Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Paraclinical Sciences

Philosophiae Doctor (PhD)
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Effects of the brain-infecting parasite *Pseudoloma neurophilia* in laboratory zebrafish (*Danio rerio*)

Effekter av den hjerneinfiserende parasitten *Pseudoloma neurophilia* i sebrafisk (*Danio rerio*)

Helene Louise Eghave Midttun

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Abbreviations

AS	Aerobic scope
CNS	Central nervous system
DEGs	Differentially expressed genes
GO	Gene ontology
hpi	Hours post infection
HPLC	High-performance liquid chromatography
IFN	Interferon
IL	Interleukin
KEGG	Kyoto Encyclopedia of Genes and Genomes
MHC	Major histocompatibility complex
MMR	Maximum metabolic rate
MR	Metabolic rate
Pv	Parasitophorous vacuole
qPCR	Real-time quantitative polymerase chain reaction
RNAseq	RNA-sequencing
SMR	Standard metabolic rate
SPF	Specific pathogen free
TNF	Tumor necrosis factor

Summary

Zebrafish (*Danio rerio)* are increasingly popular model animals in scientific fields ranging from behavioural ecology to neurobiology. It is therefore of increasing concern that half of all zebrafish research facilities are contaminated with the brain-infecting microsporidian parasite *Pseudoloma neurophilia*. This parasite mainly aggregates in the hindbrain, where it causes chronic yet typically sub-clinical infections. Hence, infected fish often show no obvious disease symptoms and researchers are often unaware of infection status. Previous studies indicate that *P. neurophilia* reduces growth and alters shoaling behaviour and habituation to fearful stimulus, in zebrafish. These changes in behavioural phenotype suggest that the parasite may affect stress, anxiety and sociability in zebrafish. However, effects of this parasite on host phenotype remains largely uncharted. Thus, in this Thesis, I investigated behavioural, metabolic, neurophysiological and brain transcriptional effects of sub-clinical infections with *P. neurophilia* in the zebrafish host. The first aim of the Thesis was to identify behavioural effects of *P. neurophilia* infection in zebrafish across a range of contexts. To this end, infected and uninfected zebrafish were tested in commonly used behavioural paradigms, namely social preference, mirror biting, open field and light/dark preference tests. I found infection to not alter classic behavioural outputs such as sociability and aggression. However, infected individuals displayed reduced activity in all arenas. Furthermore, in accordance with previous studies, infection negatively affected growth, indicating that *P. neurophilia* is energetically costly for the zebrafish host. This cost is likely related to immune responses mounted by the host. Moreover, behavioural changes may indicate that the parasite has direct effects on the nervous system in zebrafish. The second aim of the Thesis was therefore to study brain transcriptional changes caused by infection. Specifically, I aimed to characterise the immune responses to infection and identify biological processes affected by the parasite. In line with my predictions, RNA-sequencing analysis revealed that the parasite induces a pro-inflammatory response in the zebrafish brain. However, a distinct downregulation of specific immune-related genes also suggests that the parasite takes advantage of specific immune evasion strategies. Surprisingly, *P. neurophilia* infection had no significant effects on genes related to nervous system function. The initial findings that *P. neurophilia* reduces growth and activity and induces pro-

inflammatory responses in the brain, indicate that infection constitutes a considerable metabolic cost for the host. In addition, certain neurophysiological (*e.g*. monoaminergic) responses to infection may not be detectable by RNA-sequencing. Thus, the third aim of the Thesis was to determine metabolic and neurophysiological responses following acute and long-term *P. neurophilia* infections. In line with my predictions, *P. neurophilia* infection increased metabolic rate in zebrafish. However, the increase was highest three days after acute exposure (independent of whether the fish had an established infection or not) and mitigated again on day six. Furthermore, acute parasite exposure increased serotonergic and dopaminergic activity, but only in zebrafish with no previous history of infection (naïve). The results suggest that the metabolic and neurophysiological effects of *P. neurophilia* depends on time post last exposure and previous infection status and that metabolic costs are higher with acute compared to established infection. Since zebrafish frequently encounter infectious spores in their environment, repeated acute infections may represent a substantial metabolic cost to laboratory zebrafish. Taken together, the results obtained in this Thesis indicate that infection is associated with decreased activity and growth, a pro-inflammatory immune response and elevated metabolism in zebrafish. This phenotype is reminiscent of sickness behaviour (a condition in which acutely infected individuals adopt energy reducing strategies in order to fight infection). This thesis provides evidence that *P. neurophilia* can affect multiple biological aspects, that potentially have severe consequences for research outcomes. Hence, the findings highlight the importance of proper and standardised health monitoring in animal research facilities, not only for improving animal welfare, but also for ensuring research reproducibility.

Sammendrag

Sebrafisk (*Danio rerio*) har i løpet av de siste tiårene blitt en av de mest brukte og populære dyremodellene i biovitenskapelig forskning og brukes i dag i en rekke forskningsfelt, inkludert atferdsbiologi og hjerneforskning. Det er derfor svært bekymringsverdig at halvdelen av alle forskningsfasiliteter, som holder sebrafisk, er kontaminert med den hjerneinfiserende mikrosporidia-parasitten *Pseudoloma neurophilia*. Denne parasitten angriper primært hjernen, der den etablerer kroniske, dog oftest subkliniske infeksjoner. Det betyr at infiserte fisk ofte ikke viser tegn på sykdom, og at forskere derfor ofte jobber med infisert fisk uten at de vet det. Tidligere studier har indikert at *P. neurophilia* reduserer vekst, påvirker fiskegruppedynamikk og hvordan sebrafisk responderer på faretruende stimuli. Disse funnene indikerer at denne hjerneparasitten påvirker atferdsparametre som stressrespons, angst og sosiabilitet hos sebrafisk, men stort sett er effektene av denne parasitten på vertens fenotype ukjente. I denne avhandlingen undersøker jeg effekter av subklinisk *P. neurophilia*-infeksjon på atferd, metabolisme, nevrofysiologi og genuttrykk i hjernen hos sebrafisk. Det første delmålet i denne avhandlingen var å identifisere og kartlegge effekter av *P. neurophilia*-infeksjon på atferd hos sebrafisk på tvers av flere kontekster. Til dette formålet ble både infiserte og ikke-infiserte sebrafisk testet i atferdstester som er mye benyttet av sebrafiskforskere. Disse testene brukes til å måle atferdsparametre, som for eksempel sosial preferanse, aggressivitet, angstatferd og dristighet. Til tross for parasittens privilegerte plassering i hjernen, fant jeg ingen effekter av infeksjon på disse klassiske atferdsparametrene. På den annen side fant jeg at infiserte individer viste nedsatt aktivitet i alle atferdsarenaene de ble testet i. I tråd med tidligere studier, fant jeg også at *P. neurophilia*-infeksjon reduserte vekst. Samlet sett tyder redusert aktivitet og vekst på at *P. neurophilia* utgjør en betydelig kostnad for sebrafiskens energiressurser. Antageligvis er denne kostnaden relatert til sebrafiskens immunrespons til infeksjonen. Atferdsendringene kan også skyldes at parasitten påvirker nervesystemet og hjernen mer direkte. Det andre delmålet i denne avhandlingen var derfor å karakterisere transkripsjonelle responser på *P. neurophilia*-infeksjon i hjernen. Mer spesifikt ville vi karakterisere immunrespons på infeksjon og identifisere biologiske prosesser påvirket av parasitten. RNA-sekvenseringsanalyse av hjernevev avslørte, ikke helt overraskende, at

parasitten induserer en pro-inflammatorisk respons i sebrafiskehjernen. På den annen side tyder nedregulering av spesifikke immunrelaterte gener på at parasitten også utnytter spesifikke unnvikelsesstrategier for å unnslippe immunsystemet. En slik unnvikelsesstrategi kan være avgjørende for parasittens evne til å etablere kroniske infeksjoner. Til tross for at parasitten invaderer nerveceller i hjernen, fant vi ingen effekter på gener involvert i nervesystemets funksjon. Disse foreløpige funnene som viser at *P. neurophilia* reduserer aktivitet og vekst, og induserer en kraftig immunrespons i sebrafisk, peker på at infeksjonen er kostbar og kan øke sebrafiskens metabolske krav. Noen potensielle effekter på nervesystemet kan man heller ikke detektere ved hjelp av RNA-sekvensering (f.eks. monoaminresponser). Det tredje delmålet i avhandlingen var derfor å bestemme metabolske og nevrofysiologiske responser på akutt versus etablert infeksjon. I samsvar med våre forventninger fant jeg at *P. neurophilia* øker metabolsk rate hos sebrafisk. Økningen var riktignok størst tre dager etter akutt eksponering for *P. neurophilia*-sporer og uavhengig av tidligere infeksjonsstatus (ikke tidligere infisert versus etablert infeksjon). Interessant nok ble denne økningen i metabolisme reversert seks dager etter eksponering. Videre så jeg at akutt men ikke etablert parasitteksponering, økte monoaminerg (serotonerg og dopaminerg) aktivitet i hjernen til sebrafisken. Resultatene tyder på at metabolske og nevrofysiologiske effekter av *P. neurophilia* avhenger av tid etter siste eksponering og at akutte infeksjoner er mer kostbare enn etablerte infeksjoner. I og med at sebrafisk stadig utsettes for infeksiøse parasittsporer i akvariet, kan gjentatte akutte infeksjoner utgjøre en betydelig metabolsk kostnad for sebrafisk i forskningslaboratorier. Samlet viser forsøkene i denne avhandlingen at *P. neurophilia*-infeksjon reduserer aktivitet og vekst, induserer en kraftig immunrepons og øker fiskens metabolske omkostninger. Denne fenotypen kan minne om det man ser ved sykdomsatferd, en velkjent tilstand der dyr og mennesker tillegger seg energireduserende strategier for å bekjempe akutte infeksjoner. Resultatene viser også at *P. neurophilia* kan påvirke mange biologiske aspekter hos sebrafisk, som potentielt kan ha alvorlige konsekvenser for forskningsresultater, der man bruker sebrafisk med subklinisk *P. neurophilia*-infeksjon. Derfor fremhever resultatene viktigheten av ordentlig og standardisert helseovervåkning i forsøksfasiliteter som holder sebrafisk. Dette vil ikke bare forbedre dyrevelferd, men vil også være nødvendig for reproduserbarhet av forskning som benytter sebrafisk som forsøksdyr.

List of papers

Paper I

Behavioural effects of the common brain-infecting parasite *Pseudoloma neurophilia* in laboratory zebrafish (*Danio rerio*)

Helene L.E. Midttun, Marco A. Vindas, Lauren Nadler, Øyvind Øverli and Ida B. Johansen
Scientific Reports 2020, 10:8083, DOI: https://doi.org/10.1038/s41598-020-64948-8

Paper II

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

Helene L.E. Midttun, Marco A. Vindas, Paul J. Whatmore, Øyvind Øverli and Ida B. Johansen
Journal of Fish Diseases/in press

Paper III

Metabolic and neurophysiological effects of a microsporidian parasite infection depend on previous infection status

Lauren Nadler, Helene L.E. Midttun, Marco A. Vindas, Shaun S. Killen, Øyvind Øverli and Ida B. Johansen
Manuscript

1. Introduction

1.1 Effects of pathogens in laboratory animals

Humans have been using animal models since the dawn of medicine for medical progress (Franco, 2013). In fact, model animals have been crucial for our understanding of disease, anatomy, physiology and development (Insel, 2007). Two of the most used animal models, the domesticated rat (*Rattus norwegicus*) and mouse (*Mus musculus*), were introduced to European and American laboratories about 100 years ago. However, these rodents were often infected with countless pathogens (Weisbroth, 1999). Researchers soon realized that pathogens affected physiological and immunological functions, which created variation in research outcomes and high mortality rates (Nicklas et al., 1999). Increased interest in effects of pathogens on experimental animals gave rise to several publications on infectious diseases in rodent facilities, which aided the control of pathogen infections and resulted in the eradication of several of these from research facilities (Weisbroth, 1999). In the 1950's the book "The Principles of Humane Experimental Technique" was published, which represents a turning point for proper care and use of laboratory animals. Furthermore, the publication laid the ethical foundation for what we now know as the 3 R's (Replacement, Reduction, Refinement), which are guidelines to help ensure high ethical and welfare standards in the maintenance of animals used for research (Richmond, 2000). In the 1960's the desire to properly control for specific pathogen infection led to the introduction of the first germ-free lines of rodents. Subsequently, pathogens became even less prevalent in animal research facilities in the 1980's thanks to further advances in animal husbandry and diagnostic testing (Baker, 1998). However, rodent facilities still struggle with pathogen infections such as pinworms (*Aspiculuris tetraptera*, *Syphacia* spp.), parvovirus and bacteria such as *Helicobacter* spp. (Pritchett-Corning et al., 2009).

One of the reasons for the prevalent pathogen problem in research facilities is that many pathogens are latent or induce subclinical diseases (*i.e.* infected animals show no visual symptoms of disease). Subclinical infections can nevertheless affect a variety of study outcomes. For example, subclinical infection with the pinworm *Syphacia* spp. increases hematopoiesis (*i.e.* production of the cellular components of blood) in mice, ultimately affecting a variety of biological systems (*e.g.* the cardiovascular and immune system).

Pathogens that affect vital biological systems are very likely to interfere with studies that relate to these systems directly, as well as integrated functions and wider regulatory networks, potentially confounding result interpretation on a broad scale (Bugarski et al., 2006). Thus, it is crucial for facilities to perform proper health monitoring procedures (Nicklas, 2007), which includes defining infection status, detecting and mitigating infection as early as possible in addition to preventing new pathogens from entering the facilities. However, not all animal facilities carry out proper health monitoring (Nicklas, 2008). Even more problematic is that health monitoring has largely been the focus of research conducted in rodent facilities. For example, it is only within the last two decades that health monitoring practices for husbandry and care of zebrafish (*Danio rerio*) have been established. Zebrafish are by now one of the most used and popular vertebrate models (ONS, 2019). To which extents facilities practice the proposed guidelines and procedures however remains unclear (Collymore et al., 2016, Lidster et al., 2017, Alestrom et al., 2019).

1.2 Zebrafish (*Danio rerio*), the new model animal

The *Cyprinidae* family member, the zebrafish, is a small teleost species native to South Asia. Natural habitats include rivers, paddy fields, small streams and channels, all with stagnant or slow-moving water. In the wild, zebrafish has a preference for relatively clear water, and temperatures ranging from 10 to 40 °C (Engeszer et al., 2007, Arunachalam et al., 2013). The zebrafish was used as laboratory animal for the first time in the 1960's, yet it is only within the last few decades that the species gained its momentum as a prominent new animal model in fields such as neurobiology and development (Grunwald and Eisen, 2002, Fontana et al., 2018, Meyers, 2018).

The reasons for the increasing popularity of this teleost species are manifold. Firstly, maintenance of zebrafish has a relatively low cost, they have a short generation time, produce hundreds of offspring per week and breed year-round. Second, the eggs are fertilized and develop outside the mother and since the embryos are transparent, organ development and structures can easily be studied. Third, being a vertebrate, zebrafish has fundamental resemblances to human organogenesis and physiology that makes it valuable

as a comparative model in translational and biomedical research (Gerlai, 2003, Rubinstein, 2003, Lieschke and Currie, 2007, Meeker and Trede, 2008, Kalueff et al., 2016, Meyers, 2018)

Endocrine and neural signalling systems of zebrafish resemble that of mammals by expressing many of the same major brain structures, neurotransmitters, hormones and receptors (Panula et al., 2010). Furthermore, the complete zebrafish genome has been sequenced and shows approximately 70% similarity to the human reference genome (Howe et al., 2013). These assets combined has resulted in the use of zebrafish in more than 3600 research institutions worldwide in 2013 (Kinth et al., 2013). In addition, approximately 5000 scientific publications from numerous scientific fields such as developmental biology, toxicology, immunology and neuroscience were published on zebrafish in 2016 alone (Meyers, 2018).

Human and zebrafish sensory pathways share an overall homology. Furthermore, with a few notable exceptions, the organization of the major brain components and pathways are highly conserved throughout the vertebrate lineage (Tropepe and Sive, 2003). Thus, zebrafish are particularly gaining popularity as behavioural models within biomedical research, for example in translational neuroscience. Here, zebrafish behaviour is used for studying a myriad of processes and topics, from responses to different drug treatments to complex brain disorders (Stewart et al., 2015, Kalueff et al., 2016). Sophisticated video-tracking tools for recording and analysing both larvae and adult zebrafish behaviour give researchers the potential to use zebrafish for high-throughput screenings (Gerlai, 2010, Lessman, 2011, Varga et al., 2018). However, because the species is relatively novel within neurobehavioural research, the behavioural terminology is not as developed and consistent as in other animal models, such as that for rodents or primates. In this context, a comprehensive catalogue of zebrafish behaviour was developed to help researchers improve, standardise and interpret behavioural outputs from the most commonly used behavioural tests (Kalueff et al., 2013).

By studying behavioural outputs researchers can understand how the zebrafish interacts with its environment and link this to specific biological traits (Orger and de Polavieja, 2017). Considering that behaviour by now is one of the most important endpoints in zebrafish research, it is of particular concern that 74% of all zebrafish facilities submitting fish to the

diagnostic pathology service at the Zebrafish International Research Center (ZIRC) in 2010 tested positive for the brain-infecting, microsporidian parasite *Pseudoloma neurophilia* (Murray et al., 2011). In comparison to other pathogens, parasites are infamous for affecting host behaviour in multiple ways (further described in section 1.4.1), and such infections can potentially give rise to misleading and biased research outcomes. This Thesis will focus on behavioural, molecular and physiological effects caused by *P. neurophilia* in the zebrafish host, as described below.

