.

Based on our first observations of high eye fluke infections in bream sampled in Utterslev Mose 2015, following a major reduction of the cyprinid populations, we established the hypothesis that massive invasions of young fish by cercariae, released from intermediate host snails, may influence fish health and/or survival in confined water bodies. First of all, the infection pressure may be very high as the potential for cercarial shedding from pulmonate freshwater snails such as a single specimen of *Lymnaea stagnalis* was found to release up to 60,000 cercariae (genus *Diplostomum*) during a 24-hr period (Lyholt & Buchmann, 1996). Secondly, evidence of a lethal effect of massive cercarial penetration of fish surfaces was presented by Larsen et al. (2005) showing that 1,000 *Diplostomum* cercariae killed juvenile rainbow trout within 24 hr. Likewise, Wesenberg-Lund (1932) described clouds of *Diplostomum* cercariae released from *L. stagnalis* killing crucian carp within five hour (Wesenberg-Lund, 1932). Our experimental exposure study showed that exposure of zebrafish to 600 or 1,000 cercariae/fish is fatal within minutes. The chronical effect of eye fluke infections on fish is well documented. The parasites decrease the size of the eye lens, cause cataract and exploit energy resources that would otherwise be used by the host for normal eye functioning (Karvonen & Seppälä, 2008). The resulting behavioural and physiological stress (Thatcher, 1979) may then prove lethal (Ostrand et al., 2006). Furthermore, the migration of larval parasites (diplostomules) through fish tissues may cause a series of physical damages, which can cause non-specific stress responses characterized by increased oxygen consumption, increased metabolic rate and decreased total lipid content in the body (Lemly & Esch, 1984). Together, this evidence supports the notion of a potential devastating effect of eye fluke infection on freshwater fish populations.

The different fish species may be exposed differently as some host specificity occurs for some eye fluke species. However, *T. clavata* seems to exhibit a low host specificity and may target all fish species. Roach (*Rutilus rutilus*) showed the highest trematode richness, both in Lyngby Sø and Bromme Lillesø, compared with other fish species. This does not imply that this species is more affected by penetration than others are. It could be hypothetized that other fish species, such as bream, although invaded by the same number of cercariae, may trap the cercariae (termed diplostomules) during their migration in the host towards the eye. It may be hypothesized that entrapment of diplostomules in the vascular system or central organs may lead to pathological reactions and possibly death of the host. In such a case, the parasites would not be recorded in the fish host because the metacercariae never reached the eye. A notion which should be further investigated experimentally.

4.2 | Immunological reactions in the fish

The pathological events in the fish may also be reflected by the immune reactions. We used *D. pseudospathaceum* to infect zebrafish for assessment of pathogenicity and immune response. The sublethal infection dosages stimulated the fish immune system significantly, but apart from an initial inflammatory reaction (involving IL-4/13B, IL-6 and IL-8) at 3 hpi the downregulation of many immune genes was noteworthy. Thus, this study indicated that zebrafish establish an inflammatory reaction shortly after penetration with *D. pseudospathaceum* cercariae, but subsequently a series of immune genes encoding effector molecules become downregulated.

These effects may be indirect due to interactions between the different cytokines. Thus, IL-4 mediates many specific pathways, including fine-tuning of the Th2 response through its ability to initiate, perpetuate or inhibit the response through the activation of multiple signalling pathways (Wills-Karp & Finkelman, 2008). IL-4 and IL-13 suppress inflammatory responses by antagonizing production of TNFα, IL-1β, IFN-γ and other pro-inflammatory mediators (Bottiglione et al., 2020; Wang et al., 2016). This could explain the down-regulation of genes encoding TNFα, IFN-γ and IL-1β. Interleukin-6 (IL-6) is a pleiotropic cytokine, produced by various cells to regulate haematopoiesis, inflammation, immune responses and bone homeostasis, and principal mediator of the acute phase protein response (Heath et al., 1993; Hirano, 2010). This may explain our observations of the upregulated expression of the IL-6 gene at 3 and 8 hpi, and the downregulation of the acute phase protein (SAA) at 8hpi, as it can be interpreted an IL-6-based regulation of SAA.

