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Effects of *Pseudoloma neurophilia* infection on the brain transcriptome in zebrafish (*Danio rerio*)

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Abstract

Laboratory zebrafish are commonly infected with the intracellular, brain-infecting microsporidian parasite *Pseudoloma neurophilia*. Chronic *P. neurophilia* infections induce inflammation in meninges, brain and spinal cord, and have been suggested to affect neural functions since parasite clusters reside inside neurons. However, underlying neural and immunological mechanisms associated with infection have not been explored. Utilizing RNA-sequencing analysis, we found that *P. neurophilia* infection upregulated 175 and downregulated 45 genes in the zebrafish brain, compared to uninfected controls. Four biological pathways were enriched by the parasite, all of which were associated with immune function. In addition, 14 gene ontology (GO) terms were enriched, eight of which were associated with immune responses and five with circadian rhythm. Surprisingly, no differentially expressed genes or enriched pathways were specific for nervous system function. Upregulated immune-related genes indicate that the host generally show a pro-inflammatory immune response to infection. On the other hand, we found a general downregulation of immune response genes associated with anti-pathogen functions, suggesting an immune evasion strategy by the parasite. The results reported here provide important information on host–parasite interaction and highlight possible pathways for complex effects of parasite infections on zebrafish phenotypes.

KEYWORDS

health monitoring, immune evasion, immune system, microsporidium, RNA sequencing

1 | INTRODUCTION

Animal research models are crucial for generating new fundamental knowledge in life sciences. For example, studies utilizing animal models can help researchers identify disease mechanisms and develop novel therapeutic agents in human medicine (Insel, 2007). The usefulness of animal models in biological research hinges on study animals being healthy and free of pathogens. Pathogens like viruses, bacteria and parasites are known to influence physiology, immune mechanisms and behaviour, all of which can cause bias in study outcomes (Baker, 1998; Nicklas et al., 1999). Adding to the complexity, there is large interspecific variation in how animals respond to certain pathogens (Ehret, Torelli, Klotz, Pedersen, & Seeber, 2017). Animal research facilities have struggled with pathogen infections since animals were first brought into use by modern science, but concerns about how the spread of pathogens and infectious disease could confound research results were first raised in the mid-1900s (Baker, 2003; Nicklas, 2007). Since then, regular health monitoring

in for example rodent research facilities has improved drastically and many pathogens have been systematically eradicated from these systems (Weisbroth, 1999).

Among the vertebrate lineage, teleost fish (e.g. zebrafish; *Danio rerio*, medaka; *Oryzias latipes* and goldfish; *Carassius auratus*) are now rapidly complementing or even replacing rodent models in scientific disciplines like neurobiology, toxicology and immunology. In particular, zebrafish are increasingly popular laboratory animal models. These fish are easy and less expensive to maintain (compared to rodents), have short generation time and are viable for genetic manipulation. There is, indeed, a rapidly expanding availability of genomic resources for this species (Kinth, Mahesh, & Panwar, 2013; Lieschke & Currie, 2007; Meyers, 2018). Unfortunately, there has been minute focus on possible implications of common infectious agents that colonize laboratory fish. Consequently, there has also been little focus on treatment and eradication of such agents, and standard health monitoring programmes to prevent the introduction of pathogens in fish facilities are not widely practiced (Collymore, Crim, & Lieggi, 2016; Crim & Riley, 2012; Marancik, Collins, Afema, & Lawrence, 2019). Yet, numerous viruses, bacteria and parasites have been detected and characterized in many fish research facilities (Kent et al., 2009).

Of particular worry, in 2010 the Zebrafish International Research Center (ZIRC) found that more than 70% of all tested zebrafish facilities held fish infected with the brain-dwelling, intracellular microsporidium parasite *Pseudoloma neurophilia* (Murray et al., 2011). The high prevalence of this parasite in zebrafish facilities is alarming for several reasons. First, *P. neurophilia* infections are generally subclinical (*i.e.* no visible signs) (Kent & Bishop-Stewart, 2003; Matthews et al., 2001) and infection status of the fish is therefore often unknown to the researcher. Second, despite the high prevalence, very little is known about how the parasite affects the zebrafish host and thus its possible implications in study outcomes. As the specific epithet implies, spores and parasite clusters of *P. neurophilia* are primarily found in neurons of the central nervous system (CNS), such as the spinal cord and hind brain. In the brain, the parasite has the potential to influence a myriad of biological processes. Although the parasite was discovered in the 1980s (Kinkelin, 1980), it is only within the last two decades that possible implications of the infection on research outcomes has begun to be investigated.

Infection with *P. neurophilia* has so far been shown to alter shoaling behaviour and startle responses in zebrafish (Spagnoli, Sanders, & Kent, 2017; Spagnoli, Xue, & Kent, 2015). Moreover, it negatively affects growth (Ramsay, Watral, Schreck, & Kent, 2009a; Sanders, Monteiro, Martins, Certal, & Kent, 2020) and general activity (Midttun, Vindas, Nadler, Øverli, & Johansen, 2020). The spore stage of the parasite has been shown to induce inflammation in the brain, spinal cord, meninges and occasionally in the muscles. However, parasite clusters—part of the sporogenic development when immature spores cluster in isolated vacuoles (Cali, Kent, Sanders, Pau, & Takvorian, 2012)—do not appear to provoke severe inflammation (Spagnoli, Xue, Murray, Chow, & Kent, 2015). This apparent ability of *P. neurophilia* to limit inflammatory responses at this life stage makes it particularly interesting to characterize neuroimmune interactions that may be at play at the molecular level. Immune suppression is a well-known approach for many parasites to avoid elimination from their host (Maizels, Smits, & McSorley, 2018). Whether the moderate inflammatory response observed in the CNS of infected zebrafish reflects *P. neurophilia*-induced suppression of certain immune pathways thus needs to be explored. Furthermore, to what degree *P. neurophilia* affects other biological processes in the nervous system is unknown.

In theory, *P. neurophilia* infection may affect a wide variety of fields, such as neurobiology, toxicology and pharmacology. However, with the current knowledge we are not yet able to predict what scientific disciplines may be affected by subclinical *P. neurophilia* infections. Nevertheless, given previous reports on behavioural effects associated with *P. neurophilia* infection and the location of this parasite inside CNS neurons, we suspect the parasite to affect neural signalling pathways. Moreover, other intracellular and extracellular parasites have been found to affect numerous host biological processes in mammals and fishes. For example, in mice (*Mus musculus*) the parasite *Leishmania major* disrupts circadian rhythm in immune cells (Kiessling et al., 2017), while the brain-encysting trematode *Euhaplorchis californiensis* alters CNS neurotransmitter levels in California killifish (*Fundulus parvipinnis*) (Shaw et al., 2009; Shaw & Øverli, 2012).