1.3 The microsporidian parasite *Pseudoloma neurophilia*

1.3.1 The microsporidian life cycle

Microsporidia are unicellular, obligate intracellular parasites. This group of spore-forming parasites are known to infect protists, vertebrates and invertebrates alike and make up approximately 1400 species, distributed over 200 genera (Lee et al., 2008, Capella-Gutiérrez et al., 2012, Szumowski and Troemel, 2015, Han and Weiss, 2017). Parasitism is defined as a relationship in which one of the participants, the parasite, lives in or on the other participant, the host, from which the parasite derives its nutrients (Poulin and Morand, 2000, Roberts et al., 2013). Microsporidian parasites pose immense health threats and socioeconomic burdens. For example, the microsporidium *Nosema* spp. nearly destroyed the silkworm industry in the 17th century and has later led to great economic losses in the honey bee industry (reviewed by (Didier et al., 2004)). In addition, microsporidian parasites have emerged as opportunistic pathogens infecting immunocompromised and AIDS patients, causing chronic diarrhoea. Furthermore, microsporidian parasites are commonly found in a number of laboratory animals (reviewed by Didier et al., 2000).

Most microsporidia have a simple life cycle, only needing one host. However, some few species have an indirect lifecycle and thus require a minimum of two hosts of different species in a specific order (Poulin and Randhawa, 2015). For example, the microsporidium *Amblyospora indicola* has two hosts, namely the mosquito *Ochlerotatus* (*Aedes*) *cantator* and the copepod *Acanthocyclops vernalis* (Sweeney et al., 1990). The microsporidian life cycle, including that of *P. neurophilia*, generally consists of two life stages, the meront stage and a

the infectious spore stage (Figure 1) (Matthews et al., 2001, Cali et al., 2012). This parasite has no active life stages outside of the host. However, due to a thick chitinous wall, the spores are environmentally resistant and can survive outside the host, which aids transmission between hosts (Han and Weiss, 2017). Microsporidian spores contain a unique coiled polar tube that, once inside a suitable host and under the right conditions, will be expelled from the spore. The polar tube then penetrates the host cell and injects the infective sporoplasms (reviewed by Franzen and Müller, 1999, Weiss and Becnel, 2014). The sporoplasms multiplies extensively to meronts by merogony (binary fission) or schizogony (multiple fission). This process can happen inside either parasitophorous vacuoles (PVs) or in direct contact with the host cell cytoplasm (Franzen and Müller, 1999, Bigliardi, 2001). The meronts will further develop into sporonts, then sporoblasts and finally into mature spores by sporogony. Once the infected host cell cytoplasm is completely full of spores it will burst and release the spores, which are then ready to infect new cells (Franzen and Müller, 1999, Franzen, 2004, Weiss and Becnel, 2014).

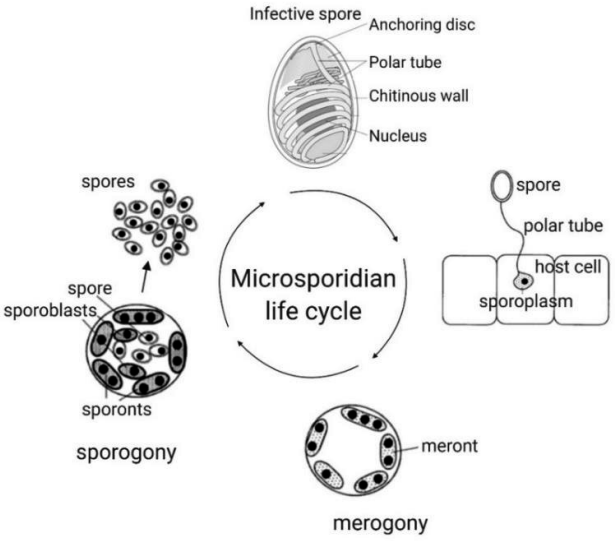

Figure 1. The microsporidian lifecycle (Edited from Franzen and Müller 1999, Franzen 2004).

Although microsporidia are classified as eukaryotes, they lack some eukaryotic characteristics, such as a typical Golgi apparatus. In addition, the microsporidian mitochondria has been reduced to mitosomes unable to generate adenosine triphosphate (ATP) and thus renders the parasite reliant on the host for energy metabolism, *i.e.* oxidative phosphorylation (Franzen and Müller, 1999, Bigliardi, 2001, Bass et al., 2018). It is therefore critical for microsporidia to have a successful host-host transmission as they rely on intracellular resources of the host for reproduction. Spores are transmitted from host to host via horizontal and/or vertical transmission routes. Some microsporidia, like *P. neurophilia*, take advantage of both routes to increase transmission success (Kent and Bishop-Stewart, 2003, Sanders et al., 2013). During horizontal transmission, the host ingests spores, which then spread to other tissues via the gut. Parasites using horizontal transmission are dependent on a relatively large parasite burden, because spores must be released into the environment for further transmission. The higher parasite burden results in increased virulence and often host death. Vertical transmission, on the other hand, involves the parasite passing through the host from generation to generation. Therefore, the parasite hinges on the reproduction and survival of the host, and natural selection thereby favours reduced virulence. Parasites which exploit both types of transmission strategies, favour high virulence during vertical transmission, and low virulence during horizontal transmission. Because horizontal transmission is only viable between mother and offspring, this transmission mode is suggested to favour high virulence in males leading to increased death in this group. This ultimately increases the number of spores released from male carcasses, which can then be consumed by females and thereby transmitted to the next generation via horizontal transmission (Dunn and Smith, 2001).

1.3.2 Transmission, distribution and pathology of *P. neurophilia*

Pseudoloma neurophilia is commonly found in domesticated zebrafish (Kent et al., 2011). Despite its prevalence in animal facilities, the parasite is most likely not a natural pathogen of zebrafish as it has not been described in wild-caught zebrafish thus far (Sanders et al., 2016). Many zebrafish used for research have been obtained from retail pet stores, from where the zebrafish could get infected from other fish species. In fact, the known range of hosts for *P. neurophilia* has been expanded to include seven other fish species, namely the

siamese fighting fish (*Betta splendens*), platy (*Xiphophorus maculatus*), giant danio (*Devario aequipinnatus*), fathead minnow (*Pimephales promelas*), medaka (*Oryzias latipes*), goldfish (*Carassius auratus*) and neon tetra (*Paracheirodon innesi*), all of which are commonly found in pet stores (Sanders et al., 2016).

As suggested by the name, this parasite has a preference for infecting neural tissue. Once in the brain, *P. neurophilia* develops intracellularly by creating parasite clusters inside nerve fibres, before rupturing and releasing new infectious spores (Cali et al., 2012). Because infections are mostly subclinical and intracellular, it is difficult to ascertain the infectious status of fish within facilities and it is therefore necessary to perform specific pathology tests of sampled, euthanized fish (*i.e.* histology or real-time polymerase chain reaction, qPCR) (Sanders and Kent, 2011, Kent et al., 2012, Miller et al., 2019). For this reason, it is common that infections with this parasite remain undetected in many fish facilities.

When infectious spores of *P. neurophilia* are released from females during spawning, they can be ingested directly by the next host, since zebrafish readily eat their own eggs. Moreover, zebrafish also feed on carcasses of their conspecifics, increasing the likelihood of transmission (Murray et al., 2011). Once the spore is ingested it moves to the intestine, where mature spores have been detected 12 hours post infection (hpi). From here, the spores infect host cells and proliferative stages (*i.e.* meronts) have been found in pancreas and kidneys 36-48 hpi. After 72 hours, meronts have made their way to the spinal cord and skeletal muscles, and at 96 hpi the first mature spores are found in the liver, spinal cord and skeletal muscle. The parasite has been observed in the brain at 120 hpi (Fig. 2) (Sanders et al., 2014).

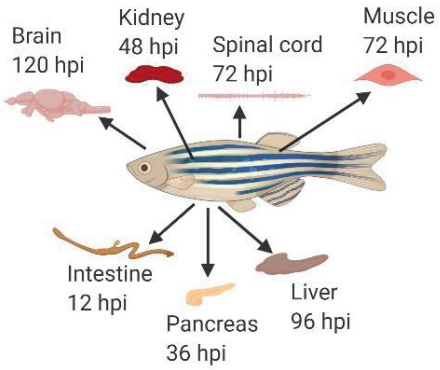

Figure 2: Tissue distribution of *Pseudoloma neurophilia* in zebrafish, hours post infection (hpi). Figure created with BioRender.

Although infection with *P. neurophilia* in zebrafish is largely subclinical, clinical signs including emaciation and spinal deformation (*i.e.* lordosis and scoliosis) are common once infection reaches a mature state (Matthews et al., 2001, Murray et al., 2011). Furthermore, *P. neurophilia* negatively affects the growth of zebrafish (Ramsay et al., 2009a). Chronic clinical and subclinical infections are characterised by inflammation in muscles, meninges and spinal cord tissue (Spagnoli et al., 2015b). Notably, recent findings indicate that the parasite have sex-specific effects. For example, the parasite reduces body condition (width/length ratio) in females due to reduced gonadal area and thus reduces fecundity (Ramsay et al., 2009a, Sanders et al., 2020). Furthermore, males appear to be more susceptible to infection compared to females (Chow et al., 2016), possibly suggesting increased mortality rate in males. Even though the parasite induces moderate inflammation and infects the brain and spinal cord, studies on how *P. neurophilia* affects its host phenotype remain sparse.

1.4 Behavioural and physiological effects of *P. neurophilia*

1.4.1 Behaviour and other aspects of host phenotype

Few studies have examined the effect of *P. neurophilia* on zebrafish behaviour, despite the fact that other microsporidian parasites have previously been found to affect behaviour, physiology and immune mechanisms of their hosts. For example, the microsporidium *Nosema ceranae* suppresses immune responses in the bee host (*Apis mellifera*) (Antunez et al., 2009), and advances maturation resulting in premature death (Goblirsch et al., 2013). *Glugea anomala*, a common microsporidium of three-spined sticklebacks (*Gasterosteus aculeatus*), makes the fish host more social (Petkova et al., 2018) and more likely to shoal (Ward et al., 2005). Furthermore, the microsporidium *Tubulinosema kingi* causes reduced fecundity in the fruit fly host *Drosophila melanogaster* (Futerman et al., 2006). In fact, parasites are infamous for altering host behaviour, and it is often assumed that parasite-induced phenotypic alterations benefit the parasite by increasing transmission and thus fitness (Moore, 2002). Yet, hosts have also developed strategies to avoid/fight parasites, such as displaying behavioural fever, *i.e.* elevation in body temperature based on acute change in thermal preference, which helps the host eliminate parasites (Hart and Hart, 2019).

Some changes in host phenotype are simply caused by side effects of infection (*i.e.* pathology) and possibly does not benefit neither host nor parasite (Poulin et al., 1994, Poulin, 1995, Moore, 2002). Some of the most dramatic behavioural effects of parasites are found in indirect lifecycle parasites. For example, the trematode *Dicrocoelium dendriticum* will make the ant host leave the colony and climb to the tip of grass blades. Here, the parasite causes the ant to lock its jaws on the blade increasing the likelihood of it being predated by grazing mammals, the definitive host for the parasite (reviewed by Moore, 2002, Rajan, 2002) . Interestingly, altered host behaviour has been found to be a strategy of parasites that utilize direct lifecycles as well (Moore, 2002). For instance, after being ingested by a cricket (*Nemobius sylvestris*), the hairworm larvae (*Paragordius tricuspidatus*) will grow inside the cricket host. Once fully mature, the parasite will induce migration by crickets into an aqueous environment, and then escape the host. The aqueous environment is fatal for the cricket but is vital for the reproduction of the worm (Thomas et al., 2002). The examples reviewed here demonstrate more dramatic impacts on hosts as a result of parasite-induced behavioural

alterations. However, parasites often have less extreme behavioural effects such as moderately affecting activity levels of the host (Moore, 2002). For example, many hosts of direct lifecycle parasites compensate for the cost of having a parasite by altering their behaviour (Binning et al., 2017). This is seen in hosts like the three-spined stickleback. Once infected with the energy-draining tapeworm *Schistocephalus solidus*, the stickleback increases time spent foraging for food to meet energy demands of harbouring a parasite infection (reviewed by Barber et al., 2000, Binning et al., 2017).

Since it may be assumed that parasites actively manipulate their hosts to increase transmission, the potential strategies they use to do so have been grouped into three different strategies; proteomic- and genomic-based, immunological and/or neuropharmacological (Adamo, 2013). Parasites altering behaviour by taking advantage of proteomic- and genomic-based mechanisms, do so by affecting gene expression. For example, by inactivating a gene associated with circadian rhythm in the caterpillar host, the *Lymantria dispar* nucleopolyhedrovirus can alter feeding behaviour. This results in the host not descending from the tree it is feeding on. While the host is stuck in the top of the tree, the virus can better spread its viral particles down onto new hosts (reviewed by Adamo, 2013).

Neuropharmacological mechanisms are defined by parasite secretion of molecules that interact with the central nervous system (CNS) and neuronal activity. For instance, this mechanism is utilized by the jewel wasp (*Ampulex compressa*) that injects its venom into the cockroach (*Periplaneta Americana*). The venom blocks acetylcholine- and gamma-aminobutyric acid (GABA) mediated synaptic transmission and contains dopamine and/or a dopaminergic agonist which increases the host's dopamine levels. The increase in dopamine results in excessive grooming by the host, during which the wasp searches for a nest. The wasp then returns and guides the docile "zombie" cockroach to the nest, where it lays its eggs on the cockroach's legs. Once hatched, the larvae enter the body and feed on the cockroaches internal organs (reviewed by Libersat et al., 2009, Libersat and Gal, 2014).

Immunological mechanisms involve parasite-induced alteration of communication between the immune system and the CNS. For example, the intracellular parasite *Toxoplasma gondii* encysts in the brains of rats (*Rattus norwegicus*), the intermediate host. In the brain, *T. gondii*

increases host dopamine metabolism (Prandovszky et al., 2011) and promotes the release of specific cytokines that are toxic to neurons. Cytokine release leads to microglia activation and release of nitric oxide (NO), a well-known neuromodulator. Coinciding with increased dopamine and NO release, infected rats then become attracted to feline urine, increasing the likelihood of the parasite reaching the feline definitive host (Reviewed by Herbison, 2017). In this context, it is important to point out that parasites can affect immune responses in multiple ways. For example, pathogenic infections often result in the release of cytokines as a part of an inflammatory process, which can induce sickness behaviour (Dantzer, 2004), mostly benefitting the host. Yet other parasites alter immune mechanisms in order to evade elimination from the host (reviewed by Herbison, 2017).

Pseudoloma neurophilia mainly infects the hind brain of zebrafish, specifically regions associated with motor function and emotional and cognitive functions such as anxiety and fear learning (Fig. 3) (Spagnoli et al., 2015a, Spagnoli et al., 2015b). However, only two studies have examined behavioural effects of *P. neurophilia* on zebrafish thus far (Spagnoli et al., 2015a, Spagnoli et al., 2017). The first study found infection to affect behaviours associated with anxiety, fear and stress (Spagnoli et al., 2015a). These results were obtained from a tap-test, where infected zebrafish showed smaller reduction in startle velocity to a fearful stimulus. In the second study, effects of *P. neurophilia* on social interactions were assessed by studying inter-individual distances in shoals of infected and non-infected zebrafish (Spagnoli et al., 2017). Infected zebrafish showed reduced inter-fish distances, resulting in closer shoal formations. Increased shoal cohesion has been associated with stress and the authors suggested that the two studies taken together indicate that *P. neurophilia* increases stress or anxious behaviours in the zebrafish host (Spagnoli et al., 2017). However, shoal cohesion could also reflect other behavioural correlates, such as sociability (Pham et al., 2012). Therefore, in order to identify behavioural correlates (*e.g.* sociability, anxiety etc.) affected by the parasite, it is necessary to study behaviours across a range of contexts. By assessing the same behavioural traits in different settings, it is easier to determine the effects of the parasite. Moreover, zebrafish are used as a model animal in tests assessing several other behavioural correlates (*e.g.* aggression, exploration, boldness) none of which have been studied so far.

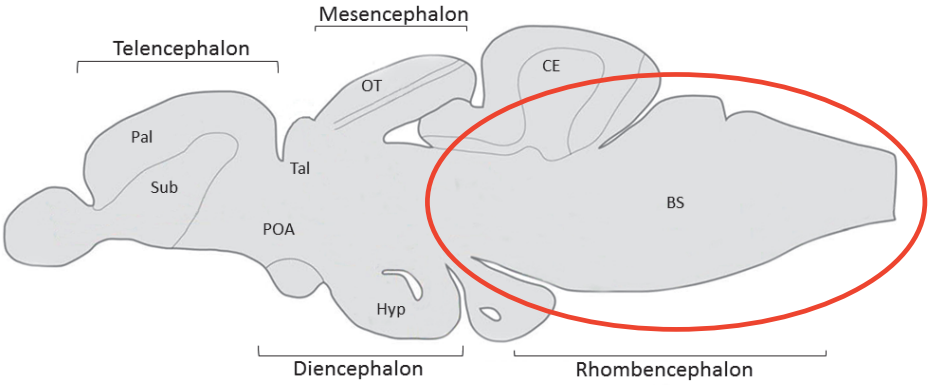

Figure 3: Schematic overview of the zebrafish brain. The red circle encompasses the areas of the brain where *Pseudoloma neurophilia* mainly aggregates (Edited from Parker et al., 2013).