The downregulation may benefit the survival of migrating diplostomules in the fish on their way to the immune-privileged lens of the eye. A previous study indicated that the IL-4R and IL-8 genes were upregulated after repetitively challenging stickleback for 7 weeks with *D. pseudospathaceum* (Haase et al., 2016b). However, in our short-term study other genes encoding cytokines were downregulated at different sampling points. IL-1β is an important pro-inflammatory cytokine in fish (Sigh et al., 2004), and from mammalian studies, it is known that nearly all inflammatory reactions are followed by production of among others this cytokine and TNF (Titus et al., 1991). We noted downregulation of various isoforms of IL-17-playing central roles in responses as a bridge between innate and adaptive immunity in the head kidney and intestine (Takahashi et al., 2020). Previous studies frame that parasitic pathogens may downregulate the host response as seen for genes encoding GATA3 and MHC II beta appeared after *D. pseudospathaceum* infection of stickleback (Haase et al., 2014). This was supported by the present study. Type-2 immunity processes initiated by IL-4 and IL-13 are fundamental for immune defence against helminth parasites (Bao & Reinhardt, 2015), and in fish, these two paralogs are located in different chromosomes and were named Il-4/13A and IL-4/13B (Ohtani et al., 2008). Genes encoding these cytokines along with IL-6, a pro-inflammatory cytokine, and IL-8, chemoattractant for immune cells (Conti et al., 2020), were upregulated in the initial infection phase but later silenced. All other immune genes investigated were found downregulated or upregulated at 3 hpi and 8 hpi. This applies for the immune regulating IL-10, promoting B-cell differentiation and IgM antibody secretion in an antigen-specific manner in fish (Zou & Secombes, 2016). Depression was also noted for the IgM and IgZ/T encoding genes themselves, the antigen presenting surface molecule MHC II, transcription factors GATA3, T-bet, the regulator FoxP3 (Shevach, 2009), the acute phase reactant SAA, an all-round innate protective protein (Gruys et al., 2005; Uhlar & Whitehead, 1999) and the central pattern recognition receptors Toll-like receptors 2 initiating immune responses (Medzhitov & Janeway, 1997). Transcription of IgM was downregulated at 8 days post-infection in *Oncorhynchus mykiss* infected by the nematode larva *Anisakis simplex* (Haarder et al., 2013), and SAA expression may be highly variable upon parasite challenge (Kovacevic et al., 2015; Tadiso et al., 2011).

In conclusion, the present study contributes to a better understanding of ecological aspects of the natural infection level of eye flukes and their early/acute phase impacts during cercarial invasion in the fish host. Focus on the occurrence of the snail intermediate host is central as the intermediate hosts determine the dynamics of the infection level in fish. Further experimental studies illustrated that the penetration of cercariae into the fish is highly pathogenic and may be directly lethal. Non-lethal infections by *D. pseudospathaceum* suppress the fish host immune response as only three of the investigated genes (IL 4/13B, IL-6 and IL-8) were upregulated and merely at the initial infection time point. The immune depression may be a strategy of the parasite, during the migration in the host, for raising the probability of reaching the immune-privileged site (the eye).

ACKNOWLEDGMENTS

We thank the Chinese Scholarship Council (No. CSC201706910086) for the financial support by a stipend to YJD. We thank Hannah Malene Jensen (University of Copenhagen), Henrik Carl and Marcus A. Krag (Statens Naturhistoriske Museum) for assistance with field sampling of fish for this study.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

ETHICS APPROVAL STATEMENT

The infection experiment was conducted at the Laboratory of Aquatic Pathobiology fish infection facilities at the University of Copenhagen (Frederiksberg C, Denmark). Animal care and investigations were performed according to license 2020-15-0201-00724 (The Experimental Animal Inspectorate under the Ministry of Food, Agriculture and Fisheries).

PATIENT CONSENT STATEMENT

There is no any patient consent statement.

PERMISSION TO REPRODUCE MATERIAL FROM OTHER SOURCES

The manuscript does not contain reproduced material from other sources.

CLINICAL TRIAL REGISTRATION

There is no clinical trial in this study.