Here, we aimed to characterize the differential expression of genes (DEGs) in response to *P. neurophilia* infection in the zebrafish brain, by means of RNA-sequencing (RNAseq). This method not only allows for identification of gene expression changes of single genes, but also identification of novel regulatory and functional networks involved in biological processes that may be affected by parasite infection. This study is, as far as we are aware, the first attempt to identify effects of *P. neurophilia* in zebrafish at the molecular level and will help provide new and important insights into our understanding of the wider range of effects of microsporidian infections on host phenotype.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

This work was approved by the Norwegian Animal Research Authority (NARA), following the Norwegian laws and regulations controlling experiments and procedures on live animals in Norway (permit number 11241).

2.2 | Experimental animals and facilities

Zebrafish were reared at the Norwegian University of Life Sciences, campus Adamstuen (Oslo, Norway). Five male and five female adult AB zebrafish were obtained from the *P. neurophilia* specific pathogen free (SPF) facility Sinnhuber Aquatic Research Laboratory (SARL), Oregon State University, USA. The fish were kept in a

quarantine room in a 25-L glass tank (40 cm × 25 cm × 25 cm; L × W × H) for two months in order to acclimate. Tanks were provided with UV-treated and filtered freshwater throughout this period, and 50% of the water was changed twice weekly. Water was kept at 28°C, 7.4–7.6 pH and 500µS conductivity; furthermore, the photoperiod was kept at 14:10 light/dark following recommendations from the Zebrafish International Research Center (ZIRC) (Westerfield, 2007). Fish were fed flake food twice daily (Special Diets Services; SDS; Witham, United Kingdom) and live brine shrimp (Ocean Nutrition, Essen, Belgium) once per day. Following the acclimation period, the fish were transferred to a recirculating system (Techniplast, Buguggiate, Italy) and kept at a density of 5 fish/L, with water conditions maintained as described above.

To breed more SPF fish, the adult fish were transferred to 1-L standard breeding tanks (Techniplast, Buguggiate, Italy) overnight once per week. Males and females were separated by a divider, which was removed the following morning. Fish were then allowed to spawn for up to four hours, before being transferred to their respective holding tanks, according to ZIRC recommendations (Westerfield, 2007). Eggs were collected, rinsed with autoclaved water, counted and maintained at a density of 50 eggs/30ml in Petri dishes (95 × 15 mm; Heger, Rjukan, Norway) at 28°C for five days post-fertilization (dpf). During this period, dead eggs were removed, and water was changed daily. Zebrafish larvae were transferred to 1-L plastic beakers (VWR, Radnor Pennsylvania, USA) with UV-treated and filtered water at 5 dpf. Larvae were maintained at a density of 1 fish/6ml of water and were fed twice daily with freeze dried rotifers and small-grained dry food (SDS). Water was changed daily. At 21 dpf, juvenile zebrafish were transferred to a recirculating system with water quality and feeding routines kept as described above. The light:dark cycle was always kept at 14-hr light:10-hr dark.

2.3 | Experimental design

Approximately 5 months after hatching, 252 zebrafish from the F1 generation were moved to an infection room, where experimental infections were conducted over a period of 10 weeks. Zebrafish were divided into 30 tanks (23 × 15.3 × 16.5 cm; L × W × H) (Exo Terra, Montreal, Canada), 15 control and 15 infected, by using a random number generator (https://www.random.org/), and keeping a female:male ratio of 1:1 in each tank. Eight fish were placed in each tank, keeping a density of 5 fish/L. Water was kept at 26–28°C and was continuously aerated using air pumps (Eheim, Stuttgart, Germany) and air stones. Three times a week, 50% of the water was changed, and all water was substituted once biweekly. Fish were fed flake food twice daily (SDS). Simultaneously, two extra donor groups of approximately 80 fish were kept in 25L (40 cm × 25 cm × 25; L × W × H) tanks, with one group consisting of zebrafish positive for only *P. neurophilia* and one with SPF zebrafish. These fish were kept under same conditions as described above and were used for the control infections as explained below.

For experimental infections, 100 ml of water from each tank was substituted with 100 ml water from either the donor tank containing *P. neurophilia*-infected fish, or from the donor tank containing SPF fish daily for 10 weeks. Furthermore, zebrafish in the *P. neurophilia*-treated group were exposed to infectious spores four times as described by Peneyra et al. (2018) with at least two weeks between each exposure. Briefly, CNS tissue and spinal cords were removed from fish from the donor group and macerated by passing the samples through sterile needles with decreasing gauge size (18, 23, 26 G) (Braun Medical, Sempach, Switzerland). The samples were then mixed with brine shrimp to increase ingestion by the zebrafish before being added to the tanks. This was controlled by conducting the same procedure with CNS and spinal cord tissue from SPF controls and feeding it to control groups. During the infection study, 12 fish from the *P. neurophilia*-treated group died from *P. neurophilia* infection, while two fish from the control group died of swim bladder disorder.

2.4 | DNA extraction and qPCR

To test for the presence of *P. neurophilia*, 20 fish from each of the donor tanks and 50% of the fish from the infection study were euthanized in an overdose (1g/L) of Tricaine methanesulfonate (MS-222; Sigma, St. Louis Missouri, USA). Brains were removed and homogenized by two minutes of sonication at 55W (QSonica Sonicators, Connecticut Newtown, USA) before immediately being placed on ice. Between each sample, the sonicator probe was decontaminated with 100% ethanol. To extract DNA, the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) was used according to manufacturer's protocol. The qPCR protocol and a prior overnight proteinase K and lysis buffer digestion at 56°C was conducted following protocol by Sanders and Kent (2011). Briefly, a concentration of 900nm of forward and reverse primers was used, with the addition of 250nM hydrolysis probe, 1X TaqMan and 2 µl DNA sample to a total of 25 µl per reaction. Forward and reverse primers as well as the hydrolysis probe were as follows: 5'-GTAATCGCGGGCTCACTAAG-3', 5'-GCTCGCTCAGCCAAATAAAC-3' and 5'-6-carboxyfluorescein (FAM)-ACACACCGCCCGTCGTTATCGAA—3'-Black Hole Quencher 1 (BHQ1), respectively. The following qPCR programme was used: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15s, 60°C for 1 min on a LightCycler 96 instrument (Roche, Basel, Switzerland) and analysed using the LightCycler 96 software. Only fish from the *P. neurophilia*-treated group tested positive for the presence of the parasite.