Since little is known about the behavioural effects of *P. neurophilia*, it remains unknown what causes the observed behavioural alterations (*i.e.* closer shoal-formation and altered habituation). Given the reported behavioural alterations and the prime location of *P. neurophilia* in the zebrafish brain, it is tempting to speculate that this parasite manipulates its host. Exploring behaviour across a range of contexts can thereby contribute to detecting more specific effects of the parasite infection and contribute to a better understanding of this parasite-host interaction.

1.4.2 Immune response

Infectious spores of *P. neurophilia* have been found to induce inflammation in brain, meninges, spinal cord and muscles of zebrafish. However, parasite clusters (containing immature spores) induce little to no inflammation (Spagnoli et al., 2015b). Studies have suggested that the intensity of inflammation in response to *P. neurophilia* is linked to stress and immunity. For example, stressed fish experience earlier onset of disease and more severe inflammation (Ramsay et al., 2009a). Simultaneously, immunocompromised

zebrafish infected with *P. neurophilia* suffer from increased parasite load, more intense inflammation and higher mortality rates (Spagnoli et al., 2016). Moreover, zebrafish infected with the bacterium *Mycobacterium marinum* are more likely to acquire *P. neurophilia* infection than healthy zebrafish. Because *Mycobacterium spp*. is known for suppressing immunity, it has been suggested that the parasite is more prevalent in immune-suppressed individuals (Ramsay et al., 2009b). While previous studies clearly indicate that *P. neurophilia* induces an immune response in zebrafish, this immune response has not yet been studied at the molecular level. Examining immunological effects of the parasite-host interaction could aid in understanding the effects already found on behaviour, as immune responses can lead to different behavioural alterations (Klein, 2003).

Energetic drainage: One way to affect behaviour is by activating host immune responses, which can be an energetically costly process for the host (Lochmiller and Deerenberg, 2000). In fact, parasitic infections have been found to increase metabolic rate in both fish and mammals (Binning et al., 2013, Garrido et al., 2016), likely reflecting the high cost of infection. Yet, draining the host for energy can also benefit parasite transmission. For example, the tapeworm *Echinococcus granulosus* increases its chances of reaching the final canid host, by making the intermediate moose (*Alces alces*) host sick and thus an easier prey (Joly and Messier, 2004, and see review by Øverli and Johansen, 2019). Contrary, energy drainage by infection can benefit the host by inducing sickness behaviour, a process where energy resources are redirected to fight infection (Dantzer and Kelley, 2007).

Sickness behaviour: Acutely ill animals are typically described as lethargic, depressed and anorexic. Behavioural alterations like this usually helps the host redirect its own energy resources towards immune responses, thus increasing its chances of overcoming disease by fighting the invading pathogen (Hart, 1988, Dantzer and Kelley, 2007). In this context, proinflammatory cytokines have been found to act on the brain, where they induce non-specific infection symptoms such as fever and sickness behaviour (Kelley et al., 2003). Since sickness behaviours are characterised by reduced sociability (Hennessy et al., 2014, Eisenberger et al., 2017, Kirsten et al., 2018b), anorexia (Exton, 1997), depression (Miller et al., 2009), reduced mobility and decreased libido (reviewed by Shattuck and Muehlenbein,

2015), such behavioural changes are often not benefitting the parasite. In fact, sickness behaviours often negatively affect contact rates and thus spread of the parasite from one host to another. For example, infected and healthy rats and mice avoid contact with each other, while shoals of three-spined sticklebacks avoid models of parasitized fish (reviewed by Adelman and Martin, 2009). Still, depending on transmission modes, sickness behaviour can benefit the parasite when this is relying of the host to be eaten by the next host, as described in moose the example above. Even though this behavioural strategy can be beneficial for animal hosts when avoiding/fighting infection, numerous parasites have also evolved strategies to reduce the host's immune inflammatory processes to increase their own survival.

Immune evasion strategies: Parasites from all major groups use immune evasion strategies to prevent the host from forming immune memory (Schmid-Hempel, 2008). Depending on species, size and niche, parasites have developed different evasion strategies. One of the most well-studied strategies is antigenic variation by the malarial parasite *Plasmodium falciparum*. This parasite has the ability to express several different kinds of tightly regulated surface proteins, which they can express specifically in response to avoid host antibodies, immune memory and thus evade elimination (Miller et al., 1994, Scherf et al., 1998, Craig and Scherf, 2001, Hisaeda et al., 2005). A different strategy is that used by the protozoa *Trypanosoma cruzi*, which downregulates the major histocompatibility complex class I (MHC-I). By downregulating this molecule that is crucial for immune regulation, the parasite can hide from the host immune response and avoid elimination from the host (Overtvelt et al., 2002).

Importantly, the parasites often need to maintain a balance with their host in order to stay alive for as long as possible. Therefore, parasites must modulate the immune system to not activate mechanisms resulting in its own elimination, but simultaneously not induce severe immunosuppression leading to the death of the host by other infectious diseases (Wu et al., 2017), although depending on life cycle some parasites can benefit from more severely sick hosts. Helminths are incredibly successful in maintaining this balance and often cause more harmless, chronic infections that can last up to a lifetime if untreated. These parasites employ

active immunomodulation and depending on the helminth species, they act on specific phases of the host immune response (Maizels et al., 2018). For example, helminths such as *Echinococcus multilocularis*, *Trichinella spiralis*, *Trichuris suis* and *Taenia crassiceps* all suppress the host pro-inflammatory response by regulating cytokine expression (reviewed by (McSorley et al., 2013). The nature of immunological responses to *P. neurophilia* in zebrafish remains largely unexplored. However, seeing that clusters of *P. neurophilia* are associated with surprisingly little inflammation in surrounding tissues, it can be speculated that this parasite takes advantage of immune evasion strategies as well. Exploring zebrafish immune responses to *P. neurophilia* at the molecular level can provide important insights into this parasite-host interaction.

1.5 Knowledge gaps

Previous studies have found *P. neurophilia* infection in zebrafish to alter shoaling behaviour and habituation to fearful stimuli. Still, behavioural outputs in infected fish have not been studied across a range of contexts. Thus, the full range of behavioural traits affected by this parasite remains uncharted. Furthermore, it is known that *P. neurophilia* induces chronic infections as well as inflammation in the spine, brain and meninges. However, the molecular immune responses associated with this inflammation have yet to be elucidated. Studying the whole brain transcriptome will reveal immune responses that might be activated, and whether the parasite takes advantage of an immune evasion strategy, which is currently unknown. Parasites and hosts alike can redirect energy needs from *e.g.* growth and fitness for their own survival, and *P. neurophilia* has previously been found to reduce weight and fecundity in infected individuals. Yet, the energetic cost of infection has not been explored. Studying the metabolic and neurophysiological response to acute exposure in fish naïve to infection and those with an established infection can help reveal how zebrafish modulates their energetic response according to infection history, and in response to new infections. Lastly, implications of subclinical *P. neurophilia* infections on study outcomes in research have been indicated but remains largely uncertain. Studying parasite-host interactions at multiple levels will provide important insights on how and where subclinical infections may represent a critical threat to reproducibility and reliability of research results.

2. Aims

This Thesis sets out to investigate and obtain a better understanding of behavioural, transcriptional and physiological effects of subclinical *P. neurophilia*-infections in laboratory zebrafish. In order to achieve this objective, three sub-aims were formulated

Sub aims:

1. Describe behavioural effects of *P. neurophilia* infection in zebrafish across a range of standardised laboratory tests (Paper I)

2. Identify biological processes affected by *P. neurophilia*-infection by describing brain transcriptional changes associated with infection (Paper II)

3. Quantify metabolic and brain monoaminergic effects of acute and long-term *P. neurophilia*-infection in zebrafish (Paper III)

3. Summary of papers

Paper I: Behavioural effects of the common brain-infecting parasite *Pseudoloma neurophilia* in laboratory zebrafish (*Danio rerio*)

Previous studies have found that *P. neurophilia* induces altered habituation to fearful stimuli and shoaling behaviour in zebrafish. However, possible effects of the parasite on zebrafish behaviour across a range of contexts, in commonly used behavioural tests have not been described. In this study, the behavioural effects of *P. neurophilia* were examined in four behavioural tests commonly used by the zebrafish research community, namely the light/dark preference, mirror biting, open field and social preference tests. These tests are developed to measure behavioural correlates of emotional and cognitive states such as anxiety, aggression, exploration, locomotor activity and sociability. Contrary to my expectations, I found that *P. neurophilia* infection does not appear to affect behavioural correlates of sociability, aggression or anxiety. Instead, infected fish were characterised by immobility in the open field and mirror biting test, decreased distance moved in the social preference test and decreased crossings in the light/dark preference test, all indicative of reduced general activity. In line with previous studies, I also found infection to reduce body weight and length. Taken together, my findings suggest that the parasite affects general activity, while also affecting fish growth. These effects are possibly indications of general sickness behaviour, which is characterised by lethargy and anorexia, and is a way for the host to reallocate energy resources towards immune responses.

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

Pseudoloma neurophilia primarily aggregates in the hindbrain of the zebrafish host, where it leads to chronic infections. Infectious spores have been found to induce inflammation in meninges, brain and spinal cord, however parasite clusters (*i.e.* immature spores clustered in isolated vacuoles) induce little histological evidence of inflammation. The immune response to *P. neurophilia* has not been investigated at the molecular level, thus it remains unknown why these parasite clusters do not elicit a more evident inflammatory response. Moreover, because this intracellular parasite infects neurons and induces specific behavioural alterations, it is likely that the parasite affects neural functions. However, effects of *P. neurophilia* on the nervous system remain completely unexplored. In this study I investigated the effects of *P. neurophilia* on whole brain gene transcript abundance (RNA sequencing), to identify genes associated with biological processes possibly affected by the parasite. *Pseudoloma neurophilia*-infection resulted in 175 upregulated and 45 downregulated genes when compared to uninfected controls. A Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis identified four pathways to be enriched by the parasite, all of which were associated with immune functions. Additionally, a gene ontology (GO) analysis revealed 14 affected GO terms, eight of which were associated to immune responses and five to circadian rhythm. Contrary to my expectations, none of the differentially expressed genes or enriched pathways were specific for nervous system function. Rather, the main effects of this parasite on the brain transcriptome in zebrafish appears to be on genes associated with immunity. More specifically, several of the upregulated genes were associated with the pro-inflammatory branch of the immune system, suggesting a strong pro-inflammatory response to the parasite. More interestingly though, I found a distinct downregulation of a major histocompatibility complex II gene, *mhc2dab* and several circadian rhythm genes associated with anti-pathogen functions. These findings thus suggest that *P. neurophilia* may take advantage of different immune evasion strategies to avoid being removed by its host and allow it to maintain chronic infections.

Paper III: Metabolic and neurophysiological effects of a microsporidian parasite infection depend on previous infection status

Parasite infections commonly induce costly immune responses in the host. Thus, in the course of an infection, animals reallocate energy resources from *e.g.* fecundity, activity and growth to the immune system. Still, after reallocation of energy resources, increased energy expenditure caused by parasite infection may exceed the animal's total energy budget, resulting in increased metabolic demand. Reduced growth and activity observed in zebrafish chronically infected with the brain-dwelling parasite *P. neurophilia* (Paper I) indicate that the cost of this parasite infection may exceed the total energy budget of zebrafish. Moreover, since zebrafish are frequently exposed to new parasite spores in their environment, considerable costs of infection could arise already during and immediately following each new parasite exposure. Metabolic costs of *P. neurophilia* infection on the zebrafish host and the relative importance of acute versus established infections has not been studied previously. Therefore, the metabolic cost of acute versus established *P. neurophilia* infection in zebrafish was investigated using intermittent flow respirometry. This included measuring how acute parasite exposure alters metabolic rate in naïve versus previously infected hosts, and whether these effects are accompanied by changes in behaviour and major neurophysiological systems involved in the regulation of energetic and behavioural responses to parasite infection (serotonergic and dopaminergic signalling systems). The results indicate that established *P. neurophilia* infection causes a marginal, not statistically significant, increase in metabolic rate. Acute parasite exposure, on the other hand, resulted in a more pronounced increase in metabolic rate three days following exposure (regardless of previous infection status), which was mitigated by post-exposure day six. Brain serotonergic and dopaminergic activity also increased with acute parasite exposure, but only in naïve fish, three days post-exposure. Contrary to acute infection, established infection did not affect monoaminergic signalling. Lastly, established *P. neurophilia* infection was associated with increased activity levels (number of 180° turns in the respirometry chamber) six days post exposure regardless of acute infection status (parasite exposure versus sham exposure). Taken together, these results suggest that *P. neurophilia* infection entails the highest metabolic cost in the first days following exposure, particularly in naïve

individuals. In support of this, the parasite affects serotonergic and dopaminergic responses in naïve zebrafish only following first exposure. Lastly, increased activity in the respirometry chambers in fish with an established infection, suggest increased stress responsiveness with long-term infection and a complex interplay between long-term infection and exposure to multiple stressors which certainly deserves further scientific scrutiny.

4. Methodological considerations

4.1 Experimental animals

Zebrafish from the facilities at the Norwegian University of Life Sciences (NMBU) were initially planned to be used for the work in this Thesis. However, after conducting a thorough screening of this population prior to the start of the experimental infections I found that the fish tested positive for *P. neurophilia*, making it impossible for me to use these as a source for non-infected fish. Thus, five pairs of adult AB zebrafish were ordered from a specific pathogen free (SPF) facility in the United States (Sinnhuber Aquatic Research Laboratory (SARL), Oregon State University). A potential disadvantage of only having five pairs as the parental generation is the reduced genetic diversity in my study population. Small gene pools, as a result of inbreeding, can affect fertility, survival, birth weight and resistance to disease (Keller and Waller, 2002, Nasiadka and Clark, 2012), among other traits. According to SARL, each new generation comes from different crossings of parental lines, resulting in larger gene pools. Yet, in order to circumvent a potential problem with low genetic variance in the offspring, I used different combinations of males and females for every mating to reach the needed number of fish for my studies. In addition, I only used the F1 generation to avoid any further probability of inbreeding. I therefore obtained the highest genetic variance possible from the parental generation. In addition, the F1 generation of zebrafish used for the experiments were all bred within three weeks of each other (March 2018), so that the fish would be approximately the same age and size when the infection study was initiated (August 2018). The zebrafish larvae and juveniles were nursed according to the standardised protocols used at the zebrafish facilities at NMBU. Still, I experienced higher mortality rates in the offspring from the SPF strain (~50%), compared to standard AB fish maintained at the NMBU facilities (~10%). Moreover, the larvae did not grow as fast, or to the same size, as the AB strain at NMBU. Zebrafish growth can be affected by different factors, such as food and water quality, temperature and genetics (Singleman and Holtzman, 2014). Notably, the F1 generation were sexually mature at three months of age, which is common for laboratory-reared zebrafish (Singleman and Holtzman, 2014). Thus, the slower growth is suspected to be caused by genetics rather than husbandry or environmental factors. I initially expected to have 600–1000 SPF fish for my studies, but due to the higher mortality

rate among these fish, I settled with a final population of approximately 300 adult SPF zebrafish.

Numerous laboratory-bred zebrafish strains, as well as wild-caught zebrafish, are being used for research. The different strains have been found to react differently to behavioural tests. For example, larvae from the AB strain habituate faster to acoustic stimuli than Tupfel Longfin (TL) larvae, while Tübingen (TU) larvae are more active than AB larvae (Vignet et al., 2013, van den Bos et al., 2017). In addition, *P. neurophilia* has been found to affect zebrafish strains differently, increasing mortality in the AB strain compared to the TL strain (Ramsay et al., 2009a). I only tested the AB strain and therefore possible differences of *P. neurophilia* infection in other strains was not assessed in this work. However, since the AB strain is by far the most widely used strain in scientific research since the 1970's (Holden and Brown, 2018), these results are still highly relevant to the zebrafish research community and for the general usage of zebrafish as a translational model. Still, the results should be put into context by studying other strains since behavioural and physiological studies characterizing strain differences suggest that, it is likely some strains will be more affected by *P. neurophilia* infections than others. However, the comprehensive studies performed in this Thesis show that this parasite affects multiple biological traits in zebrafish, indicating the importance of proper health screening no matter the strain of zebrafish used.

4.2 Infection study

In order to carry out an infection protocol to infect the SPF strain with *P. neurophilia*, I first had to obtain enough infected donor fish from which I could later collect infective spores. Therefore, to get a stock of infected donor fish, I consistently exposed 100 retired zebrafish from the NMBU facility to spores over a period of 10 months (October 2017-August 2018). The spores were obtained from euthanized, clinically infected donor fish from the NMBU facility. Briefly, central nervous system (CNS) tissue and spinal cords were macerated by being passed through sterile needles with decreasing gauge size. The samples were then mixed with brine shrimp to increase ingestion by the zebrafish before being added to the tank. Although feeding spinal cords and brains to zebrafish can appear cruel, it is important

to point out that zebrafish cannibalize eggs, larvae and moribund zebrafish (Lawrence, 2007, Spagnoli et al., 2015b). Furthermore, this method was successfully used by other researchers, resulting in an approximately 85% infection rate (Peneyra et al., 2018). Throughout the exposure period, fish were sampled and analysed for *P. neurophilia* via qPCR in order to test the efficacy of the infection method. In my studies, all exposed fish that were analysed, tested positive for infection; thus, the method was deemed successful. The tank with the stock of infected donor fish, as well as a tank with SPF fish, were transferred to an infection room one week prior to the infection study.