DATA AVAILABILITY STATEMENT

The raw data that support the findings of this study are available from the corresponding authors upon reasonable request. Supplementary data (four tables and one figure) associated with this article can be found in the supplementary files.

ORCID

Louise von Gersdorff Jørgensen https://orcid.org/0000-0002-6005-3249
Kurt Buchmann https://orcid.org/0000-0002-2747-237X

REFERENCES

Bao, K., & Reinhardt, R. L. (2015). The differential expression of IL-4 and IL-13 and its impact on type-2 immunity. *Cytokine*, *75*(1), 25–37. https://doi.org/10.1016/j.cyto.2015.05.008

Bottiglione, F., Dee, C. T., Lea, R., Zeef, L. A. H., Badrock, A. P., Wane, M., Bugeon, L., Dallman, M. J., Allen, J. E., & Hurlstone, A. F. L. (2020). Zebrafish IL-4–like cytokines and IL-10 suppress inflammation but only IL-10 is essential for gill homeostasis. *The Journal of Immunology*, *205*(4), 994–1008. https://doi.org/10.4049/jimmunol.2000372

Buchmann, K. (2007). *An introduction to fish parasitological methods: Classical and molecular techniques*. Biofolia, Forlaget Samfundslitteratur.

Buchmann, K., Monies, S., Uldal, A., & Bresciani, J. (1997). Different seasonal infection dynamics of two species of eye flukes (*Diplostomum spathaceum* and *Tylodelphys clavata*) in rainbow trout (*Oncorhynchus*

mykiss). *Bulletin of the European Association of Fish Pathologists*, *17*(5), 165.

Buchmann, K., & Uldal, A. (1994). Effects of eyefluke infections on growth of rainbow trout (*Oncorhynchus mykiss*) in a mariculture system. *Bulletin of the European Association of Fish Pathologists*, *14*(3), 104–107.

Bush, A. O., Lafferty, K. D., Lotz, J. M., & Shostak, A. W. (1997). Parasitology meets ecology on its own terms: Margolis et al revisited. *The Journal of Parasitology*, *83*(4), 575–583. https://doi.org/10.2307/3284227

Christiansen, A. Ø., Olsen, A., Buchmann, K., Kania, P. W., Nejsum, P., & Vennervald, B. J. (2016). Molecular diversity of avian schistosomes in Danish freshwater snails. *Parasitology Research*, *115*(3), 1027–1037. https://doi.org/10.1007/s00436-015-4830-3

Conti, P., Ronconi, G., Caraffa, A., Gallenga, C., Ross, R., Frydas, I., & Kritas, S. K. (2020). Induction of pro-inflammatory cytokines (IL-1 and IL-6) and lung inflammation by Coronavirus-19 (COVID-19 or SARS-CoV-2): Anti-inflammatory strategies. *Journal of Biological Regulators & Homeostatic Agents*, *34*(2), 1.

Désilets, H. D., Locke, S. A., McLaughlin, J. D., & Marcogliese, D. J. (2013). Community structure of *Diplostomum* spp. (Digenea: Diplostomidae) in eyes of fish: Main determinants and potential interspecific interactions. *International Journal for Parasitology*, *43*(11), 929–939. https://doi.org/10.1016/j.ijpara.2013.07.002

Duan, Y., Al-Jubury, A., Kania, P. W., & Buchmann, K. (2021). Trematode diversity reflecting the community structure of Danish freshwater systems: Molecular clues. *Parasites & Vectors*, *14*(1), 1–15. https://doi.org/10.1186/s13071-020-04536-x

Dzika, E., Kuształa, M., & Kozłowski, J. (2008). Metazoan parasite fauna of fish species from lake kortowskie. *Fisheries & Aquatic Life*, *16*(1), 75–86. https://doi.org/10.2478/s10086-008-0006-4

Erasmus, D. A. (1959). The migration of Cercaria X Baylis (Strigeida) within the fish intermediate host. *Parasitology*, *49*(1), 173–190. https://doi.org/10.1017/S0031182000026810