2.5 | Sampling

Five randomly selected zebrafish from five different tanks in each group (infected and control) were euthanized in an overdose of MS-222 as described above. For both groups, two males and three females were used. Fish were weighed and measured before brains

were dissected out and divided into telencephalon (Tel), optic tectum (OT), hypothalamus (Hyp) and brain stem (BS) under a dissecting scope. Brain parts were transferred to 100 µl RNAlater (Thermo Fisher Scientific, Waltham Massachusetts, USA) and immediately placed on dry ice before being stored at −80°C until further analysis.

2.6 | RNA extraction

In order to obtain a sufficient concentration of RNA required for RNA sequencing (RNAseq) analysis, extracted brain parts were pooled (*e.g.* all control Tel were pooled) for control and infected fish so that the following samples were sequenced and analysed: control Tel, infected Tel, control OT, infected OT, Control Hyp, infected Hyp, control BS and infected BS. Unfortunately, this pooling did not allow for analysis of gene expression in individual fish. However, sequencing of different brain parts allowed for analysis of consistency of transcriptional changes throughout all brain areas. The RNA extraction was done using a RNeasy® Plus Micro Kit according to manufacturer's protocol (Qiagen, Hilden, Germany). Concentration of the samples was measured using NanoDrop (Thermo Fisher Scientific, Waltham Massachusetts, USA), and RNA integrity (RIN score) was quantified using the Agilent RNA 6000 Pico Kit according to manufacturer's protocol (Agilent, Santa Clara California, USA), with scores between 7.1 and 8.8. RNA samples were kept at −80°C until further analysis.

2.7 | Transcriptome sequencing

Sequencing of total RNA was completed by NovoGene (Beijing, China). After additional quality testing at Novogene, total RNA samples were enriched with oligo(dT) magnetic beads for extraction of mRNA. First-strand cDNA was synthesized by randomly fragmenting the mRNA in fragmentation buffer, combining with random hexamers and assembling with M-MuLV reverse transcriptase. Complementary strands were then synthesized by nick translation using a custom (Illumina) synthesis buffer containing dNTP's, Rnase H and *Escherichia coli* polymerase I. The resultant cDNA library underwent adapter ligation, terminal repair, poly A-tailing, size selection and PCR enrichment, before a final quality assessment—concentration by Qubit 2.0 fluorometer (Life Technologies), insert size by Agilent 2,100 Bioanalyzer and quantification by qPCR. Libraries were sequenced as 150bp, paired-end reads on an Illumina Hiseq 2,500 instrument. For sequencing analysis, the four samples for each group (n = 4) were compared to each other in order to find common regulated genes throughout all brain parts.

2.8 | Read mapping and quantification

Reads were mapped to both the NCBI zebrafish reference genome (GRCz11; ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/000/002/035/GCF_000002035.6_GRCz11/) and the *Pseudoloma neurophilia* reference genome (ASM143216v1; ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/001/432/165/GCA_001432165.1_ASM143216v1/). HISAT2 v2.1.0 (Kim, Paggi, Park, Bennett, & Salzberg, 2019) was used to map reads to the reference genomes. FeatureCounts v1.6.5 was used to quantify the number of reads that mapped to gene regions, said regions being defined by the general feature format (GFF) annotation files for each genome. Quantification generated a table of read counts per gene which was used in downstream (beginning with differential expression) analysis, completed in R version 3.6.1 (R Developer Core Team, 2019).

2.9 | Differential expression analysis and functional annotations

The DEGs were initially defined by their Entrez (RefSeq) gene identifiers, which were then annotated to gene descriptions and symbols using the AnnotationHub (v2.16.1) package. For functional clarity, gene symbols are presented in this paper, though Entrez IDs were used as input for GO term and KEGG pathway enrichment analysis. The R package DESeq2 (Love, Huber, & Anders, 2014) was used for the identification of DEGs, using the read count table generated by featureCounts. DESeq2 initially performs library size and RNA composition normalization based on per-gene geometric mean between samples, then estimates DE using a negative binomial generalized linear model. Significantly DEGs were identified with a Wald test and a significance cut-off of less than 0.05 false discovery-adjusted (Benjamini-Hochberg) p-value. A fold change cut-off was not introduced, as DESeq2 is designed to identify small, true differences and accurately control for false positives (Love et al., 2014). Enrichment of KEGG pathways and GO terms was estimated using the clusterProfiler package v3.12.0 (Yu, Wang, Han, & He, 2012). An over-representation test was used to estimate enrichment, with significantly enriched (q-value < 0.05) pathways or terms identified by Fisher's exact test.

3 | RESULTS

3.1 | RNAseq results

As stated in the methods section, sequence reads were mapped to both zebrafish and *Pseudoloma neurophilia* reference genomes. Zebrafish were sampled from tanks where the presence or absence of the parasite was confirmed as described in methods. As the goal of this study was to examine gene expression in zebrafish in response to *Pseudoloma neurophilia* infection, we carried out differential expression (DE) and other downstream analysis only on the zebrafish-mapped sequence results for the fish in this study. However, mapping to the *Pseudoloma neurophilia* reference genome provided validation of the presence of the parasite in infected animals: 5,269 reads from infected animals mapped to the parasite genome, compared to 0 reads from uninfected animals.

TABLE 1 Differently expressed genes (DEGs) associated with immune responses between *Pseudoloma neurophilia*-infected zebrafish and uninfected controls