After breeding and nursing SPF zebrafish for approximately 5 months, 252 fish were transferred to the infection room. The fish were divided into 30 tanks by using a random number generator, with an approximate 1:1 ratio of males and females. The tanks were split evenly into control or exposed treatment groups. To assure that control fish would not get infected by accident, the room was divided into two zones. The optimal design of the study would include randomized placings of exposed and control tanks; however, I have not been able to come up with a logistical design that allows for this without possible cross-contamination. Using separate zones can in theory result in zone-specific differences due to different areas possibly having different noise levels, light exposure or even temperatures. However, I made sure the room was kept at a constant temperature at all time and measured temperature in multiple locations in the room. The light was also evenly distributed throughout the entire room, while the room was only 3x4m, decreasing the possibility of zone-specific differences. The results obtained suggest a decrease in activity and growth, as well as an increase in immunological responses and metabolic rate in the exposed treatment group (*i.e.* infected group). These results are highly unlikely to be caused by the placement of treatment groups in different zones of the room, however the results must be seen in the light of this experimental setup.

The experimental infection of SPF fish was carried out over a period of 10 weeks. The daily infections included adding water from donor fish with known *P. neurophilia*-infection (from the stock of infected donor zebrafish) to the exposed treatment group to mimic natural transmission conditions (Spagnoli et al., 2017). Additionally, fish from the exposed group received infected CNS tissue four times during the 10 weeks, as described above. The control

group received the same treatment, but with water and CNS tissue from a batch of non-infected SPF fish. Spores were not quantified before being added to the tanks; hence this protocol does not allow for a controlled infection as such. If the spores were to be quantified, CNS samples should first be passed through a cell strainer (40µm) before being counted in a hemocytometer. However, when trying this method, I experienced low numbers of spores, and therefore I decided to rather use the entire CNS sample to avoid loss of spores. With the goal of standardising the infections, the same amount of infected tissue and water was added to each tank, ensuring that all individuals within a treatment group were equally exposed. Since it was not possible to quantify infection intensity following the infection study (as discussed below), I was not able to compare infection intensity between individuals in the exposed treatment group. Hence, some zebrafish might have had higher parasite loads than others. However, to my knowledge, no publication has established what the realistic parasite loads of *P. neurophilia* is in zebrafish, although it is established that infection results in variating intensities (Ramsay et al., 2009a). Because it remains uncertain whether experimental infections resembled those commonly occurring in infected zebrafish facilities, I only used fish that did not display any clinical symptoms of disease for the behavioural and physiological studies.

4.3 Gene expression

Real-time polymerase chain reaction (qPCR) is a method for amplification and quantification of target DNA and RNA. This method is used for studying expression levels of specific genes of interest. Commonly, RNA extractions from tissue samples is used and RNA is first DNase treated and then copied to complementary DNA (cDNA)(Kubista et al., 2006). Primers are designed to flank and amplify the target DNA by repeated cycles of polymerase chain reaction (PCR). Specific for qPCR is that the target DNA is labelled with a fluorescent tag which is measured after each cycle (Bustin, 2000). The detection value of the target, cycle threshold (C_t), indicates when the fluorescent intensity of the reaction is greater than that of the background. Thus, greater quantities of the target DNA result in lower C_t values (Heid et al., 1996). The measure of fluorescence can then be quantified by comparison to a standard curve, allowing for the determination of the concentration of specific genes (Wong and

Medrano, 2005). To detect *P. neurophilia*, I followed the protocol developed by Sanders and Kent (2011), in which primers are designed to detect the small subunit ribosomal ribonucleic acid (SSUrRNA) of *P. neurophilia*. Prior to qPCR, brains were homogenized, and DNA was extracted using the DNeasy® Blood & Tissue Kit (Qiagen). Because I did not have a standard curve with known spore concentrations, it was not possible to quantify the parasite load. However, for the purpose of this study I was only interested in whether zebrafish were infected or not. To this end, C_t values below 38 were accepted as positive markers for infection to avoid false-positive results. However, in diagnostics the results can be interpreted as positive after just one copy of the pathogen molecule, meaning C_t values around 40, with the negative standards resulting in no C_t values (Purcell et al., 2011). Although it would also be interesting to study the effect of parasite load, it was beyond the scope of the current project to develop a quantitative qPCR protocol. Previous studies have utilized histology to detect spores using different staining methods (Peterson et al., 2011, Sanders et al., 2014). However, histology only determines parasite load in a semi-quantitative way due to clusters being easily detected, while individual spores might be too small. Yet, semi-quantitative detection is still the most precise way to determine the parasite load of *P. neurophilia*. With this in mind, I still did not take advantage of this method due to the excessive number of samples. As explained above, for the sake of this study, I was mainly interested in the infection status, and qPCR is a fast, reliable and more cost-efficient way to assess infection than the discussed alternatives.

Where qPCR only allows for detection of known sequences, RNA-sequencing (RNAseq) gives the opportunity to perform a hypothesis-free detection of novel genes and characterization of a whole gene expression changes. This method is appropriate for detection of highly expressed genes, however low expressed genes are more difficult to detect and can thus get lost in the analysis (Halvardson et al., 2012). For RNAseq, extracted RNA must first be fragmented into small cDNA sequences by for instance chemical fragmentation (enzyme based, alkaline buffer or divalent cations). The RNA is then combined with random hexamer primers, before complementary strands are synthesized (Hrdlickova et al., 2017). These strands can then be sequenced using a high-throughput platform, such as Illumina/Solexa, Life/APG and Roche/454 (Metzker, 2010). The sequences are then mapped to the species-

specific genome and expression counts are estimated. All mapped data is normalized, and by using statistical methods the differentially expressed genes (DEGs) between contrast groups are determined. The DEGs can then be evaluated in a biological context (Costa-Silva et al., 2017). In **Paper II,** RNAseq was performed to study the whole brain gene transcriptome in zebrafish infected with *P. neurophilia* compared to uninfected controls. I extracted RNA from four different brain areas of five infected and five control zebrafish: the hypothalamus, telencephalon, optic tectum and brain stem. Samples were then sent to Novogene, a company that provides genomic services, for the RNAseq analysis using the Illumina platform. However, since Novogene requests a specific concentration of RNA in order to perform this analysis and due to low tissue RNA yields from the zebrafish brain samples, I had to pool brain areas (*e.g.* five telencephalon areas were pooled into one). The sequenced data was analysed in *R* (R Developer Core Team, 2019), and DEGs were functionally annotated based on Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Gene Ontology (GO) terms by using the package clusterProfiler (Yu et al., 2012). When performing the RNAseq analysis, the four different brain areas in one treatment group were compared to each other. In this way, the whole brain transcriptome of infected fish could be compared with that of control fish. Pooling samples is not optimal for RNAseq analysis as this can result in data bias. For example, it is impossible to know whether the observed transcriptional changes are driven by changes in one or a few samples or whether it is an overall effect of the treatment (Rajkumar et al., 2015). Therefore, a more appropriate method would be to study several individual samples of just one brain area. However, the results obtained showed that the gene expression was consistent throughout all samples in a treatment group (infected or control), suggesting the effect to be caused by treatment. These data indicate that the brain stem appears to be particularly susceptible to infection (*i.e.* by far more *P. neurophilia* sequence reads in this brain area compared to the others). Hence, brain stem samples from multiple individuals would possibly result in more DEGs than found in this study. Having more DEGs could help elucidate other mechanisms or pathways affected by *P. neurophilia* infection. Yet, the current study remains exploratory and is the first to investigate biological processes affected by *P. neurophilia* infection based on whole brain transcriptional changes. Importantly, the work performed here detected effects of *P. neurophilia* on biological

processes like immune function and has opened new research avenues for the study of *P. neurophilia* infection in zebrafish and other fish research models.

4.4 Behavioural experiments

The behaviour of infected and uninfected zebrafish was assessed across a range of contexts in **Paper I**. The study set out to elucidate possible behavioural outputs altered by *P. neurophilia* infection, and through that possible implications of subclinical infection on results obtained from behavioural studies. To this end, four different tests were utilized, namely: open field, mirror biting, light/dark preference and social preference (Fig. 4). The tests were chosen because they are commonly used in zebrafish research and target a range of behavioural correlates of emotional states on zebrafish (*e.g.* sociality, aggression, anxiety etc.). Furthermore, the protocols used in this Thesis have been comprised in the book "Zebrafish Protocols for Neurobehavioural Research" (Kalueff and Stewart, 2012) with the aim of standardising behavioural tests for zebrafish. Despite this, protocols between research groups tend to differ on several levels. For example, protocols can differ in the size and shape of the arena used, duration of test, acclimation time prior to testing or light intensity. Moreover, behavioural data collection may be obtained manually by a trained observer, or by using a tracking software. Tracking software provides a more sensitive assessment of behaviour, and thereby the mode of tracking may also lead to bias and inconsistencies in studies (Desland et al., 2014). For example, human error and variability can result in incorrect results when data is only tracked manually (Cachat et al., 2011).

The setup for the behavioural tests was based on recommended size of arenas (Fig. 4) and followed the suggested observation periods described in the aforementioned zebrafish protocol book. However, acclimation time was extended to five minutes per test for the light/dark preference and social preference tests. Although five minutes acclimation period has been considered extremely short by some researchers (Melvin et al., 2017), the protocol for the light/dark preference test suggests three minutes (Araujo et al., 2012), while an acclimation time of only 30 seconds is proposed for the social preference test (Pham et al., 2012). With acclimation periods of five minutes or less, the test may assess behavioural

responses to acute stress and a novel environment and not necessarily reflect actual individual preferences at basal conditions. Importantly, since I wanted to study the effect of *P. neurophilia* in commonly used behavioural tests and protocols, I decided to keep a relatively short acclimation time.

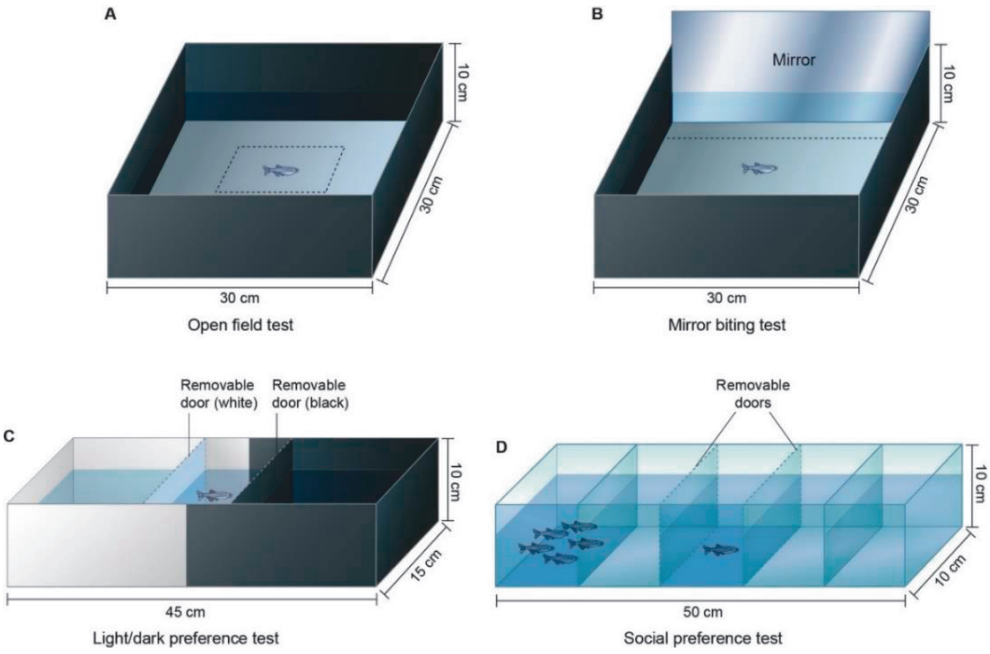

Figure 4: Behavioural tests and arenas used for studying behavioural outputs between control and infected fish (**Paper I**).

Several behavioural tests used for zebrafish have been modified from paradigms commonly used in rodent models (Champagne et al., 2010). For example, the light/dark preference test is based on nocturnal rodents displaying light aversion behaviour as a correlate of anxiety. It is then suggested that zebrafish display anxiety-like behaviour by preferring the dark compartment as well. However, zebrafish are diurnal, hence the biology and their respective preferences are very dissimilar to those of rodents. It is therefore possible that zebrafish instead prefer a brighter environment. Biological differences like this can ultimately affect the interpretation of study outcomes, as seen in for example the light/dark preference test, where researchers struggle to obtain consistent results, and zebrafish have been suggested to prefer both the dark (Maximino et al., 2010) and light (Champagne et al., 2010) compartments.

Furthermore, the interpretation of zebrafish behaviours still needs proper standardisations, as the terminology is less developed and inconsistent for zebrafish compared to other animal models, such as rodents. For these reasons, results obtained in behavioural tests often have varying interpretations. A behavioural catalogue has been developed to improve interpretations of zebrafish behaviour (Kalueff et al., 2013), which has been used for **Paper I** whenever possible. Although there are different interpretations for behavioural traits which makes it difficult to fully understand the behavioural endpoints measured, my study is aimed at circumventing this challenge by analysing behaviours across a range of contexts. For example, in **Paper I** I observed increased freezing behaviour in the open field and mirror biting tests. Considering the behaviour observed in the other tests (*e.g.* light/dark and social preference) where activity was generally reduced, increased freezing in the open field and mirror biting tests likely reflects immobility rather than anxiety-like behaviour (also see section 5.1.1 in Discussion).

Lastly, I used the software Ethovision XT 13 for a more objective and sensitive tracking of behaviour. With this in mind, all trials were video recorded before being analysed. Unfortunately, I experienced that the background lighting of the room interfered with the recordings and made it difficult for the tracking system to track parameters like distance moved and velocity in two out of three arenas. Therefore, I decided to first analyse videos using Ethovision, before manually tracking behaviour. This was done to test for conformity

and to make sure data obtained using Ethovision was correct, despite the lower quality of videos. Behaviours that were tracked manually were quantified a minimum of three times to reduce potential observer bias and all videos were blinded to the observer. My results showed that manually and software tracked data correlated.

4.5 Respirometry

In **Paper III** the energetic cost of acute versus long-term *P. neurophilia* infection was examined. To this end, metabolic rate in uninfected (naïve), acutely infected and long-term infected (established infection) zebrafish was measured using intermittent flow respirometry. This method measures oxygen uptake by individuals in sealed chambers, that are periodically flushed (Svendsen et al., 2016). Prior to oxygen measurements, zebrafish were stressed until they ceased burst swimming and were additionally air exposed for one minute. This was done to measure maximum metabolic rate (MMR). This measure indicates the maximal rate at which an animal can transport oxygen from the environment to the mitochondria, *i.e.* the maximum rate of oxygen consumption that a fish can achieve under certain conditions (Norin and Clark, 2016). Fish were then transferred to chambers composed of end-capped cylindrical glass tubes immediately after (Fig. 5).

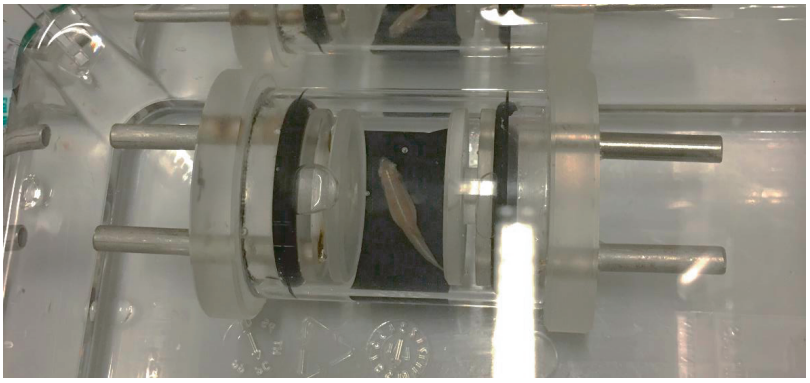

Figure 5: Respirometry chamber with adult zebrafish

The chambers were connected to a pump that flushed oxygen-saturated water through the chambers every 13th minute to keep air saturation above 80%, preventing hypoxia (Svendsen et al., 2016). Uninfected and long-term infected (10 weeks) zebrafish were exposed to infectious spores or a sham treatment on day zero after being transferred to the chamber. Oxygen concentrations were measured over a period of 24 hours using a Fire-sting fibre-optic oxygen meter starting on day zero, and additionally on day three and six post-treatment (parasite exposure or sham exposure). The measurements were used to quantify MMR, standard metabolic rate (SMR) and aerobic scope (AS). These measures indicate different relevant metabolic rates in fishes. For example, SMR indicates the lowest rate of oxygen needed to sustain life under specific conditions (Chabot et al., 2016), while aerobic scope refers to the capacity of the fish to increase its aerobic metabolism rate and is calculated as the difference between MMR and SMR (Norin and Clark, 2016).