Flink, H., Behrens, J. W., & Svensson, P. A. (2017). Consequences of eye fluke infection on anti-predator behaviours in invasive round gobies in Kalmar sound. *Parasitology Research*, *116*(6), 1653–1663. https://doi.org/10.1007/s00436-017-5439-5

Galazzo, D. E., Dayanandan, S., Marcogliese, D. J., & McLaughlin, J. D. (2002). Molecular systematics of some North American species of *Diplostomum* (Digenea) based on rDNA-sequence data and comparisons with European congeners. *Canadian Journal of Zoology*, *80*(12), 2207–2217. https://doi.org/10.1139/z02-198

Glöer, P. (2019). *The freshwater gastropods of the West-Palaearctis*. Biodiversity Research Lab.

Gopko, M., Mikheev, V. N., & Taskinen, J. (2015). Changes in host behaviour caused by immature larvae of the eye fluke: Evidence supporting the predation suppression hypothesis. *Behavioral Ecology and Sociobiology*, *69*(10), 1723–1730. https://doi.org/10.1007/s00265-015-1984-z

Gopko, M., Mikheev, V. N., & Taskinen, J. (2017). Deterioration of basic components of the anti-predator behavior in fish harboring eye fluke larvae. *Behavioral Ecology and Sociobiology*, *71*(4), 68. https://doi.org/10.1007/s00265-017-2300-x

Gruys, E., Toussaint, M., Niewold, T., & Koopmans, S. (2005). Acute phase reaction and acute phase proteins. *Journal of Zhejiang University SCIENCE*, *6B*(11), 1045. https://doi.org/10.1631/jzus.2005.B1045

Haarder, S., Kania, P. W., Bahlool, Q. Z., & Buchmann, K. (2013). Expression of immune relevant genes in rainbow trout following exposure to live *Anisakis simplex* larvae. *Experimental Parasitology*, *135*(3), 564–569. https://doi.org/10.1016/j.exppara.2013.09.011

Haase, D., Rieger, J. K., Witten, A., Stoll, M., Bornberg-Bauer, E., Kalbe, M., & Reusch, T. B. (2014). Specific gene expression responses to parasite genotypes reveal redundancy of innate immunity in vertebrates. *PLoS One*, *9*(9), e108001. https://doi.org/10.1371/journal.pone.0108001

Haase, D., Rieger, J. K., Witten, A., Stoll, M., Bornberg-Bauer, E., Kalbe, M., & Reusch, T. B. (2016a). Immunity comes first: The effect of parasite genotypes on adaptive immunity and immunization in three-spined sticklebacks. *Developmental & Comparative Immunology*, *54*(1), 137–144. https://doi.org/10.1016/j.dci.2015.09.008

Haase, D., Rieger, J. K., Witten, A., Stoll, M., Bornberg-Bauer, E., Kalbe, M., Schmidt-Drewello, A., Scharsack, J. P., & Reusch, T. B. (2016b). Comparative transcriptomics of stickleback immune gene responses upon infection by two helminth parasites, *Diplostomum pseudospathaceum* and *Schistocephalus solidus*. *Zoology*, *119*(4), 307–313. https://doi.org/10.1016/j.zool.2016.05.005

Heath, D. I., Cruickshank, A., Gudgeon, M., Jehanli, A., Shenkin, A., & Imrie, C. W. (1993). Role of interleukin-6 in mediating the acute phase protein response and potential as an early means of severity assessment in acute pancreatitis. *Gut*, *34*(1), 41–45. https://doi.org/10.1136/gut.34.1.41

Hirano, T. (2010). Interleukin 6 in autoimmune and inflammatory diseases: A personal memoir. *Proceedings of the Japan Academy, Series B*, *86*(7), 717–730. https://doi.org/10.2183/pjab.86.717

Jensen, H. J. (2015). Utterslev Mose. Environmental condition 2015. Fish Ecological Laboratory. Report to the Municipality of Copenhagen, November 2015. 22 pp. (in Danish).