Log2Fold change	Adjusted p-value	Gene ID	Description
−8.32	$4.3*10^{-10}$	30762	Major histocompatibility complex class II DAB gene
−2.98	$2.5*10^{-2}$	360143	Myxovirus (influenza) resistance B
0.98	$5.7*10^{-3}$	791453	Major histocompatibility complex class I ZBA
1.2	$6.3*10^{-3}$	30645	CD74 molecule, Major histocompatibility complex, class invariant chain B
1.32	$2.7*10^{-2}$	445073	Suppressor of cytokine signalling 1a
1.49	$2.7*10^{-4}$	447809	T-cell activation Rho GTPase activating protein b
1.71	$3.0*10^{-2}$	793819	CD40 ligand
1.87	$1.4*10^{-6}$	103910066	B- and T-lymphocyte attenuator-like
2.16	$5.8*10^{-3}$	368967	T-cell receptor alpha constant
2.34	$6.3*10^{-10}$	654692	Chemokine (C-X-C motif) receptor 3, tandem duplicate 1
2.53	$7.7*10^{-3}$	360145	Myxovirus (influenza virus) resistance C
2.62	$1.0*10^{-4}$	561000	Tumour necrosis factor receptor superfamily member 5-like
2.62	$1.3*10^{-2}$	798906	IL2-inducible T-cell kinase
2.69	$5.4*10^{-3}$	795887	Interferon-induced protein 44
2.72	$9.1*10^{-4}$	103909973	Interferon-induced protein 44
2.83	$1.4*10^{-9}$	100537088	Chemokine (C-C motif) ligand 36, duplicator 1
2.85	$6.0*10^{-3}$	100135062	Colony stimulating factor 2 receptor, beta, low affinity (granulocyte–macrophage)
3.08	$4.1*10^{-7}$	101886833	C-X-C motif chemokine 11-like
3.09	$2.1*10^{-2}$	100329726	Chemokine (C motif) receptor 1b, duplicate 3
3.21	$4.8*10^{-6}$	108190761	Tumour necrosis factor ligand superfamily member 14-like
3.68	$1.1*10^{-2}$	405790	Interferon gamma 1
3.75	$1.6*10^{-3}$	567656	C-X-C motif chemokine 11–6-like
3.83	$4.6*10^{-6}$	798043	Immunoglobulin C1-set domain
3.83	$3.5*10^{-3}$	101884219	Cytotoxic and regulatory T-cell molecule
3.96	$3.2*10^{-2}$	798119	TNF superfamily member 14
4.38	$7.0*10^{-3}$	100006534	cd8 beta
4.66	$1.9*10^{-3}$	60652	Novel immune-type receptor 4a
4.78	$2.4*10^{-4}$	101884895	Immunoglobulin light 4 variable 8
4.95	$7.5*10^{-35}$	556621	Chemokine (C-C motif) ligand 34b, duplicate 4
5.90	$6.2*10^{-9}$	677754	CD8a molecule
5.95	$7.2*10^{-7}$	100150591	Immunoglobulin kappa variable 1–9-like
6.11	$3.1*10^{-3}$	60647	Novel immune-type receptor 2b
6.19	$5.8*10^{-3}$	101887143	CD27 molecule
7.00	$3.1*10^{-5}$	101884556	Interferon-induced very large GTPase 1-like

In order to examine how *P. neurophilia* affects the zebrafish brain at the molecular level, we measured transcript abundance in experimentally infected and uninfected fish using the Illumina sequencing platform (n = 4 per group). A total of 39,701 genes were identified. For *P. neurophilia*-infected zebrafish, the total reads per sample ranged from 20.01 to 23.88 million (mean = 21.81 million), while for uninfected controls the reads ranged from 21.58 to 22.32 million (mean = 22.07 million). Mapping rate percentage, that is the proportion of sample sequences that matched to reference genome, in *P. neurophilia*-infected zebrafish was between 71.17 and 72.57, while it was between 71.91 and 73.62 for uninfected controls (Table S1).

3.2 | Differential gene expression analysis

Differential expression analysis indicated that between uninfected controls and *P. neurophilia*-infected zebrafish brains, 220 genes were significantly differentially expressed (0.55% of all identified genes, $p < .05$). Of these 220 genes, 175 were upregulated, while

45 were downregulated (Fig. S1), with cluster of differentiation 27 (*cd27*), *cd8a*, *cd8b* being some of the most upregulated genes, while major histocompatibility complex II DAB (*mhc2dab*) was one of the most downregulated genes. All differentially expressed genes (DEGs) can be found in Table S2. Notably, out of the 220 DEGs, we found that 34 genes were associated with the immune response system (Table 1). Figure 1 shows a heatmap of the top 40 DEGs.

3.3 | KEGG over-representation analysis

Over-representation analysis identified four significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Enriched pathways are grouping of genes participating in same cellular biological systems, containing an over-represented number of significantly DEGs in the analysed samples ($p < .05$) compared to the background number of genes in each pathway. The pathways are proteasome, cytokine–cytokine receptor interaction, cell adhesion molecules (CAMs) and herpes simplex virus 1 infection. All pathways had a gene count of 7 regulated genes, except herpes simplex virus 1 infection with 6 regulated genes (Table 2). For all affected pathways, a minimum of one gene showed a connection to an immune response. A map of the KEGG pathway cell adhesion molecules (Figure 2) and the genes involved indicated that no major neural systems were affected by *P. neurophilia* infection, except for immune neural responses. Maps for the remaining pathways can be found in supplementary material (Fig. S2).

3.4 | Gene ontology over-representation test

To identify biological processes affected by *P. neurophilia*, a Gene Ontology (GO) over-representation analysis was performed by