Because of the small size of zebrafish, respirometry chambers had to be relatively small. One disadvantage with this is, the chambers then have a high surface area to water volume ratio, resulting in high background bacterial respiration. In order to combat build-up of bacteria in the system, all tubing and chambers were periodically cleaned in a weak bleach solution before each trial. It is possible to use a flow-through water system to avoid bacterial growth, however this was not possible in the facilities available on campus. Rather, a closed system was used, where water was UV-sterilised. The system allowed for only 10 UV-wattage, which prevents moderate bacterial growth. Ideally the UV-wattage should have been considerably higher to eliminate bacteria in the water and thereby the high bacterial background respiration. Because bacterial respiration was still present in the system, several chambers without fish were run for 24 hours. This allowed for the calculation of the oxygen uptake caused by bacterial growth in the analyses and subtract this background respiration. It is commonly assumed that bacteria follow a linear increase in growth for the calculations of background respiration (Rodgers et al., 2016). However, due to the high bacterial respiration measured in the small chambers, the 24-hour measurements obtained from empty chambers allowed for a more accurate exponential regression equation between bacterial respiration and time. This equation was then used to estimate bacterial respiration for each trial, to determine a more correct metabolic rate for the zebrafish. Of note, zebrafish are social

animals that commonly form shoals. Thus, confinement can result in increased stress and results obtained from a setup like this one can potentially reflect metabolic rates under stress conditions (Rey et al., 2015). Previous studies have used setups where shoal-mates could move around the chamber, decreasing the stress of social isolation for the tested individual (Nadler et al., 2016). In this setup, however, it was unfortunately not possible to test metabolic rate with shoal-mates. Hence, the results obtained reflect differences in metabolic rate in socially isolated zebrafish as caused by *P. neurophilia*. The results provide a detailed insight into the effects of infection following a stressful stimulus, and at different time points following infection.

4.6 High-Performance Liquid Chromatography

An HPLC with electrochemical detector was used to quantify monoamine neurochemistry in **Paper III.** That is, in this paper results on quantification of concentrations of the monoamines serotonin (5-hydroxytryptophan; 5-HT) and dopamine (3,4-dihydroxyphenethylamine; DA) and their respective catabolites 5-hydroxyindoleacetic acid (5-HIAA) and 3,4-dihydroxyphenylacetic acid (DOPAC) in *P. neurophilia*-infected and uninfected zebrafish are presented. This technique separates compounds in a sample in order to identify and quantify their concentration, by calculating against a known standard concentration in a certain amount of tissue sample. I sampled the telencephalon, hypothalamus, optic tectum and brain stem immediately after the open field and mirror biting test. Brain areas were then homogenized in a buffer before being injected into the HPLC system. In the system, the samples are passed through a reverse phase column, meaning that the column attracts non-polar solvents, resulting in polar solvents to travel through the column faster. Additionally, bigger molecules also have increased friction and thus higher retention time. The retention time is amplified by a detector and refers to the time it takes for the compound to pass through the column. The compounds can then be analysed and quantified by comparing to a standard solution with known concentrations of the compounds of interest (Malviya et al., 2010). This method is well-established, relatively fast and inexpensive, however the results obtained only reveal the concentration of the compound at a specific time point. Other methods, such as optogenetics, allow the researcher

to visualise and track living neural circuits using genetic targeting of specific neurons or proteins (Deisseroth et al., 2006). Because of this, neurotransmitter activity can be observed over a period of time giving a broader understanding of the neural functions (Guru et al., 2015). However, methods such as optogenetics can be challenging to use in zebrafish, in part because transgenic fish are needed (Zhu et al., 2009). Perhaps transgenic fish behave differently in response to infection and in general. This is why it is valid to first study common AB zebrafish before utilizing more time-consuming and expensive techniques. Thus, HPLC remains a reliable and standardised method to obtain data on neural functions in *P. neurophilia*-infected and uninfected control zebrafish.

5. Discussion

In this Thesis I have investigated behavioural, transcriptomic and physiological effects of the common brain-infecting parasite *Pseudoloma neurophilia* in laboratory zebrafish. Here, I found infection to have no effects on commonly measured behavioural correlates of emotional states like sociability and aggression. However, *P. neurophilia* infection generally reduced activity in zebrafish across a range of contexts, in addition to negatively affecting growth. Moreover, infected zebrafish were found to display an upregulation of genes associated with a pro-inflammatory immune response, but also more specific downregulation of genes involved in anti-pathogen functions. *Pseudoloma neurophilia* infection also resulted in increased metabolic rate in zebrafish following acute exposure to infectious spores regardless of previous infection state. These results taken together indicate that *P. neurophilia* is costly for the host and this might be compensated for by sickness behaviour, a condition where acutely infected individuals allocate energy resources in order to fight infection. Although sickness behaviour is clasically expressed in response to acute infections, the phenotype was observed in zebrafish after long-term infection. Thus, the results obtained in this Thesis may suggest that sickness behaviour can persist even in chronic infections, or that *P. neurophilia* induce a phenotype resembling sickness behaviour that reflects a long-lasting effect of chronic infection. Furthermore, the data suggest that *P. neurophilia* may utilize immune evasion strategies to avoid being eliminated by the host immune response and induce chronic infections. Lastly, the results highlight the many biological processes that can be affected by a subclinical, microsporidian infection, and how these may have implications for research outcomes.

5.1 Are *P. neurophilia*-induced alterations indicative of sickness behaviour in zebrafish?

Parasites, by definition, are costly for the host. By deriving their nutrients from the host they incur an energetic demand, which can help explain the negative impacts of parasitism, such as reduced fecundity, growth and survival (Dallas et al., 2016). Simultaneously, hosts can

redirect energy resources to cope with infectious pathogens, thus an increase in pro-inflammatory immune responses can result in specific alterations of behaviour known as sickness behaviour (Dantzer, 2001). Sickness behaviour is defined as a set of adaptive behavioural changes in acutely sick animals (Prather, 2013), characterised by lethargy, decreased activity and social interactions in addition to reduced appetite. Although the zebrafish in this study were infected with *P. neurophilia* for 10 weeks, the results obtained reflect a syndrome resembling sickness behaviour. Thus, below I discuss whether our findings support the notion that *P. neurophilia* induce sickness behaviour in laboratory zebrafish even in the chronic phase of infection.

5.1.1 *P. neurophilia* infection reduces overall activity levels in zebrafish host

Sick animals tend to spend more time sleeping, while spending less time being active and eating. By decreasing activity, vital resources can be saved for the immune response in order to fight infection (Hart and Hart, 2019). In **Paper I**, multiple behaviours were studied across a range of contexts. I found that infection reduces the total distance moved in the social preference test and decreases crossings between compartments in the light/dark preference test. Distance moved and crossing between compartments are both behavioural traits commonly used for measuring activity (Egan et al., 2009, Maximino et al., 2011, Tran and Gerlai, 2013). In addition, infected individuals displayed increased freezing behaviour, *i.e.* immobility, in the open field and mirror biting tests. Increased freezing behaviour is commonly interpreted as an anxiety-related behaviour in zebrafish (Egan et al., 2009). However, freezing and immobility are difficult to distinguish from each other and are used as synonyms in zebrafish research (Kalueff et al., 2013). Importantly, all freezing is a correlate of immobility but not all immobility is freezing. For example, orienting is an immobility state that occurs in response to a novel situation or stimulus, and as opposed to freezing is subject to habituation (Roelofs, 2017). Furthermore, freezing in zebrafish is accompanied by an increase in opercular movement, whereas other correlates of immobility are not (Kalueff et al., 2013). This is a relatively subtle difference that makes it difficult for tracking software, as well as trained observers, to distinguish and interpret the behaviour. Notably, tracking software such as Ethovision is not able to track opercular movements and cannot distinguish immobility from freezing, thus the programme only refers to immobility.

Considering the general reduction in activity observed across behavioural tests, I believe that the general reduction in movement observed in my experiments is indicative of immobility. With this clarification in mind, the data suggest that *P. neurophilia* infection reduce activity in the zebrafish host, which is one of the central characteristics of sickness behaviour.

Contrary to the findings in **Paper I**, *P. neurophilia* infection was associated with increased activity (mean number of 180° turns per minute) six days post exposure to spores in **Paper III**. However, in this study fish were exposed to several stressors simultaneously, such as social isolation, confinement and repeated handling stressors (in connection with respirometry), so that increased activity in this test possibly reflects an interaction between stress and infection rather than sickness behaviour. Yet, the underlying mechanisms for increased activity following repeated stressors remain unknown. It would be interesting to further study how infection and stress interact and affect study outcomes if zebrafish were exposed to the behavioural test mentioned above multiple times. The results obtained suggest that only novel testing results in reduced activity whereas multiple testing result in increased activity, highlighting how this parasite can affect consistency of behavioural tests.

Sickness behaviour is further associated with reduced social interaction (Kelley et al., 2003). Nevertheless, I found no effects of *P. neurophilia* infection in the social preference test, hence the results do not provide evidence that zebrafish display decreased sociability once infected with *P. neurophilia*. However, zebrafish are social animals that actively form shoals, thus by studying shoal formation the overall social behaviour can be analysed (Pham et al., 2012). Previously, *P. neurophilia*-infected zebrafish were found to form tighter shoals, suggesting that the parasite affects social behaviour (Spagnoli et al., 2017). Interestingly, altering social interactions, such as increasing host sociability, has been suggested as a parasite strategy to increase transmission success in other host-parasite interactions. For example, more social contact with conspecifics resulted in higher transmission rates of the parasite *Gyrodactylus turnbulli* in guppies (*Poecilia reticulata*) (Johnson et al., 2011). Since I found *P. neurophilia* to reduce distance moved and velocity (Fig. 6) in the social preference test, one can speculate whether reduced activity can explain closer shoal formations. Indeed, slow-moving three-

spined stickleback (*Gasterosteus aculeatus*) form more cohesive groups compared to fast-moving individuals (Jolles et al., 2017).

Alternatively, the observed increased shoal cohesion may represent host-induced parasite manipulation. Theoretically, a closer shoal cohesion could aid the parasite by increasing the chances of zebrafish ingesting infectious spores released by shoal-mates. Although no studies indicate how long spores of *P. neurophilia* can survive in water, other microsporidia have been found to have reduced longevity in temperatures ranging from 25-30°C (Li et al., 2003). Because laboratory zebrafish are kept at a constant 28°C, the parasite might be dependent on reaching the next host relatively fast. Decreasing activity and inter-fish distances could therefore suggest a parasitic strategy aimed at increasing transmission rates. Regardless of the underlying mechanism, the closer shoal formations induced by *P. neurophilia* probably increase transmission success and consequently benefits the parasite, while the energy saved on activity benefits the host.

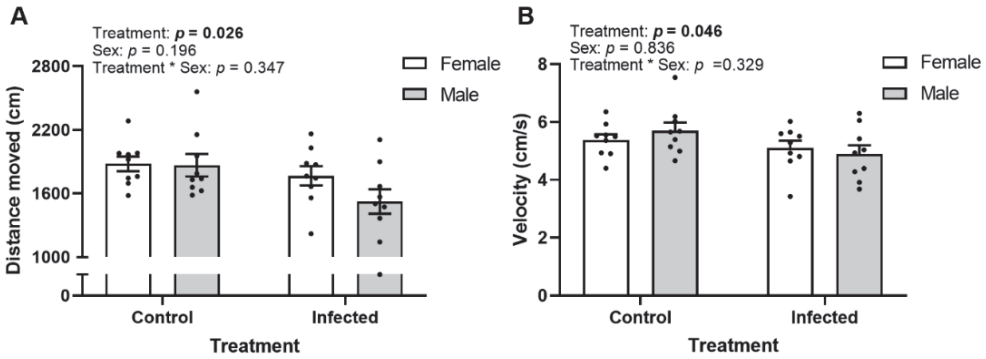

Figure 6: behavioural correlates of activity in zebrafish infected with *Pseudoloma neurophilia*. Infected individuals are negatively affected and display reduced (**A**) average distances and (**B**) velocity in the social preference test. *n* = 18 for both treatment groups.

The first description of sickness behaviour in zebrafish was provided by Lee et al. (2015). In this article researchers found that zebrafish infected with the bacterium *Edwardsiella tarda* displayed reduced activity across several correlates. To further study this, researchers later stimulated the immune response in zebrafish by inoculating them with formalin-inactivated *Aeromonas hydrophila* bacterin before studying their behaviour. They found a higher expression of pro-inflammatory cytokines which was associated with reduced activity as well as a decrease in social interactions in the social preference test (Kirsten et al., 2018a). Hence, these studies suggest that immune responses and behaviour are linked in zebrafish, resembling the sickness behaviour previously described in mammals.

Findings of decreased activity and reduced growth taken together suggest that some correlates of sickness behaviour are met, and that the behavioural differences found in **Paper I** are most likely caused by the cost of fighting infection. Despite sickness behaviour being characterised by reduced sociability, the parasite did not appear to affect this behavioural correlate. However, in this Thesis zebrafish could only use visual cues in the social preference test, whereas several sensory modalities are present in nature in social preferences, such as olfactory and auditory cues (Nunes et al., 2020). Thus, other tests for sociability in response to *P. neurophilia* would be interesting to study further. It is however also interesting to speculate whether reduced sociability would be disadvantageous to *P. neurophilia* regarding transmission, explaining the closer shoal formation. Indeed, other studies looking at sickness behaviour often use lipopolysaccharide (LPS), which is derived from gram negative bacteria (Alexander and Rietschel, 2001), and not live parasites. Hence, the motivational aspects of parasitic strategies are usually not accounted for. Alternatively, *P. neurophilia* modulate immune responses so that infection is less energetically costly than classic infections (*e.g.* LPS induced infection) and thus does not affect sociability. Therefore, to further understand the mechanisms behind the behavioural effects of this parasite, it is necessary to study the biological processes and immunological responses to *P. neurophilia* infection.

5.1.2 Immunological responses to *P. neurophilia* infection

In mammals, sickness behaviour is mediated by an increase in pro-inflammatory cytokines such as interleukin (IL) 1α and 1β, IL6 and tumor necrosis factor-α (TNFα) particularly

following acute infection (Dantzer et al., 1998, Bluthé et al., 2000, Huang et al., 2008, Maes et al., 2012). The immune response in zebrafish displaying sickness behaviour characteristics, *i.e.* reduced activity and social interactions, was recently found to resemble that of mammals. That is, zebrafish with activated immune responses (by inoculation of formalin-inactivated *Aeromonas hydrophila* bacterin) were found to have increased levels of IL1β, IL6 and TNFα in the brain (Kirsten et al., 2018b). Thus, to identify effects of *P. neurophilia* in the zebrafish host at the molecular level, an RNA-sequencing analysis on CNS tissue was performed in **Paper II**.

Although multiple genes associated with a pro-inflammatory immune response was found to be upregulated in infected fish, no affected genes associated with the above-mentioned cytokines were detected. Instead, an upregulation of TNF superfamily member 14 (*tnfsf14*, otherwise known as LIGHT), major histocompatibility complex I ZBA (*mhcIzba*), two isoforms of cluster of differentiation 8 (*cd8a* and *cd8b*) and interferon gamma 1 (*ifng1*) was observed. These genes taken together indicate a strong activation of pro-inflammatory immune responses in zebrafish after 10 weeks of infection. Notably, *ifng1*, the gene coding for the pro-inflammatory cytokine IFNγ, is upregulated in response to the parasite. Due to sickness behaviour being hitherto defined as a set of syndromes following acute infection, the immune response observed here probably differs from what is normally associated with acute infection and sickness behaviour. Studying the transcriptomics of zebrafish acutely infected with *P. neurophilia* would possibly lead to a different set of differentially expressed genes and perhaps genes encoding for other cytokines. In fact, metabolic rate increases following acute exposure to *P. neurophilia* as shown in **Paper III**, which suggest that acute infection results in activation of innate immune responses. Importantly, activation of immune responses is energetically costly (Lochmiller and Deerenberg, 2000). Therefore, what can be assumed to be a chronic activation of pro-inflammatory responses in long-term infected zebrafish is likely to affect activity in the same way as innate immune responses, *i.e.* upregulation of IL1, IL6 and TNFα, and thus the decreased activity (**Paper I**) possibly reflects that sickness behaviour is also found in response to chronic infection.

As mentioned above, most research on sickness behaviour has focused on acute inflammation responses. This has been done by studying the immune responses and

behaviour following LPS treatment. LPS interacts with the immune system by stimulating toll-like receptor 4 (TLR4), which, in turn, releases pro-inflammatory cytokines, such as IL1 and TNFα (Beutler, 2000, Lu et al., 2008). Sickness behaviour in response to chronic microsporidian infection has to my knowledge not been examined previously. It is possible that zebrafish would display an increase in the cytokines normally associated with sickness behaviour in the days following the first infection, which would also affect behaviour differently. For example, mice with autoimmune allergic encephalomyelitis (EAE) had increased levels of the cytokines IL1β, IL6 and TNFα in the acute phase of the disease, but the cytokine expression profile was attenuated in the chronic phase (Okuda et al., 1998). Interestingly, EAE mice also displayed behavioural symptoms of sickness behaviour such as anorexia and reduced social interactions in the acute phase, which recovered in later phases of the disease. Notably, EAE mice remained underweight even in the recovery phase, possibly due to alterations in their metabolism (Pollak et al., 2000). Thus, the state of the infected zebrafish possibly resembles that of EAE mice in the chronic phase of disease by displaying reduced weight, different cytokine expression and what appears as normal social behaviour (at least within the studied parameter) following 10 weeks of infection.