Kalbe, M., & Kurtz, J. (2006). Local differences in immunocompetence reflect resistance of sticklebacks against the eye fluke *Diplostomum pseudospathaceum*. *Parasitology*, *132*(1), 105. https://doi.org/10.1017/S0031182005008681

Karvonen, A., Kirsi, S., Hudson, P., & Valtonen, E. (2004). Patterns of cercarial production from *Diplostomum spathaceum*: Terminal investment or bet hedging? *Parasitology*, *129*(1), 87. https://doi.org/10.1017/S0031182004005281

Karvonen, A., Paukku, S., Valtonen, E., & Hudson, P. (2003). Transmission, infectivity and survival of *Diplostomum spathaceum* cercariae. *Parasitology*, *127*, 217. https://doi.org/10.1017/S0031182003003561

Karvonen, A., Savolainen, M., Seppälä, O., & Valtonen, E. T. (2006). Dynamics of *Diplostomum spathaceum* infection in snail hosts at a fish farm. *Parasitology Research*, *99*, 341–345. https://doi.org/10.1007/s00436-006-0137-8

Karvonen, A., & Seppälä, O. (2008). Eye fluke infection and lens size reduction in fish: A quantitative analysis. *Diseases of Aquatic Organisms*, *80*, 21–26. https://doi.org/10.3354/dao01918

Karvonen, A., Seppälä, O., & Valtonen, E. (2004). Eye fluke-induced cataract formation in fish: Quantitative analysis using an ophthalmological microscope. *Parasitology*, *129*, 473. https://doi.org/10.1017/S0031182004006006

Kovacevic, N., Hagen, M. O., Xie, J., & Belosevic, M. (2015). The analysis of the acute phase response during the course of *Trypanosoma carassii* infection in the goldfish (*Carassius auratus* L.). *Developmental & Comparative Immunology*, *53*(1), 112–122. https://doi.org/10.1016/j.dci.2015.06.009

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, *35*(6), 1547–1549. https://doi.org/10.1093/molbev/msy096

Lafferty, K. D., & Morris, A. K. (1996). Altered behavior of parasitized killifish increases susceptibility to predation by bird final hosts. *Ecology*, *77*(5), 1390–1397. https://doi.org/10.2307/2265536

Larsen, A., Bresciani, J., & Buchmann, K. (2005). Pathogenicity of *Diplostomum* cercariae in rainbow trout, and alternative measures to prevent diplostomosis in fish farms. *Bulletin of the European Association of Fish Pathologists*, *25*(1), 20–27.

Lemly, A. D., & Esch, G. W. (1984). Effects of the trematode *Uvulifer ambloplitis* on juvenile bluegill sunfish, *Lepomis macrochirus*: Ecological implications. *Journal of Parasitology*, *70*(4), 475–492. https://doi.org/10.2307/3281395

Loy, C., & Haas, W. (2001). Prevalence of cercariae from *Lymnaea stagnalis* snails in a pond system in Southern Germany. *Parasitology Research*, *87*(10), 878–882. https://doi.org/10.1007/s004360100462

Lyholt, H., & Buchmann, K. (1996). *Diplostomum spathaceum*: Effects of temperature and light on cercarial shedding and infection of rainbow trout. *Diseases of Aquatic Organisms*, *25*(3), 169–173. https://doi.org/10.3354/dao025169

Marcogliese, D. J., Dumont, P., Gendron, A. D., Mailhot, Y., Bergeron, E., & McLaughlin, J. D. (2001). Spatial and temporal variation in abundance of *Diplostomum* spp. in walleye (*Stizostedion vitreum*) and white suckers (*Catostomus commersoni*) from the St. Lawrence River. *Canadian Journal of Zoology*, *79*(3), 355–369. https://doi.org/10.1139/cjz-79-3-355

McCloughlin, T. (2016). A sight for sore eyes: *Diplostomum* and *Tylodelphys* in the eyes of fish. *Iranian Journal of Parasitology*, *11*(3), 429–430.