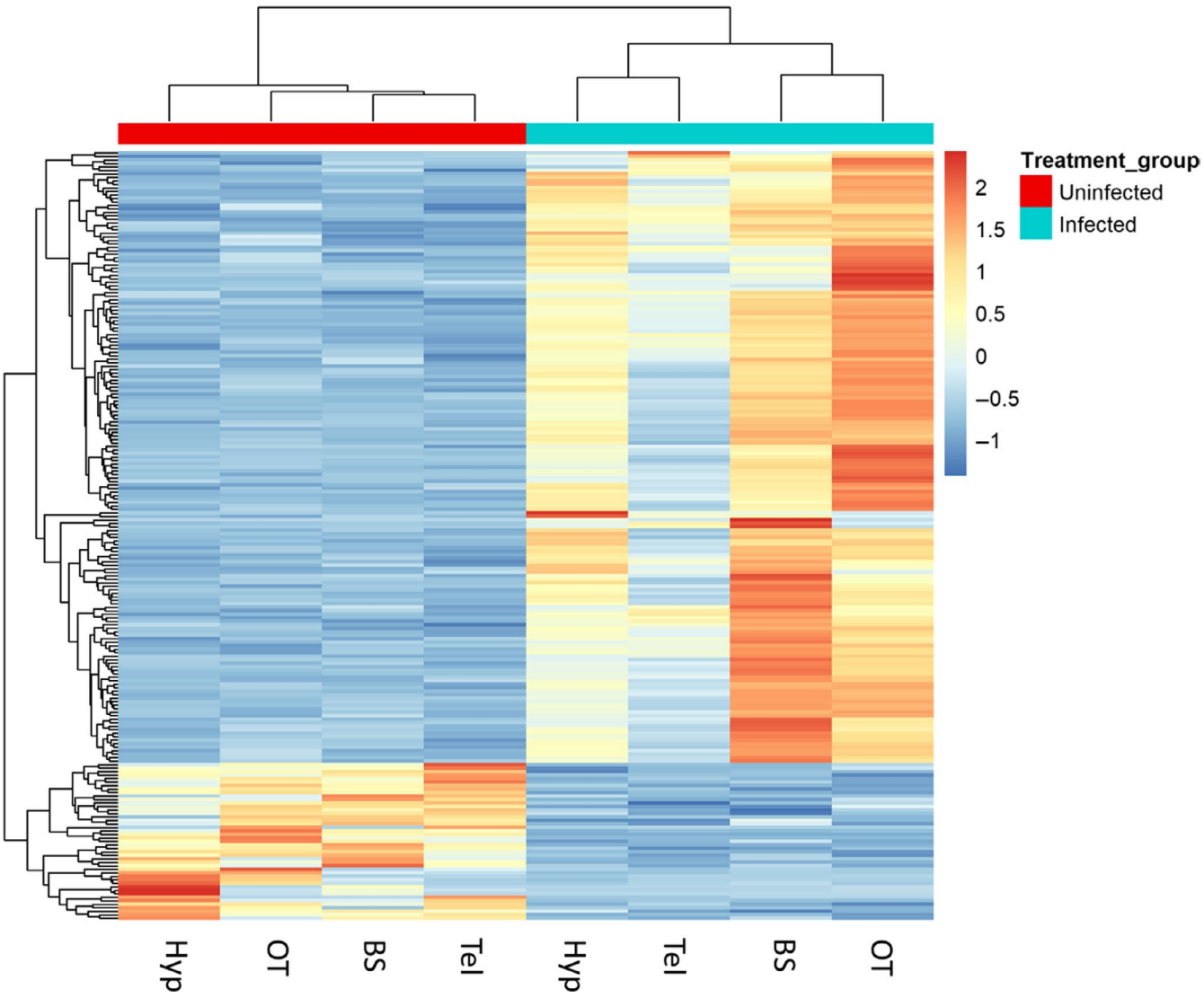

FIGURE 1 Heatmap of top 40 differentially expressed genes between zebrafish experimentally infected with the microsporidian parasite *Pseudoloma neurophilia* and uninfected controls. Brain parts from four control and four infected samples were compared, where Hyp = hypothalamus, BS = brain stem, OT = optic tectum, Tel = telencephalon

TABLE 2 Enriched KEGG pathways in the brain of zebrafish infected with *P. neurophilia* compared to uninfected control. Pathways were found based on significantly differentially expressed genes ($p < .05$)

KEGG ID	KEGG pathway	Gene count	Adjusted *p*-value	Log2fold	Gene ID	Name
dre03050	Proteasome	7	6,66E+06	3.675	405790	Interferon gamma 1[a]
				1.293	30647	Proteasome activator subunit 2
				1.237	83917	Proteasome 20S subunit alpha, like
				2.226	30666	Proteasome 20ssubunit beta 8A
				1.968	64280	Proteasome 20S subunit beta 13a
				1.535	64279	Proteasome 20S subunit beta 12
				1.266	30665	Proteasome 20S subunit beta 9a
dre04514	Cell adhesion molecules (CAMs)	7	0.0016	−8.324	30762	Major histocompatibility complex class II DAB gene[a]
				4.138	100322456	si:dkey−11f4.20
				1.511	557797	Integrin, beta 2
				1.705	793819	CD40 ligand[a]
				4.377	100006534	cd8 beta[a]
				5.903	677754	CD8a molecule[a]
				1.816	559154	Protein tyrosine phosphatase receptor type c
dre04060	Cytokine-cytokine receptor interaction	7	0.0085	3.213	108190761	Tumour necrosis factor ligand superfamily member 14-like[a]
				2.845	100135062	Colony stimulating factor 2 receptor, beta, low-affinity (granulocyte−macrophage)
				3.956	798119	TNF superfamily member 14[a]
				3.675	405790	Interferon gamma 1[a]
				1.705	793819	CD40 ligand[a]
				2.336	654692	Chemokine (C-X-C) receptor 3, tandem duplicate 1[a]
				6.192	101887143	CD27 molecule[a]
dre05168	Herpes simplex virus 1 infection	6	0.0302	3.213	108190761	Tumour necrosis factor ligand superfamily member 14-like[a]
				3.956	798119	TNF superfamily member 14[a]
				1.366	30400	Beta−2-microglobulin
				3.675	405790	Interferon gamma 1[a]
				−8.324	30762	Major histocompatibility complex class II DAB gene[a]
				0.716	100034470	TAP binding protein (tapasin), tandem duplicate 2

[a]Indicate genes associated with immune function

functionally annotating all DEGs. We found 14 Gene Ontologies terms to be over-represented, where eight were associated with immune responses, five with circadian rhythm and one with protein degradation (Figure 3a). Genes associated with immune responses were generally upregulated, such as *cd27*, interferon gamma 1 (*ifng1*), TNF superfamily member 14 (*tnfsf14*) and *mhclzba*. Genes associated with circadian rhythm were mostly downregulated, for example the genes *period circadian clock 1b* (*per1b*) and *nuclear receptor subfamily, group d, member 1* (*nr1d1*) (Figure 3b). All GO terms and genes affected can be found in Table S3.

4 | DISCUSSION

Our findings demonstrated that established *P. neurophilia* infections induced major transcriptional changes and affected several

pathways and networks in the zebrafish brain. Four KEGG pathways (i.e. grouping of genes participating in the same biological systems) were significantly altered by the parasite, all of which are associated with immune mechanisms, namely proteasome, cell adhesion molecules (CAMs), cytokine–cytokine receptor interaction and herpes simplex virus 1 infection. When we further scrutinized the biological processes affected by the parasite using GO analysis, we found 11 enriched GO terms. Eight of these were associated with immune function and five with circadian rhythm. Since infection generally induces an immune response (Medzhitov & Janeway Jr, 1997), it was not surprising to find that several immune pathways were significantly regulated in response to parasite infection. Interestingly, parasite infection was associated with a distinct downregulation of MHC II gene, namely *mhc2dab*, which is critical for the functioning of the immune system (Neefjes, Jongsma, Paul, & Bakke, 2011). Because MHC II is important for antigen presentation and hence clearance of pathogens (Forsyth & Eisenlohr, 2016), the downregulation of this gene could suggest a parasite evasion strategy to avoid being recognized/expelled by the zebrafish immune system response. Contrary to our predictions, we found no effect of the parasite on genes associated with nervous system functioning.