These findings suggest that harbouring intracellular parasites induce chronic inflammation in the host. For example, I found a pro-inflammatory immune response to be activated in the zebrafish following long-term *P. neurophilia* infection. Chronic inflammation has previously been found to manifest in animals displaying the cachexia syndrome, which is characterised by anorexia, lethargy and increased catabolism, often resulting in higher mortality rates (reviewed by Burfeind et al., 2018). Simultaneously, constant immune signalling to the brain can also lead to depression-like states in sick individuals (Dantzer et al., 2008). Interestingly, mice chronically inoculated with *Bacillus Calmette-Guerin* showed a sustained upregulation of both IFNγ and TNFα after three weeks of infection. Here, sickness behaviour lasted only 5 days, and was followed by depression-like behaviour, including immobility (Moreau et al., 2008).

Consequently, it is possible that upregulated pro-inflammatory responses together with the reduced activity and growth observed in this work, reflect symptoms of syndromes caused

by long-term infection in the wake of sickness behaviour. However, I can also speculate whether *P. neurophilia* modulates the immune response to benefit its own survival. Acute inflammatory processes with the release of a myriad of pro-inflammatory cytokines will unquestionably make it difficult for the parasite to survive and maintain chronic infection. By modulating the immune response to only induce specific inflammatory processes, and thus specific correlates of sickness behaviour, the parasite could increase, not only its chances of survival, but also its transmission to the next host. Indeed, possible manipulations of the immune system with the purpose of evading the immune system is discussed in section 5.2 below. However, if the parasite does induce a constant pro-inflammatory response in the zebrafish host, it must be assumed that infection is chronically energetically costly, affecting both metabolism, growth and general activity.

5.1.3 Metabolic cost of *P. neurophilia* infection

Immunological responses to infection are known to be costly and can allocate resources from growth (Lochmiller and Deerenberg, 2000), physical performance (Bedhomme et al., 2005) and reproductive success (Cox et al., 2010) in the host. I therefore hypothesised that infection with *P. neurophilia* would result in an increase in metabolic rate indicating higher metabolic demands, as infection has previously been shown to negatively affect all the above-mentioned traits (**Paper I**, Ramsay et al., 2009b, Sanders et al., 2020). However, in **Paper III**, the standard metabolic rate (SMR) and maximum metabolic rate (MMR) showed only a marginal non-significant increase in infected fish, compared to controls. In addition, there was a small reduction in aerobic scope (AS) in zebrafish with long-term established infection, compared to acutely infected and non-infected controls. Interestingly, in other parasite-host systems, parasitism does not appear to affect the metabolic rate. For example, the brown trout (*Salmo trutta*) infected with the glochidia larvae (*Margaritifera margaritifera*), an obligate parasite, display an SMR resembling that of uninfected individuals (Filipsson et al., 2017), while the bot fly *Cuterebra emasculator* has no effect on RMR in adult chipmunks (*Tamias striatus*) (Careau et al., 2010). This suggests that parasites utilize host energy by yet unknown mechanisms, or alternatively that parasitized hosts can allocate energetic resources to fight infection by other means. For example, infection with the parasite *Diplostomum* spp. in the Arctic charr (*Salvelinus alpinus*), resulted in a lower

SMR due to a higher liver mass (a side effect of infection) which changes energetic demands (Seppänen et al., 2009).

Intriguingly, metabolic rate was increased to a much larger extent on day three following exposure to the parasite (=$MR_{exposure}$, **Paper III**), regardless of previous infection status of the fish. Extrapolating reports that acute inflammatory processes are costly, the increase in metabolic rate suggest a stimulation of acute inflammatory responses. However, this increase was mitigated again by day six, indicating that *P. neurophilia* induces transient increases in metabolic demand. Due to zebrafish being frequently exposed to new parasite spores in their environment, having this parasite in the system may incur frequent metabolic costs that can compromise growth and activity. To the best of my knowledge, it has not yet been investigated how often spores are released from infected fish. Nevertheless, it is established that spores are released during spawning (Murray et al., 2011) and zebrafish can spawn daily (Westerfield, 2007). Thus, infected zebrafish sharing a tank can theoretically ingest new spores on a regular basis. This can result in frequent increases in metabolic rate, which can further explain the observed symptoms resembling sickness behaviour (*i.e.* reduced growth and activity).

Although the neurophysiological responses to sickness behaviour to my knowledge remains unexplored, it has recently become more evident that the brain plays an important part in fighting infection. For example, many immune cells express receptors for the monoamine neurotransmitters serotonin (5-HT) and dopamine (DA). The receptors enable the immune cells to respond to these neurotransmitters and indicate that they play a part in regulating important immune functions (Matt and Gaskill, 2019, Wu et al., 2019). Seeing that a long-term established *P. neurophilia* infection results in a pro-inflammatory response, it was hypothesised that neurophysiological responses would also be affected in response to infection. However, only increased activity of dopaminergic and serotonergic activity in naïve fish exposed to an acute infection was found. Conversely, long-term infected fish displayed similar neurophysiological responses to uninfected controls both routinely and after renewed acute infection, suggesting that monoaminergic activity normalises in the chronic phase of *P. neurophilia* infections. Interestingly, dopaminergic activity has previously been shown to increase in response to acute LPS administration in rats (De

Laurentiis et al., 2002). As mentioned above, LPS is often used to induce sickness behaviour in research animals. With this in mind, it is tempting to speculate that the increase in neurophysiological responses together with the increase in metabolic rate on day three following acute exposure in naïve zebrafish reflects sickness behaviour, which is associated with a drainage of energy resources.

In summary, the results obtained in this Thesis indicate that both acute and chronic *P. neurophilia* infection represents a high energetic cost for the zebrafish host dependent on infection status. That is, while naïve fish appear to increase both metabolism, serotonergic and dopaminergic responses, long-term infected fish are characterised by chronic pro-inflammatory responses as well as reduced weight and a general reduction of activity levels. Sickness behaviour as per classical definition is commonly seen in response to acute infection, which I speculate is induced in naïve zebrafish in the first three days following infection. Notably, these results suggest that long-term infection leads to continud immune signalling in the zebrafish brain, which can result in syndromes resembling sickness behaviour or syndromes that arise following chronic infection, such as depression or cachexia, both of which induce a loss of weight, reduced activity and chronic inflammation. Although my findings do not indicate the parasite to induce "classic" sickness behaviour, it is evident that infection drains energy from the zebrafish host. As noted by Moore (2013), parasites using their host for reproduction and dispersal must minimize side-effects of infection to keep the host alive. Hence, infected individuals can appear both healthy and mobile. Indeed, most *P. neurophilia* infections remain subclinical. However, parasitic infections like this will often result in more dramatic effects, such as reduced fecundity (Moore, 2013). Interestingly, I noted that some female zebrafish were more difficult to differentiate from males following 10 weeks of infection, due to a reduced gonadal area (Fig. 7), which once again highlights the energetic costs associated with *P. neurophilia* infection in the zebrafish host.

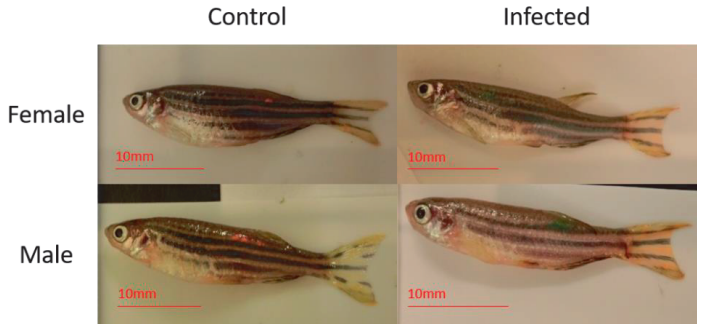

Figure 7: Zebrafish (*Danio rerio*) after 10 weeks of infection with the microsporidian parasite *Pseudoloma neurophilia*, and uninfected controls. Infected females have reduced body condition (length/width ratio) due to reduced gonadal area and thus lower fecundity (Ramsay et al., 2009a, Sanders et al., 2020).

5.2 Does *P. neurophilia* evade the zebrafish immune system?

Immune evasion is a relatively common parasitic strategy, where a parasite actively evade or manipulate the host immune system in order to increase its own survival within the host (Schmid-Hempel, 2008). In **Paper II** I found that fish infected with *P. neurophilia* showed a downregulation of specific genes associated with immune memory and anti-pathogen functions, suggesting immune evasion. Importantly, other microsporidian parasites have previously been proposed to utilize such strategies. For example, *Vavraia culicis* secretes a wide array of proteins that are suggested to suppress immune responses in the mosquito host *Anopheles quadrimaculatus* (Desjardins et al., 2015). By contrast, I found an upregulation of pro-inflammatory responses in zebrafish with established infections. In this context, previous studies suggest that immune responses following microsporidian infections depend on the life stage of the parasite. For example, following the rupture of *Loma salmonae* xenomas (*i.e.* hypertrophic host cells containing all developmental stages of the microsporidia) in the gills of the rainbow trout (*Oncorhynchus mykiss*), the cells surrounding the newly released spores are immune cells, such as macrophages and lymphocytes, reflecting a strong immune response (reviewed by Rodriguez-Tovar et al., 2011). Interestingly, only mature *P. neurophilia* spores appear to induce inflammation in

meninges, brain, spinal cord and muscles, while parasite clusters (containing immature spores) induce little to no inflammation (Spagnoli et al., 2015b). Seeing that all life stages of the parasite are present simultaneously (Cali et al., 2012), I speculate that only parasite clusters evade host immune responses, while the infectious spores and ruptured clusters stimulates pro-inflammatory responses, as was found in **Paper II**.

The findings in this Thesis suggest different possible evasion mechanisms. First, I found a strong downregulation of the gene major histocompatibility complex (MHC) II DAB (*mhc2dab*), which indicates that *P. neurophilia* takes advantage of a common immune evasion strategy. That is, interference with specific components of the MHC-II pathways is a widely used mechanism by viruses, such as the Human Immunodeficiency Virus (HIV) and Hepatitis C, in order to block adaptive immunity (Forsyth and Eisenlohr, 2016). In fact, this gene is a part of the group of MHC-II molecules, which are essential for cell-mediated immunity and provides an important step in the clearance of pathogens by initiating immune memory (Lewis et al., 2014, Rock et al., 2016). Other parasites, such as *Leishmania* spp., take advantage of manipulating the MHC-II complex to evade immune responses. The parasite uses antigen sequestration in mice macrophages and in that way hides from MHC-II to avoid elimination (Kima et al., 1996). Since *P. neurophilia* evidently results in chronic infections, much like the abovementioned pathogens, it is likely that the parasite could interfere with MHC II mechanisms to maintain infection in the host.

Moreover, I found genes associated with anti-pathogen function to be downregulated by *P. neurophilia* infection, including period circadian clock 1b (*per1b*) and nuclear receptor subfamily, group d, member 1 (*nr1d1*). Both genes have been found to be important for autophagy and consequently aid in hindering intracellular growth of pathogens (Huang et al., 2016). Intriguingly, several bacteria and viruses prevent host-immune autophagy as an evasion strategy (Orvedahl and Levine, 2009). Furthermore, the intracellular parasitophorous vacuole (PV) of the malarial parasite *Plasmodium berghei*, has been found to rely on a transmembrane protein, that inhibits essential functions of host autophagy in mice hepatic cells to evade elimination (Real et al., 2018). In addition, evasion of autophagy has been found to be an extremely important mechanism for the survival of viruses

(Orvedahl and Levine, 2008). Thus, my findings suggest that *P. neurophilia* actively manipulate pathways associated with autophagy mechanisms to evade immunity.

Although it remains unknown whether *P. neurophilia* takes advantage of immune evasion strategies, the findings show that several mechanisms and pathways are uniquely regulated by the parasite and that this may help the parasite avoid recognition by the host`s immune system. Seeing that many parasites actively evade immune responses, it is likely that this parasite manipulates specific mechanisms in order to avoid being eliminated by its host and cause chronic infections. The findings combined with previous studies suggest that parasite clusters take advantage of immune evasion strategies, while infectious spores and rupturing clusters induce a pro-inflammatory response. It was not in the scope of this Thesis to further elucidate these mechanisms or their underlying effects, therefore future studies should focus on this area of the host-parasite interaction. Studying such mechanisms would give important insights into immune responses to fish microsporidia, an area that, to date, remains largely unknown.

5.3 Can *P. neurophilia* have implications for research?

Prior to the studies performed in this Thesis, *P. neurophilia* had already been found to affect social and startle response behavioural outputs in zebrafish (Spagnoli et al., 2015a, Spagnoli et al., 2017). Furthermore, infection is associated with increased mortality rates, reduced weight and fecundity, as well as inflammation throughout muscles, brain and spinal cord tissue (Ramsay et al., 2009a, Spagnoli et al., 2015b, Sanders et al., 2020). Taken together, the results from these studies suggest that infections with *P. neurophilia* can affect study outcomes within fields such as behaviour, neurobiology, development and immunology. The results obtained in this Thesis clearly support this stance and highlight the importance of proper health monitoring of zebrafish facilities. To illustrate this, I provide below a few scenarios on the implications of how subclinical infection can affect study outcomes.

I found infection to generally reduce activity in long-term, yet subclinically infected zebrafish in **Paper I**. Activity/locomotor behaviour is an important endpoint in many scientific fields

using zebrafish as an animal model. For example, when testing the effects of chemical and pharmacological compounds on zebrafish, activity is commonly measured and used to indicate the effect on general locomotion as a correlate of health (Chen et al., 2017, Tu et al., 2017, Zhao et al., 2018). Moreover, behaviour is easily obtained in zebrafish and has provided insights into a general understanding of locomotor circuit function in vertebrates (Berg et al., 2018, Fitzgerald et al., 2019). Thus, if researchers are unaware of infection status and for example use fish with subclinical *P. neurophilia* infection in the treatment group, the observed effects on locomotion might actually reflect effects of *P. neurophilia* infection. Alternatively, if the compound of interest reduces activity and only the control group is infected, reduced activity in the treatment group may go undetected. Notably, the prevalence of *P. neurophilia* not only varies greatly between zebrafish facilities, but also between tanks within each facility (Spagnoli et al., 2015a, Spagnoli et al., 2015b).

Varying prevalence within facilities can result in severe tank effects when a study design only uses few tank replicates. A possible scenario could be that the transfer of *P. neurophilia* infected fish from an infected tank in the facility to a tank containing all the fish in the control group, resulting in healthy controls becoming sick. Since *P. neurophilia* has been found to cause more severe inflammation in immunocompromised hosts (Spagnoli et al., 2016), such tank effects can also result in more drastic outcomes. For instance, a tank with infected individuals exposed to immunocompromising drugs could result in high mortality rates and weaken the power of the study. Additionally, varying prevalence of *P. neurophilia* between zebrafish facilities could be problematic. Facilities with higher prevalence might obtain very different results compared to facilities with low or no infection. For example, if testing the effect of a compound in a facility with a high prevalence, the compound might reflect sedative effects due to reduced activity as caused by *P. neurophilia*. However, had the same compound been tested in a facility with low or no *P. neurophilia* infection only few outliers or no such effect would be linked to the compound.

Multiple genes associated with immune responses were found, that were either up- or downregulated in *P. neurophilia* infected fish (**Paper II)**. Importantly, zebrafish are crucial model organisms in immunological research and are frequently used to assess the effects of

specific pathogens on vertebrate hosts. For example, zebrafish has been used to help researchers in understanding the biology of pathogens commonly found in aquaculture, thus resulting in the improvement of disease control in such facilities (Lee-Estevez et al., 2018). However, if zebrafish that are used for such assessments have established *P. neurophilia* infections before being introduced to new pathogens, immunological responses might be wrongfully linked to the study pathogens and this might lead to wrong, or unnecessary treatment of fish in the aquaculture industry, as an example.

The results of **Paper III** revealed that acute infection with *P. neurophilia* affect both metabolic and neurophysiological responses (*i.e.* brain monoaminergic activity) in naïve zebrafish, while metabolic changes were detected in fish with long-term infection. Previously, measurements and manipulation of monoamines has been used in zebrafish research to elucidate the neural mechanisms underlying specific behaviours. For example, reduced serotonergic activity is linked to disrupted antipredator behaviour in female zebrafish (Vossen et al., 2020). Moreover, metabolic rate in zebrafish can be used to asses for example the effects of environmental pollution in aquatic animals (Zhou et al., 2018). Hence, if researchers should obtain zebrafish that has recently been exposed to *P. neurophilia*, they risk acquiring data indicating increased metabolic and neurophysiological responses that reflect underlying *P. neurophilia* infection, and not their experimental question. As an example, increased metabolic rate could get linked to environmental pollution, resulting in wrong interpretations of the effects caused by such toxins.