Medzhitov, R., & Janeway, C. A. (1997). Innate immunity: The virtues of a nonclonal system of recognition. *Cell*, *91*(3), 295–298. https://doi.org/10.1016/S0092-8674(00)80412-2

Michálková, V., & Ondračková, M. (2014). Experimental evidence for parasite-induced over-winter mortality in juvenile *Rhodeus amarus*. *Journal of Fish Biology*, *84*(5), 1377–1388. https://doi.org/10.1111/jfb.12363

Morley, N., Crane, M., & Lewis, J. W. (2005). Changes in survival characteristics of *Diplostomum spathaceum* cercariae emerged from cadmium-exposed *Lymnaea stagnalis*. *Journal of Helminthology*, *79*(1), 55. https://doi.org/10.1079/JOH2004271

Morley, N., & Lewis, J. W. (2020). Influence of an extreme climatic event on bilateral asymmetry and occurrence of eye flukes in perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) from a lake in southeast England. *Journal of Helminthology*, *94*. https://doi.org/10.1017/S0022149X19000506

Morozinska-Gogol, J. (2013). Parasite communities of European perch, *Perca fluviatilis* L. (Actinopterygii: Perciformes: Percidae) from Lake Lebsko (Central Coast, Poland). *Annals of Parasitology*, *59*(2), 89–98.

Muñoz, J. C. V., Bierbach, D., & Knopf, K. (2019). Eye fluke (*Tylodelphys clavata*) infection impairs visual ability and hampers foraging success in European perch. *Parasitology Research*, *118*(9), 2531–2541. https://doi.org/10.1007/s00436-019-06389-5

Muñoz, J. C. V., Staaks, G., & Knopf, K. (2017). The eye fluke *Tylodelphys clavata* affects prey detection and intraspecific competition of European perch (*Perca fluviatilis*). *Parasitology Research*, *116*(9), 2561–2567. https://doi.org/10.1007/s00436-017-5564-1

Näreaho, A., Eriksson-Kallio, A. M., Heikkinen, P., Snellman, A., Sukura, A., & Koski, P. (2017). High prevalence of zoonotic trematodes in roach (*Rutilus rutilus*) in the Gulf of Finland. *Acta Veterinaria Scandinavica*, *59*(1), 1–4. https://doi.org/10.1186/s13028-017-0343-7

Niewiadomska, K. (1986). Verification of the life-cycles of *Diplostomum spathaceum* (Rudolphi, 1819) and *D. pseudospathaceum* Niewiadomska, 1984 (Trematoda, Diplostomidae). *Systematic Parasitology*, *8*(1), 23–31. https://doi.org/10.1007/BF00010306

Niewiadomska, K., Czubaj, A., & Moczon, T. (1996). Cholinergic and aminergic nervous systems in developing cercariae and metacercariae of *Diplostomum pseudospathaceum* Niewiadomska, 1984 (Digenea). *International Journal for Parasitology*, *26*(2), 161–168. https://doi.org/10.1016/0020-7519(95)00106-9

Ohtani, M., Hayashi, N., Hashimoto, K., Nakanishi, T., & Dijkstra, J. M. (2008). Comprehensive clarification of two paralogous interleukin 4/13 loci in teleost fish. *Immunogenetics*, *60*(7), 383–397. https://doi.org/10.1007/s00251-008-0299-x

Ostrand, K. G., Siepker, M. J., & Cooke, S. J. (2006). Capture efficiencies of two hook types and associated injury and mortality of juvenile muskellunge angled with live baitfish. *North American Journal of Fisheries Management*, *26*(3), 622–627. https://doi.org/10.1577/M05-038.1

Pike, A., & Lewis, J. (1994). *Parasitic diseases of fish*. Samara Publishing Ltd.

Poulin, R. (2010). Parasite manipulation of host behavior: An update and frequently asked questions. *Advances in the Study of Behavior*, *41*, 151–186. https://doi.org/10.1016/S0065-3454(10)41005-0

Riley, E., & Chappell, L. H. (1992). Effect of infection with *Diplostomum spathaceum* on the internal defense system of *Lymnaea stagnalis*. *Journal of Invertebrate Pathology*, *59*(2), 190–196. https://doi.org/10.1016/0022-2011(92)90032-Y

Ruijter, J. M., Ramakers, C., Hoogaars, W. M. H., Karlen, Y., Bakker, O., van den Hoff, M. J. B., & Moorman, A. F. M. (2009). Amplification efficiency: Linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Research.*, *37*(6), e45. https://doi.org/10.1093/nar/gkp045