4.1 | Immune mechanisms

Infection with *P. neurophilia* strongly upregulated *cd8a* and *cd8b*. In mammals, CD8 function is specifically involved in the interaction with MHC I where it plays a vital part in antigen recognition (Gao & Jakobsen, 2000). CD8 has been found to play a similar crucial role in teleost species (Fischer et al., 2006; Somamoto, Koppang, & Fischer, 2014). For example, in the Japanese flounder (*Paralichthys olivaceus*) *cd8a* and *cd8b* are upregulated in response to infection by the occasional intracellular bacterium *Edwardsiella tarda* (Kato et al., 2013; Yasuike, Takano, Kondo, Hirono, & Aoki, 2010), while *Toxoplasma gondii* upregulates Cd8a in the mouse host (Tanaka et al., 2013). Furthermore, the gene *mhclzba*—encoding an MHC I molecule—was upregulated in response to *P. neurophilia infection*. In mammals, MHC I is important for antigen presentation and thus initiating an immune response towards pathogens (Dirscherl, McConnell, Yoder, & de Jong, 2014; Grimholt, 2016). The upregulation of this gene suggests that the CD8/MHC I branch of the immune system is generally upregulated by *P. neurophilia* infection. In mammals, it has been found that after activation of the CD8 + cell system, a pro-inflammatory mechanism used to eliminate or control invading pathogens is for T lymphocytes 1 cells (Th1) to secrete cytokines such as interferon gamma (IFNγ) or tumour

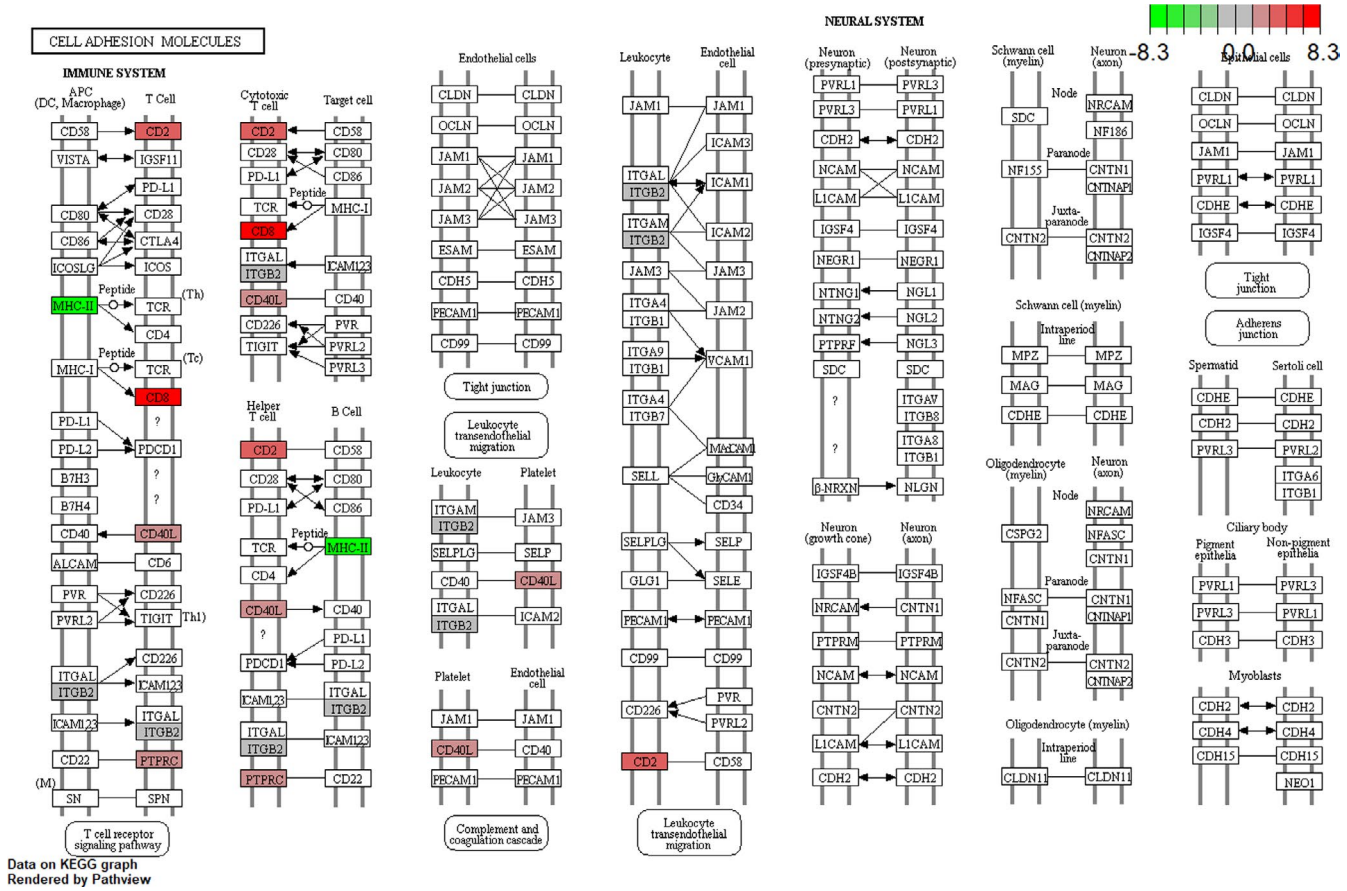

FIGURE 2 Kyoto Encyclopedia of Genes and Genomes (KEGG) map for the cellular pathway "cell adhesion molecules" containing significantly differentially expressed genes in brain tissue of laboratory zebrafish experimentally infected with the microsporidian parasite *Pseudoloma neurophilia* versus uninfected controls. Colours indicate log fold change, where red represents upregulated and green represents downregulated expression values