Besides infecting zebrafish, *P. neurophilia* has been reported to have a host range that includes siamese fighting fish (*Betta splendens*), platy (*Xiphophorus maculatus*), giant danio (*Devario aequipinnatus*), fathead minows (*Pimephales promelas*), goldfish (*Carassius auratus*), neon tetra (*Paracheirodon innesi*) and medaka (*Oryzias latipes*)(Sanders et al., 2016). In a separate study not included in **Papers I-III**, medaka were exposed to *P. neurophilia* using the same infection protocol used for zebrafish over a period of eight weeks. The medaka was then tested in the open field and mirror biting test following the same protocol as in **Paper I**. All fish were tested for the presence of *P. neurophilia* following

the infection study. Interestingly, none of the fish in the exposed group tested positive for the parasite, possibly because medaka are more resistant to *P. neurophilia* than zebrafish. It could also suggest that medaka has evolved a better mechanism to fight acute infections, (Broussard and Ennis, 2007). Surprisingly, even though *P. neurophilia* failed to infect medaka, a distinct behavioural effects of parasite exposure was observed. Contrary to what was observed in zebrafish in **Paper I**, exposure to *P. neurophilia* spores resulted in increased activity and sociability as well as decreased exploration in medaka (Fig. 8). Thus, despite failing at settling in the medaka brain, the parasite had considerably greater (and opposite) effects on medaka behaviour compared to zebrafish. Yet, the underlying mechanisms for exposure-induced alteration of behaviour in medaka remain unknown. It is also unknown whether exposure to other pathogens can induce similar behavioural effects. These knowledge gaps certainly deserve further scientific scrutiny. Nevertheless, it is important to highlight that *P. neurophilia* can have implications for studies where other species than zebrafish are used. Furthermore, the results suggest that *P. neurophilia* exposure alone can affect study outcomes regardless of whether they succeed in establishing an infection or not. Thus, if researchers are using medaka (or possibly other fish species) that share facilities with infected zebrafish, they might obtain biased results, which can affect studies as described above. Hence, all the abovementioned scenarios highlight the importance of using standardised and proper health monitoring in fish facilities.

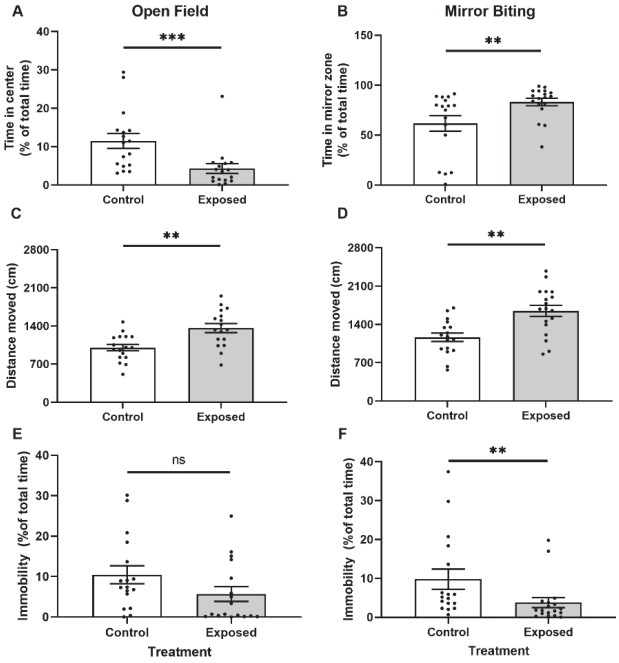

Figure 8: Medaka exposed to infectious *Pseudoloma neurophilia* spores or sham treatment. The behavioural tests (A, C, E) open field and (B, D, F) mirror biting suggest that *P. neurophilia* exposure increase (C, D, F) activity, (A) exploration and (B) sociability/aggression. *n* = 18 for both treatment groups. Mann-Whitney U test, ** = $p \leq 0.01$, *** = $p \leq 0.001$

6. Conclusion

This Thesis contributes to the growing body of literature highlighting how subclinical infections with the microsporidian parasite *P. neurophilia* affect a teleost host, the zebrafish (*Danio rerio*). These results provide a thorough examination of the influence this common parasite has on its host with regards to behavioural, transcriptomic, metabolic and neurophysiological aspects. Furthermore, the work in this Thesis also provides a more in-depth understanding on physiological and behavioural correlates of acute versus long-term infections. First, my findings provide evidence that long-term infected zebrafish display reduced activity and growth, suggesting a high cost of infection in the host. Second, I found long-term infection to result in an upregulation of genes associated with pro-inflammatory responses. However, I also detected downregulation of genes important for immune memory and autophagy, suggesting that, depending on life stage, *P. neurophilia* stimulates some immune responses, but simultaneously takes advantage of immune evasion strategies. Third, long-term infection with *P. neurophilia* leads to a marginal increase in metabolic rate. Fourth, long-term infection also results in increased activity following multiple handling stressors. On the other hand, acute exposure to new infectious spores increase the metabolic rate and monoaminergic activity in the first three days following infection. My findings taken together suggest that infection with *P. neurophilia* is costly for the zebrafish host. Where acute infection in naïve fish affect both neurophysiological and metabolic responses, long-term infection rather reduces activity and growth and induces a chronic pro-inflammatory response, resembling sickness behaviour. Sickness behaviour is often an acute response to infection, a response I now speculate can persist even in chronic infections, and which would be found in naïve zebrafish following acute infection as well. Intriguingly, the behavioural phenotype following long-term infection is also reminiscent of syndromes in response to chronic inflammation in other parasite-host systems, suggesting parasites to induce sickness behaviour, or a resembling syndrome, in both acute and long-term infections. Due to the increasing popularity of zebrafish as a model organism within numerous research fields, researchers should be aware of the implications such subclinical infections can have for their study outcomes. The findings obtained here provide strong evidence that *P. neurophilia* can

affect multiple study outcomes and highlights the importance of proper and standardised health monitoring of zebrafish facilities.

7. Future perspectives

The work in this Thesis has resulted in the identification of multiple new research questions, all of which should be addressed in future work. First, the studies on neurophysiological responses revealed that only acute infection in naïve zebrafish resulted in increased serotonergic and dopaminergic activity. I can speculate whether such alterations indicate that more severe, energy draining mechanisms are taking place in the early phase of infection. Tracking behaviour and studying gene expression in zebrafish in the days following acute exposure would give important insight into whether *P. neurophilia* does in fact induce sickness behaviour. This study should in fact be a comparative study, where the effects of *P. neurophilia* in medaka is examined simultaneously. Here, sampling of fish for neurochemical analysis, histology and gene expression at different timepoints throughout the study would highlight species-specific infection effects. Furthermore, behavioural studies should also be conducted at different timepoints after exposures. A positive control group (*e.g.* LPS injection) should be used to test how the fish react to "common" infections compared to *P. neurophilia* infections. This study would also allow researchers to examine effects in response to first and repeated exposures. Second, the results on activity in the respirometry chamber suggest a complex interplay between behaviour, *P. neurophilia* infection and stress, which is not explained by serotonergic or dopaminergic activity. Here it would be interesting to study the stress response by for example performing whole-body cortisol measurements on timepoints reflecting those used in the study. In addition, studying other monoaminergic responses, such as noradrenergic responses, could provide valuable insights to the interpretations of this specific behaviour. Third, by testing the same zebrafish before and after infection (e.g. behaviour and metabolism) one can obtain a better understanding of specific effects of the parasite and thus what implications infection might have for research outcomes. In addition, testing zebrafish infected with *P. neurophilia* in response to different drugs (*e.g.* inhibitory or excitatory) and tracking their behaviour would provide further insights. Lastly, multiple diagnostic tests for this parasite are lacking. For example, a non-lethal test would make it easier for researchers to obtain knowledge on the infection status. For example, being able to conduct the qPCR test for the presence of

P. neurophilia in tank water, would be ideal. Furthermore, a quantitative qPCR protocol could help reveal effects of infection intensity.

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9. Appendix: Papers I-III

Paper I

SCIENTIFIC REPORTS
nature research

OPEN

Behavioural effects of the common brain-infecting parasite *Pseudoloma neurophilia* in laboratory zebrafish (*Danio rerio*)

Helene L. E. Midttun✉, Marco A. Vindas, Lauren E. Nadler, Øyvind Øverli & Ida B. Johansen

Research conducted on model organisms may be biased due to undetected pathogen infections. Recently, screening studies discovered high prevalence of the microsporidium *Pseudoloma neurophilia* in zebrafish (*Danio rerio*) facilities. This spore-forming unicellular parasite aggregates in brain regions associated with motor function and anxiety, and despite its high occurrence little is known about how sub-clinical infection affects behaviour. Here, we assessed how *P. neurophilia* infection alters the zebrafish´s response to four commonly used neurobehavioral tests, namely: mirror biting, open field, light/dark preference and social preference, used to quantify aggression, exploration, anxiety, and sociability. Although sociability and aggression remained unaltered, infected hosts exhibited reduced activity, elevated rates of freezing behaviour, and sex-specific effects on exploration. These results indicate that caution is warranted in the interpretation of zebrafish behaviour, particularly since in most cases infection status is unknown. This highlights the importance of comprehensive monitoring procedures to detect sub-clinical infections in laboratory animals.

Model animal species (e.g., rodents, invertebrates and fish) are widely used in biomedicine, where study outcomes hinge on reproducibility of the results. Regular health monitoring of these animals has improved over time, as parasites and pathogens (*e.g.* microparasites, macroparasites, bacteria, viruses) are known to influence animal physiology, immune mechanisms, functional morphology, behaviour, and welfare[1,2]. However, monitoring procedures may fail to detect subclinical infections (*i.e.*, exhibiting no external signs of disease), in animals that appear otherwise healthy[3]. Thus, undetected infections can inadvertently bias results obtained from these studies, which has repercussions on many research areas, such as biomedicine. The scale of this issue is only just being uncovered. In rodents, for example, Pritchett-Corning *et al.*[4] reported the prevalence of sixteen commonly undetected pathogens in mice and rats from pharmaceutical, biotechnology, academic, and governmental institutions in North America and Europe. However, the practical impacts of these elusive infectious agents on frequently used experimental assays remain largely unknown.

Undetected parasites and pathogens can alter experimental results in model organisms in several ways. Many species of parasites seem to be particularly adapted to affect host neuroendocrine signalling and behaviour in ways which enhance parasite fitness[5–7], but other aspects of host phenotype are indeed also affected by infection. For example, the intracellular parasite *Wolbachia*, which is commonly found in laboratory *Drosophila* spp. colonies, can reduce host egg viability, confound host optimal trait expression (*i.e.*, intra-locus sexual conflict) and alter host circadian rhythms[8–10], all commonly measured traits in biomedical studies. Similar effects have been observed in rodent model systems. A common infectious agent in rodent facilities is the pathogen murine norovirus[4,11], which can induce tissue inflammation and activate cytokine signalling in murine macrophages[12–14]. In well-established model animal systems, like *Drosophila* spp. and rodents (*e.g. Mus musculus*, *Rattus norvegicus*), substantial efforts in recent years have focused on how common parasites and pathogens spread within and among laboratory facilities, as well as best practices to remove these infectious agents once established. This work has helped to successfully eliminate and prevent many infections from research facilities, improving both animal welfare and the reproducibility of study outcomes[15,16]. However, in newer model organism species (*e.g.*, zebrafish,

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Danio rerio; medaka, *Oryzias latipes*; goldfish, *Carassius auratus*), data on the prevalence of pathogens in laboratory colonies and their potential confounding effects remain limited.

The use of zebrafish as a model organism has boomed in recent years, first gaining momentum in the 1990's[17]. Due to the relatively short time period since zebrafish were introduced as a model organism, there is a scarcity of research on the pathogenesis of common infectious agents in this species. Furthermore, standard health monitoring programmes to prevent the introduction of pathogens in zebrafish facilities are not widely practiced[18–20]. In fact, many zebrafish facilities do not screen for pathogens. Further, sometimes zebrafish bought at commercial pet stores are introduced into zebrafish facilities without prior comprehensive pathogen screening[21]. One of the most common diagnoses in zebrafish submitted for health monitoring to the Zebrafish International Research Center (ZIRC) is infection with the microsporidian parasite *Pseudoloma neurophilia*. Depending on the year, more than 50% of these facilities test positive for *P. neurophilia* annually[22]. This parasite takes advantage of both horizontal (*i.e.*, transmission between conspecifics following contact) and vertical (*i.e.*, transmission from mother to offspring) transmission. Infection spreads mainly through ingestion of the infectious spore stage. Spores are released to the water from dead infected hosts or with feces and during spawning[23,24]. Infections are largely subclinical, and are often only detected in severe cases, when hosts develop spinal deformations and emaciation[25,26]. *Pseudoloma neurophilia* primarily infects the hindbrain and the spinal nerve roots of the spinal cord[27], areas commonly associated with motor function, freezing, fear-learning and anxiety[28,29]. Whether *P. neurophilia* alters emotional states like fear and anxiety in zebrafish has been suggested but remains little explored. If so, laboratories using zebrafish as animal model to study these emotional states could be critically affected by the presence of the parasite.

Recent studies report that *P. neurophilia*-infection alter startle responses (*i.e.* response to fearful stimuli)[30] and increase shoal cohesion (*i.e.* reduced inter-fish distances) in zebrafish[31]. These behavioural changes were interpreted as a parasite-induced increase in stress, fear and anxiety. It can, however, be challenging to extrapolate emotional states like fear and anxiety from behavioural outputs such as shoal cohesion. For example, the increase in shoal cohesion was interpreted as a stress/anxiety response to infection[31], but could might as well reflect increased sociability[32] or even a reduction in locomotion[33]. Thus, in order to understand how this parasite affects major behavioural outputs (*e.g.* anxiety, sociability, aggression) commonly studied by the zebrafish community, individual behavioural effects of parasite infection should be investigated across a range of contexts and preferably by using the most common neurobehavioral assays.

Here, we employed four commonly used tests to examine how *P. neurophilia* infection in zebrafish influences the following behavioural outputs: aggression, sociability and anxiety (*i.e.*, open field, mirror biting, light/dark preference, social preference). For all tests, we compared individual locomotor function and general activity in infected and uninfected fish. We hypothesised that *P. neurophilia* infection affects behavioural outputs associated with anxiety and/or sociability, given the location of the parasite in the hindbrain, and based on previous findings[27,30,31]. In the open field test (Fig. 1A), thigmotaxis (*i.e.*, maintaining proximity to the wall of an experimental arena) is quantified as a proxy for anxiety, while exploration (*i.e.*, moving in the centre of the arena) is interpreted as boldness[34,35]. In the mirror biting test (Fig. 1B), biting at or interacting with the mirror image is interpreted as aggression[36,37]. In the light-dark preference test (Fig. 1C), scototaxis (*i.e.*, aversion of bright places) is commonly interpreted as anxiety (Araujo *et al.*[38]). Lastly, the social preference test (Fig. 1D) assesses sociability, by examining an individual's tendency to associate with conspecifics versus remaining solitary[37]. In sum, there is potentially high overlap between the behavioural patterns and control systems typically addressed in biological studies, and those a parasite, pathogen, or components of the microbiome might adaptively target. Hence, by studying behavioural variation between infected and uninfected zebrafish, we can gain a better insight into how research outcomes vary with infection status, and the importance of identifying and characterizing pathogens vs commensals in model organisms.

Results

To verify infection status, we tested brain tissue from infected and uninfected fish for the presence of *P. neurophilia* by qPCR[39]. All tested fish in the parasite-exposed group tested positive for the parasite, including the random selection of fish tested already after 6 weeks of infection. Conversely, all tested fish from the uninfected treatment group were negative for infection (data pooled in Fig. S1). Fish harbouring *P. neurophilia* infection exhibited an approximately 13% lower body mass (Generalized linear model (GLM): $F_{1,115} = 14.41$, $p = 0.0002$, Fig. 2A) and 5% shorter body length (GLM: $F_{1,115} = 20.55$, $p = 0.0002$, Fig. 2B) than uninfected controls. However, Fulton's K condition factor (a weight-length relationship that is used as a health status indicator in fish[40]) was not altered by infection status ($p > 0.05$, Table S1, Fig. 2C). Although all measures of size and condition differed significantly between males and females, the interaction between sex and infection was not significant for any of these variables (Table S1).

***Pseudoloma neurophilia* infection has distinct effects on zebrafish behaviour.** Four neurobehavioral assays were conducted in which the behaviour of infected versus uninfected fish were compared (the experimental arenas used for each test are illustrated in Fig. 1). Complete details of all statistical outputs and sample sizes are summarized in Table S1 and S2.

Pseudoloma neurophilia altered time spent in the centre of the arena, *i.e.* exploration, in a sex-specific manner (Negative Binomial Generalized Linear Model, Infection*Sex Interaction: $\chi^2_1 = 3.92$, $p = 0.047$). Although uninfected and infected males exhibited similar responses, uninfected females spent substantially more time on average in the centre of the arena, *i.e.* displaying exploration, than infected females (Tukey post-hoc test: $p = 0.044$, Fig. 3A).