Scharsack, J. P., & Kalbe, M. (2014). Differences in susceptibility and immune responses of three-spined sticklebacks (*Gasterosteus aculeatus*) from lake and river ecotypes to sequential infections with the eye fluke *Diplostomum pseudospathaceum*. *Parasites & Vectors*, *7*(1), 1–10. https://doi.org/10.1186/1756-3305-7-109

Seppälä, O., Karvonen, A., & Valtonen, E. T. (2004). Parasite-induced change in host behaviour and susceptibility to predation in an eye fluke–fish interaction. *Animal Behaviour*, *68*(2), 257–263. https://doi.org/10.1016/j.anbehav.2003.10.021

Seppälä, O., Karvonen, A., & Valtonen, E. T. (2005). Manipulation of fish host by eye flukes in relation to cataract formation and parasite infectivity. *Animal Behaviour*, *70*(4), 889–894. https://doi.org/10.1016/j.anbehav.2005.01.020

Seppälä, O., Karvonen, A., & Valtonen, E. T. (2006). Host manipulation by parasites and risk of non-host predation: Is manipulation costly in an eye fluke–fish interaction? *Evolutionary Ecology Research*, *8*(5), 871–879.

Sereno-Uribe, A., López-Jimenez, A., Andrade-Gómez, L., & García-Varela, M. (2019). A morphological and molecular study of adults and metacercariae of *Hysteromorpha triloba* (Rudolphi, 1819), Lutz 1931 (Diplostomidae) from the Neotropical region. *Journal of Helminthology*, *93*(1), 91–99. https://doi.org/10.1017/S0022149X17001237

Shevach, E. M. (2009). Mechanisms of foxp3^{+} T regulatory cell-mediated suppression. *Immunity*, *30*(5), 636–645. https://doi.org/10.1016/j.immuni.2009.04.010

Sigh, J., Lindenstrøm, T., & Buchmann, K. (2004). Expression of pro-inflammatory cytokines in rainbow trout (*Oncorhynchus mykiss*) during an infection with *Ichthyophthirius multifiliis*. *Fish & Shellfish Immunology*, *17*(1), 75–86. https://doi.org/10.1016/j.fsi.2003.12.005

Sitjà-Bobadilla, A. (2008). Living off a fish: A trade-off between parasites and the immune system. *Fish & Shellfish Immunology*, *25*(4), 358–372. https://doi.org/10.1016/j.fsi.2008.03.018

Skov, J., Kania, P. W., Jørgensen, T. R., & Buchmann, K. (2008). Molecular and morphometric study of metacercariae and adults of *Pseudamphistomum truncatum* (Opisthorchiidae) from roach (*Rutilus rutilus*) and wild American mink (*Mustela vison*). *Veterinary Parasitology*, *155*(3), 209–216. https://doi.org/10.1016/j.vetpar.2008.05.011

Stanevičiūtė, G., Petkevičiūtė, R., & Kiselienė, V. (1998). Karyological analysis of two species of genus *Posthodiplostomum* Dubois, 1936 (Trematoda: Diplostomidae) with remarks on the karyological evolution of Diplostomidae. *Acta Zoologica Lituanica*, *8*(1), 41–47. https://doi.org/10.1080/13921657.1998.10541437

Tadiso, T. M., Krasnov, A., Skugor, S., Afanasyev, S., Hordvik, I., & Nilsen, F. (2011). Gene expression analyses of immune responses in Atlantic salmon during early stages of infection by salmon louse (*Lepeophtheirus salmonis*) revealed bi-phasic responses coinciding with the copepod-chalimus transition. *BMC Genomics*, *12*(1), 1–17. https://doi.org/10.1186/1471-2164-12-141

Takahashi, Y., Okamura, Y., Morimoto, N., Mihara, K., Maekawa, S., Wang, H.-C., Aoki, T., Kono, T., Sakai, M., & Hikima, J. I. (2020).