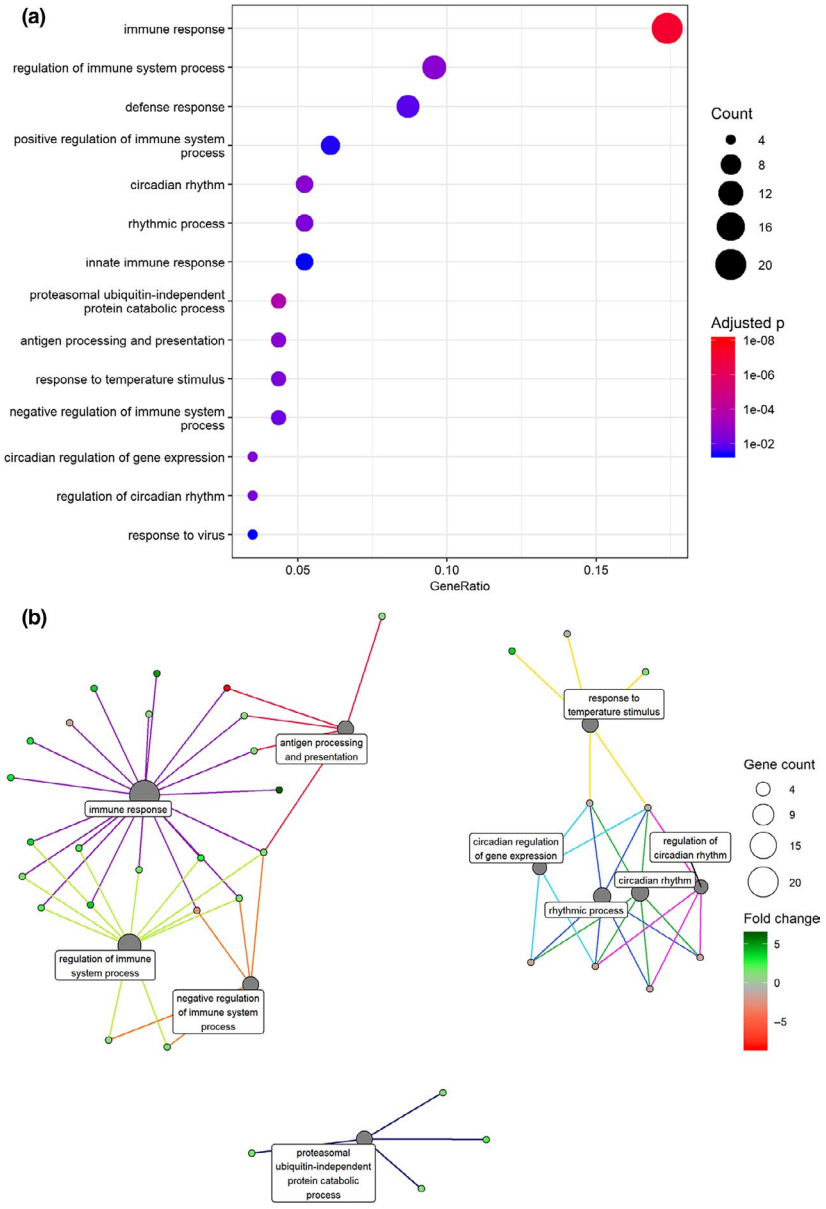

FIGURE 3 Biological processes affected by experimental infection with the microsporidian parasite *Pseudoloma neurophilia* in laboratory zebrafish compared to uninfected controls. (a) Dot plot of enriched biological process networks. Colour indicates significance (false discovery rate adjusted *p*-value). Size of dots reflects the number of differently expressed genes within each term. (b) Concept network of enriched biological processes. Only top 10 out of 14 affected biological processes are shown. Links between pathways and their associated differentially expressed genes are coloured by pathway, and coloured dots indicate fold change. Centred, grey dots indicate gene count for the specific network

necrosis factor alpha (TNFα) (Slifka & Whitton, 2000). We found that a similar mechanism appears to be activated in zebrafish in response to *P. neurophilia* infection. That is, *ifng1* was upregulated in infected zebrafish as well as *tnfsf14*. These results suggest conservation of parasite-induced activation of the MHC I branch of the immune response from fish to mammals. Taken together, our findings indicate that infected zebrafish activate a pro-inflammatory immune response against *P. neurophilia*.

In contrast to the general upregulation of immune response genes, expression of the gene *mhc2dab*, a MHC II molecule, was consistently downregulated (on average an 8.32-fold change) in infected fish compared to uninfected controls. In fact, the expression of this gene was more strongly affected by *P. neurophilia* infection than any other gene. In mammals, MHC II is crucial for initiating adaptive immune responses towards invading pathogens (Grimholt, 2016). It is broadly accepted that MHC II function is conserved across the vertebrate lineage and that it therefore has a similar function in zebrafish (Lee-Estevez et al., 2018), where *mhcIIdab* is expressed in several immune cells (Lewis, 2014). Interestingly, infection with the intracellular parasite *Toxoplasma gondii* downregulates MHC II in rodent hosts in order to evade the immune response (Lüder, Lang, Beuerle, Gross, & Immunology, 1998). A similar strategy appears to be employed by the Epstein-Barr virus, the poxvirus vaccinia and the hepatitis C virus (Forsyth & Eisenlohr, 2016). Such interference with MHC II generally inhibits activation of CD4 + cells, a crucial step for initiating immune memory and thus clearance of many pathogens (Forsyth & Eisenlohr, 2016). Importantly, all major parasite groups have been shown to take advantage of immune evasion mechanisms, with the goal of preventing the formation of immune memory (Schmid-Hempel, 2008). Thus, it is tempting to speculate that the downregulation of *mhcIIdab* could reflect an immune evasion strategy employed by *P. neurophilia* to avoid being recognized/expelled by the zebrafish immune response. If the parasite does in fact take advantage of such immune evasion strategies, it could perhaps explain why inflammation is almost absent in tissue surrounding parasite clusters (Spagnoli, Xue, Murray,

et al., 2015). Furthermore, interference with MHC II function could contribute to chronic infections despite activation of a strong Th1 immune response.

4.2 | Circadian rhythm

Zebrafish infected with *P. neurophilia* displayed a downregulation of multiple genes important for circadian rhythm as shown by the GO over-representation test. The circadian clock is a temporal 24-hr programme found in organisms from all phyla, creating structure in the diurnal and nocturnal expression of all physiological systems, from gene expression to behaviour (Roenneberg & Merrow, 2016). Continued disturbance of the circadian clock has been associated with cancer, diabetes and autoimmunity (Bass & Lazar, 2016), emphasizing how important this equilibrium is. Interestingly, the circadian rhythm genes *per1b* and *nr1d1* were recently found to play a crucial role in maintaining autophagy in zebrafish (Huang, Zhang, Ye, & Wang, 2016). Additionally, *per1b* was found to be important for expression of cytokines and recruitment of neutrophils in zebrafish (Ren et al., 2018). Host autophagy is a process hindering intracellular growth of pathogens (Evans, Sundaramurthy, & Frickel, 2018), and neutrophils and cytokines are important components of the immune response. Accordingly, we speculate that downregulation of these genes might represent another immune evasion strategy by *P. neurophilia* from the zebrafish immune system. Indeed, other pathogens have been found to take advantage of the circadian clock of their host to increase their own fitness and chances of survival. For example, the protozoan parasite *Trypanosoma brucei* alters the sleep/wake cycle in the mouse host by affecting the transcript level of specific clock genes in tissues critically important for immune and endocrine regulation, and thus enhance infection success (Carvalho Cabral, Olivier, & Cermakian, 2019; Lundkvist et al., 2010; Rijo-Ferreira et al., 2018).