Interleukin-17A/F1 from Japanese pufferfish (*Takifugu rubripes*) stimulates the immune response in head kidney and intestinal cells. *Fish & Shellfish Immunology*, *103*, 143–149. https://doi.org/10.1016/j.fsi.2020.05.016

Thatcher, T. O. (1979). A morphological defect in shiner perch resulting from chronic exposure to chlorinated sea water. *Bulletin of Environmental Contamination and Toxicology*, *21*(1), 433–438. https://doi.org/10.1007/BF01685449

Titus, R. G., Sherry, B., & Cerami, A. (1991). The involvement of TNF, IL-1 and IL-6 in the immune response to protozoan parasites. *Parasitology Today*, *7*(3), 13–16. https://doi.org/10.1016/0169-4758(91)90022-G

Tkach, V., & Pawlowski, J. W. (1999). A new method of DNA extraction from the ethanol-fixed parasitic worms. *Acta Parasitologica*, *44*(2), 147–148.

Tuomi, J. M., Voorbraak, F., Jones, D. L., & Ruijter, J. M. (2010). Bias in the Cq value observed with hydrolysis probe based quantitative PCR can be corrected with the estimated PCR efficiency value. *Methods*, *50*(4), 313–322. https://doi.org/10.1016/j.ymeth.2010.02.003

Uhlar, C. M., & Whitehead, A. S. (1999). Serum amyloid A, the major vertebrate acute-phase reactant. *European Journal of Biochemistry*, *265*(2), 501–523. https://doi.org/10.1046/j.1432-1327.1999.00657.x

Valtonen, E. T., & Gibson, D. I. (1997). Aspects of the biology of diplostomid metacercarial (Digenea) populations occurring in fishes in different localities of northern Finland. *Finnish Zoological and Botanical Publishing Board*, 47–59. https://www.jstor.org/stable/23735430

Van der Knaap, W., & Loker, E. (1990). Immune mechanisms in trematode-snail interactions. *Parasitology Today*, *6*(6), 175–182. https://doi.org/10.1016/0169-4758(90)90349-9

Vivas Muñoz, J. C. (2019). *Trematodes modulate aquatic food webs by altering host feeding behaviour*. Humboldt-Universität zu Berlin. https://edoc.hu-berlin.de/handle/18452/21420 (accessed 1 November 2019)

Wang, T., Johansson, P., Abós, B., Holt, A., Tafalla, C., Jiang, Y., Wang, A., Xu, Q., Qi, Z., Huang, W., Costa, M. M., Diaz-Rosales, P., Holland, J. W., & Secombes, C. J. (2016). First in-depth analysis of the novel Th2-type cytokines in salmonid fish reveals distinct patterns of expression and modulation but overlapping bioactivities. *Oncotarget*, *7*, 10917. https://doi.org/10.18632/oncotarget.7295

Wesenberg-Lund, C. (1932). On the occurrence of developmental stages of blood parasitizing flukes in Danish freshwaters. *Naturens Verden*, *16*, 433–446. (in Danish).

Whyte, S., Secombes, C., & Chappell, L. H. (1991). Studies on the infectivity of *Diplostomum spathaceum* in rainbow trout (*Oncorhynchus mykiss*). *Journal of Helminthology*, *65*(3), 169–178. https://doi.org/10.1017/S0022149X0001066X

Wills-Karp, M., & Finkelman, F. D. (2008). Untangling the complex web of IL-4–and IL-13– mediated signaling pathways. *Science Signaling*, *1*(51), pe55. https://doi.org/10.1126/scisignal.1.51.pe55

Wisniewski, W. L. (1958). The development cycle of *Posthodiplostomum brevicaudatum* (v. Nordmann, 1832) Kozicka, *Acta Parasitologica Polonica*. *6*(8/21).

Zou, J., & Secombes, C. J. (2016). The function of fish cytokines. *Biology*, *5*(2), 23. https://doi.org/10.3390/biology5020023

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Duan, Y., von Gersdorff Jørgensen, L., Kania, P. W., Karami, A. M., Al-Jubury, A., & Buchmann, K. (2021). Eye fluke effects on Danish freshwater fish: Field and experimental investigations. *Journal of Fish Diseases*, 44, 1785–1798. https://doi.org/10.1111/jfd.13496