4.3 | Nervous system

Clusters and spores of *P. neurophilia* primarily aggregate in the central nervous system (Matthews et al., 2001). The aggregation of the parasite in this location could therefore lead to alterations in nervous system function, which could interfere with for instance behavioural outputs. One example of such effects is provided by the California killifish and its brain-infecting parasite *Euhaplorchis californiensis*. In this parasite–host model system, from a location outside the blood–brain barrier, parasite infection alters brain serotonergic activity in the host (Shaw & Øverli, 2012), resulting in conspicuous swimming behaviour (Lafferty & Morris, 1996). Furthermore, the protozoan parasite *Toxoplasma gondii* has been found to affect the GO terms small GTPase-mediated signal transduction and cation transport in the mouse host, which are suggested to be involved in disruption of the nervous system, and thus play a part in parasite-induced behavioural changes in this model (Tanaka et al., 2013). However, our data do not support that *P. neurophilia* affects nervous system function in zebrafish, and we found no DEGs, KEGG pathways or GO terms associated with neuronal functions affected by *P. neurophilia* infection. Notably, the immune system hinges on interactions via signalling molecules such as hormones and neurotransmitters, and in addition, immune molecules are important for neuroendocrine functions (Bilbo & Schwarz, 2012). Considering that the parasite induce inflammation in the brain (Spagnoli, Xue, Murray, et al., 2015) and that previous studies (Spagnoli et al., 2017; Spagnoli, Xue, & Kent, 2015), as well as our own research (Midttun et al., 2020), show that *P. neurophilia*-infected and *P. neurophilia*-uninfected zebrafish behave differently, it is noteworthy that we found no effect of infection in parameters associated with nervous system function. However, RNAseq mainly detects highly expressed genes. Therefore, subtle yet biological relevant effects caused by *P. neurophilia* might remain undiscovered (Halvardson, Zaghlool, & Feuk, 2012). Therefore, other neurobiological sample analysis methods such as high-performance liquid chromatography (HPLC) could help elucidate possible effects of parasite infection on CNS function.

4.4 | Possible implications

Our findings suggest that at the molecular level, chronic *P. neurophilia* infection mainly affects immune system function. This finding supports our prediction that subclinical infections may affect study outcomes, particularly within immunological activation, which in turn will affect other biological functions. Notably, studies exploring immune responses to other infectious agents or pathogens may clearly be biased by underlying *P. neurophilia* infections. If both a "healthy control group" and a "pathogen exposed group" are infected with *P. neurophilia*, immunological responses to the pathogen of interest may be masked by the communal immune response to *P. neurophilia*. Even worse, if only the one group is infected with *P. neurophilia* (which may well be the case given that typically 7%–10% of rearing tanks are infected with this parasite in a zebrafish facility), it will possibly result in biased outcomes that are not liable for further scientific scrutiny. Moreover, zebrafish that are concurrently infected with other pathogens (e.g. *Mycobacterium marinum*) show higher prevalence of *P. neurophilia* (Ramsay, Watral, Schreck, & Kent, 2009b; Spagnoli, Sanders, Watral, & Kent, 2016), suggesting that either *P. neurophilia*-infected fish are more susceptible to other diseases, or alternatively other existing pathogens predispose for microsporidian infections.

Furthermore, subclinical *P. neurophilia* infections can result in higher mortality rates in treatment groups, which ultimately can affect the power of a study. Apart from the introduction of possible bias in immunological research, subclinical *P. neurophilia* infection and associated changes in immune function may affect study outcomes also in other research disciplines. Future studies should investigate whether *P. neurophilia* infection affects morbidity and mortality in response to toxicants and perhaps even metabolism of pharmacological drugs. Indeed, subclinical infections with this

parasite may affect study outcomes in a myriad of scientific disciplines. Additionally, infection with *P. neurophilia* shows reduced activity in several common zebrafish behavioural tests (Midttun et al., 2020). Reduced activity in response to infection may reflect sickness behaviour, which is mediated by host-induced upregulation of cytokines like TNFα and INFγ (Dantzer, O'Connor, Freund, Johnson, & Kelley, 2008; Kirsten, Fior, Kreutz, & Barcellos, 2018; Kirsten, Soares, Koakoski, Carlos Kreutz, & Barcellos, 2018). Thus, increased expression of cytokines in the current study supports that *P. neurophilia* induces sickness behaviour in zebrafish. A parasite that induces sickness behaviour in the study animal should be avoided in all research disciplines.

5 | CONCLUDING REMARKS

Here, we found that the zebrafish immune defence against *P. neurophilia* appears to be characterized by an upregulation of many immune-related genes and especially a pro-inflammatory Th1 response. In addition, the parasite downregulates genes associated with circadian rhythm, a mechanism often used by parasites to enhance survival. Thus, our findings indicate an activation of both innate and adaptive immune systems, but also suggest a possible immune evasion strategy by the parasite. Interestingly, *P. neurophilia* does not appear to affect neural functions, suggesting altered behaviour to be caused by other mechanisms, although more studies are needed to further elucidate possible effects. These results further indicate that infection with *P. neurophilia* can affect study outcomes within research fields such as immunology. Proper health monitoring of zebrafish facilities is thus crucial for the improvement in the use of zebrafish as a model in biomedical research. Notably, these findings likely apply to a wider range of species and model systems, since this diverse group of single celled microsporidia parasitize a wide variety of invertebrate and vertebrate animals, including insects, fish, birds and mammals (Franzen, 2004).

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CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

I.B.J. and Ø.Ø. conceived the project. H.E.M., I.B.J. and M.A.V. contributed to the design of the experiments. H.E.M., I.B.J. and M.A.V. performed the experiments and contributed to the collection of data. P.J.W. and H.E.M. conducted data analysis. H.E.M. wrote the paper. All authors edited the paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in NCBI's SRA database at http://www.ncbi.nlm.nih.gov/bioproject/633905, reference number PRJNA633905.

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SUPPORTING INFORMATION

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

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