Infection did not alter the number of bites towards the mirror image in the mirror biting test, *i.e.*, aggression (Fig. S2a, Table S2). For both open field and mirror biting tests, *P. neurophilia* infected fish exhibited approximately three times more freezing behaviour, *i.e.*, moving less than one body length/second (Negative Binomial

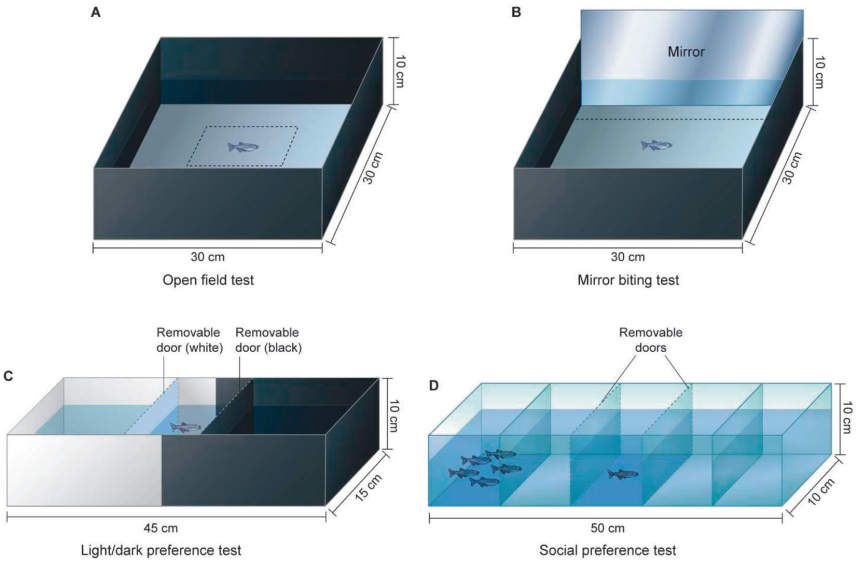

Figure 1. Behavioural test arenas. (**A**) In the open field test, zebrafish were transferred to the arena and allowed to explore for five minutes. The following behavioural outputs were measured: freezing, thigmotaxis and time spent in the centre of the arena. (**B**) In the mirror biting test, zebrafish interact with their mirror image, with aggression quantified as the amount of times the zebrafish attacks its own mirror image within six minutes. (**C**) For the light/dark preference test, zebrafish acclimated between removable doors for five minutes, before doors were removed. The fish was then able to move freely in the arena for 15 minutes. Crossings between compartments and scototaxis were measured. (**D**) In the social preference test, five conspecifics were placed in one chamber of the arena, while the chamber at the opposite end remained empty. The zebrafish acclimated for five minutes between the removable doors, before it was allowed to freely explore the arena for a total of 11 minutes. Time spent in all compartments and total distance moved were measured.

Generalized Linear Mixed-Effects Model, Infection: $\chi^2_1 = 5.40$, $p = 0.020$, Fig. 3B, Table S2), a trait typically associated with anxiety[41–43]. Neither aggression nor freezing behaviour differed significantly between the sexes (Table S2).

In the light-dark preference test, infection did not affect time spent in the white or dark compartment (Fig. S2b, Table S2). Both treatment groups spent on average approximately 50% of the trial period in each compartment. However, *P. neurophilia* infection reduced the number of crossings between compartments by five times (Zero-inflated Count Data Regression Model, Infection: $\chi^2_1 = 9.73$, $p = 0.002$, Fig. 3C), a trend typically associated with an overall decrease in locomotor activity[44].

Sociability, *i.e.*, the preference to remain close to conspecifics, was not affected by *P. neurophilia* in the social preference test (Fig. S2c, Table S2), with zebrafish spending 86% of their time in proximity to conspecifics on average. However, *P. neurophilia* infection affected locomotor activity, which was evident by a 13% reduction in distance moved relative to uninfected controls (Generalized Linear Model, Infection: $F_{1,34} = 5.49$, $p = 0.026$, Fig. 3D). There was no effect of sex on either sociability or distance moved in this test (Table S1 and S2).

Discussion

Our study demonstrates direct impacts of *P. neurophilia* infection on zebrafish behavioural responses to four commonly used tests in neurobehavioural studies. Therefore, this parasite could impact the reproducibility of study outcomes in a range of scientific fields, particularly because zebrafish infected with *P. neurophilia* exhibited reduced locomotor activity across a range of contexts. However, several behavioural traits were unchanged by infection, including sociability, aggression and thigmotaxis, indicating that in animals with unknown infection status, robust experimental results can still be gleaned with careful planning and analysis. These results illustrate the complex role of parasite infection in host behaviour and highlight the importance of examining behavioural phenotypes across several contexts to comprehensively characterize the impacts of parasite infection.

Infected individuals conducted fewer crossings between the white and black compartments in the light-dark preference test and moved a shorter distance in the social preference test, both commonly used indicators of locomotor activity[34,44,45]. These results are in line with previous literature on several host-parasite systems, in which parasite infection was associated with reduced locomotor activity[46–48]. Parasites can affect host locomotion through alterations in host morphology and/or physiology[49]. For example the trematode *Ascocotyle pachycystis* infects the heart and reduces swimming performances in the sheepshead minnow (*Cyprinodon variegatus*)[50]. In

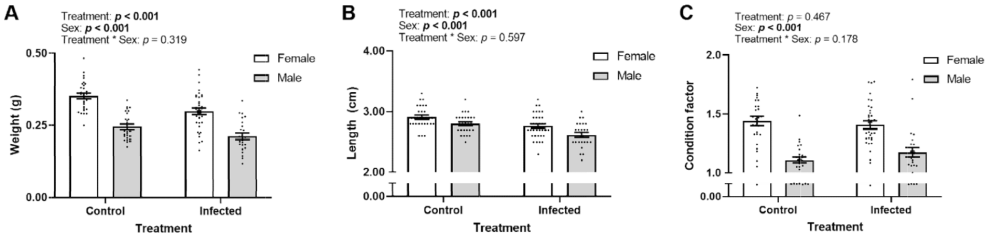

Figure 2. Weight (**A**), length (**B**) and Fulton's K condition factor (CF, **C**) of zebrafish (*Danio rerio*) experimentally infected with the microsporidian parasite *Pseudoloma neurophilia* ("Infected") or sham infected ("control") for 10 weeks. Weight and length were obtained post mortem, from which CF was calculated. All bars indicate the mean ± SEM (generalized linear model analysis). Dots indicate individual data points ($n_{control} = 57$, $n_{infected} = 60$). All graphs obtained using GraphPad Prism v8.3.1[69].

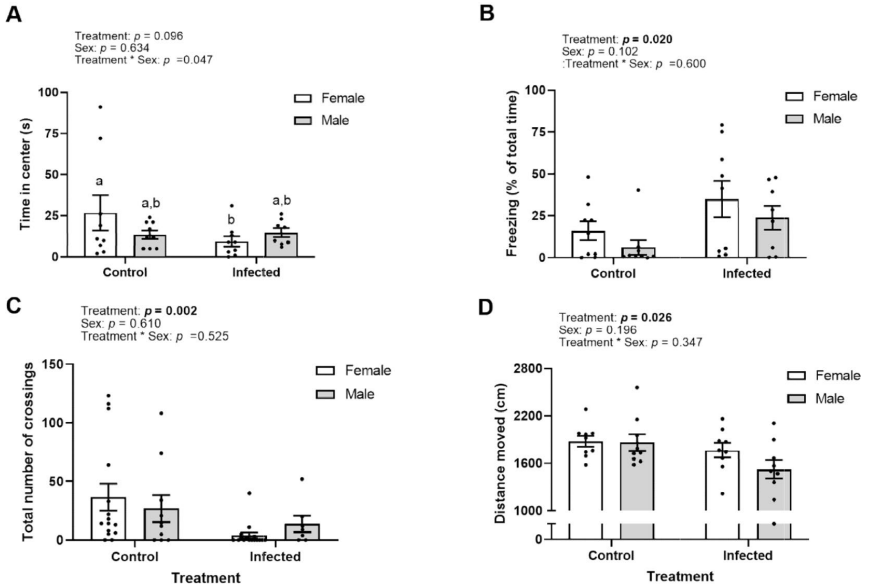

Figure 3. Behavioural effects of *Pseudoloma neurophilia*-infection in female and male experimentally infected zebrafish and uninfected controls. (**A**) Total time spent in the centre of the arena in the open field test (s ± SEM), p = 0.048, $n_{control} = 18$, $n_{infected} = 17$. (**B**) Average total time spent freezing as percent of all time in both the open field test and mirror biting test combined, *i.e.* 11 min (% ± SEM), p = 0.02, $n_{control} = 18$, $n_{infected} = 17$. (**C**) Total number of crossings between black and white compartment in the light/dark preference test (frequency ± SEM), p = 0.0018, n = 25 per treatment group. (**D**) Total distance moved in the social preference test (cm ± SEM), p = 0.026, n = 18 per treatment group. All graphs obtained using GraphPad Prism v8.3.1[69].

our study, we observed a reduction in body size (both length and mass) with infection, a trait that potentially could influence locomotion. However, Tran and Gerlai[45] found that individual differences in locomotor activity do not shift substantially with length or weight in zebrafish, and thus is unlikely to be a major driving factor in our observed effect on locomotion. Instead, *P. neurophilia* may directly affect locomotion by infecting nerve tracts controlling motor function[27,30], a scenario that deserves further investigation. Alternatively, a change in locomotor activity can be caused by the parasite's effect on host energy metabolism and immune or endocrine function. Indeed, reduced locomotion and growth in infected zebrafish can represent subtle symptoms of sickness behaviour (characterized by lethargy, anxiety and anorexia), an adaptive and organized behavioural strategy aimed at for example conserving energy[51].

Sickness behaviour is sometimes also characterized by anxiety[52]. Indeed, increased freezing behaviour observed with *P. neurophilia* infection in both the open field and the mirror biting tests (Fig. 3B) could reflect anxiety-like behaviour. Spagnoli *et al.*[27,30] recently reported that *P. neurophilia* infects brain areas associated with

anxiety and fear-learning[53]. However, freezing behaviour may not be a reliable measure of fear and anxiety in zebrafish since neither alarm pheromones nor the presence of predator cues increased freezing behaviour in zebrafish[43,54,55]. Alternatively, increased freezing in *P. neurophilia* infected zebrafish in our study could reflect immobility. In fact, the terms freezing behaviour and immobility are used interchangeably in the zebrafish literature and are difficult to differentiate[36]. Immobility is a well-known response to animal infection[56] and in line with our observations of a general reduction in locomotor activity (*i.e.*, crossings and distance moved, as described above).

If the increased freezing behaviour observed in the current study reflects a parasite-induced increase in anxiety-like behavior, we would expect reduced exploration (*i.e.* boldness) in the open field test. Although we found that *P. neurophilia* decreased exploration in female zebrafish hosts, exploration was not decreased in infected males. Exploration is a measure of boldness and risk-taking[57], here measured as the time spent in the center of the open field arena. Our results could thus indicate that infection induces a sex-specific decrease in boldness. As exploration and boldness are commonly used traits in research, including *e.g.* biomedical studies, undetected infections could have broad implications for study outcomes, particularly in studies that use either just one sex or an unequal number of males and females across treatments. These results are also in agreement with previous literature suggesting that parasite infection affects male and female zebrafish differently. For example, recent studies show that male zebrafish are more susceptible to *P. neurophilia* infection and suffer from greater parasite clusters than females[27,58]. Conversely, infected females are thinner than uninfected individuals, which is associated with a reduction in ovary size and egg development. In fact, *P. neurophilia* infection has been shown to reduce condition factor (length:width ratio) in female, but not male, zebrafish[59]. In the current study we calculated condition factor based on weight instead of width and did not observe sex-specific effects of *P. neurophilia* on condition. Nevertheless, potential sex-differentiated effects of infection could ultimately introduce uncontrolled variation into study outcomes, particularly when only one sex is employed, further reinforcing the need to carefully plan study design when infection status is unknown.

We found no change in sociability in response to infection. Microsporidia-infected zebrafish and sticklebacks (*Gasterosteus aculeatus*) have previously been shown to swim in more cohesive shoals than uninfected groups[31,60]. For example, Spagnoli *et al.*[31] found that *P. neurophilia* increases shoaling cohesion in zebrafish. Increased shoaling could reflect either stress/anxiety, increased sociability or reduced locomotion[31,32]. By studying sociability in the social preference test, we found that uninfected and infected zebrafish were uniformly social, spending 86% of their time on average with conspecifics. It is however possible that the increased shoal cohesion observed previously[31] could be the result of stress/anxiety or the general reduction in activity levels in infected zebrafish that we observe. In the Qingbo carp (*Spinibarbus sinensis*), shoal cohesion increases with decreasing locomotor activity, likely because individual repulsion radiuses increase at higher swimming speeds[33]. As illustrated by the above example, the fact that *P. neurophilia* appears to have a general effect on activity may obscure conclusions regarding the effect of this parasite on motivational or emotional states like sociability. Regardless of what mechanism increases shoal cohesion, it is tempting to speculate that a greater shoal cohesion could increase transmission rates for the parasite. Indeed, transmission of parasites with direct life cycles has previously been shown to increase with closer shoal formations in other taxa[61].

Taken together, our results illustrate that subclinical, and therefore often undetected infections, result in the alteration of behavioural outputs in a context and sex-specific manner. We show that infection alter locomotion but may also induce anxiety-like behaviour. Moreover, the parasite may also affect the behaviour of male and female hosts differently, with important implications for the reproducibility of results in studies using this model system. Since *P. neurophilia* infection appears to influence primarily energetically costly processes including growth (Fig. 2), locomotion (Fig. 3), fecundity[62] and gonad development[59], future studies should focus on effects of *P. neurophilia* infection on host energetics and characterise any costly biological processes which are stimulated by infection (*e.g.*, immune responses). Laboratory animals are still crucial for the scientific world, thus parasitic infections in zebrafish presents concerns for both animal welfare as well as reproducibility and hence impact of neurobehavioural studies.

Materials and Methods

Ethics. 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).

Fish husbandry. All experiments were performed at the Norwegian University of Life Sciences, campus Adamstuen (Oslo, Norway). Ten adult AB zebrafish (5 males and 5 females) were obtained from the Sinnhuber Aquatic Research Laboratory (SARL) at Oregon State University, a *P. neurophilia* specific pathogen free (SPF) facility. Fish were kept in a quarantine room in a 25 L tank (40 cm × 25 cm × 25 cm; L × W × H) for an acclimation period of two months. The tank was provided with filtered and UV-treated water. In addition, 50% of the water in the tank was changed twice weekly in order to further maintain high standards of water quality. Water temperature was maintained at 28 °C, pH and conductivity were kept at 7.4–7.6 and 500 µS respectively, following husbandry practices recommendations by ZIRC[63]. All fish were fed flake food twice daily (Special Diets Service, Witham, Great Britain) and live brine shrimp (Ocean Nutrition, Essen, Belgium) once per day. After two months, all fish were transferred to a standardized recirculation system, where they were kept at a density of 5 fish/L (Tecniplast, Buguggiate, Italy).

Once weekly, adult fish were placed overnight in standard 1 L crossing tanks for spawning (Techniplast, Buguggiate, Italy), with males placed at one side and females on the other side (with a 1:1 male:female ratio). The following morning, the divider was removed for up to four hours, allowing fish to spawn, according to ZIRC recommendations[63]. Following spawning, fish were placed back in their respective holding tanks and eggs

were collected. Eggs were rinsed with filtered and UV-treated water, counted and maintained in petri dishes (95 ×15 mm; Heger, Rjukan, Norway) at a density of 50 eggs/30 mL at 28 °C until 5 days post fertilization (dpf). During this period, water was changed, and dead eggs were removed daily. At five dpf, zebrafish larvae were transferred to 1 L plastic beakers (VWR, Radnor Pennsylvania, USA), at a density of 1 fish per 6 mL of filtered and UV-treated water. Two times per day larvae were fed freeze dried rotifers and small-grained dry food (Special Diets Service, Witham, United Kingdom). Water was changed daily. At 21 dpf, juvenile zebrafish were transferred to a recirculating aquarium system in which conditions (*i.e.* pH, salinity, temperature and water quality) and feeding routine were kept as described above. The light:dark cycle was always kept at 14 h light:10 h dark.

Infection protocol. At approximately 5 months post-hatch, 252 zebrafish were transferred from the F1 generation to an infection room. Here, the zebrafish were housed in 30 closed-tanks (23 ×15.3 ×16.5 cm, L x W x H) (Exo Terra, Montreal, Canada) at a density of 5 fish/L. We randomly assigned the zebrafish to treatment groups and tanks using a random number generator (https://www.random.org/), keeping a female:male ratio of 1:1 in each group. Water temperature was maintained at 26–28 °C and the water was aerated continuously, with 50% water changes conducted three times weekly and 100% water changes once biweekly. Concurrently, *P. neurophilia*-infected zebrafish from the Norwegian University of Life Sciences (NMBU) zebrafish facility were maintained in a 25 L tank (40 cm ×25 cm ×25 cm; L x W x H). The NMBU facilities did not test positive for *Mycobacterium* spp. during routine screenings of water samples and has no known history of other pathogens. Positive infection for *P. neurophilia* in zebrafish at the facility was tested via qPCR as described below.

In order to reach an infection prevalence of approximately 100% in the infected treatment, experimental infections were conducted over a 10-week period. During this time, 100 mL of the home tank water was replaced with 100 mL of water from the tank containing *P. neurophilia*-infected zebrafish on a daily basis. In addition, zebrafish were fed central nervous system (CNS) tissue from infected conspecifics four times during the course of the infection study (with a minimum of two weeks between feedings), according to the infection protocol outlined in Peneyra *et al.*[64]. Briefly, macerated CNS from infected fish was mixed with zebrafish food and subsequently fed to the study fish. In the same manner, control fish received water from a tank with spf fish and CNS tissue from uninfected fish. During the infection period, a total of 14 fish died (12 from infected and 2 from control groups). Six weeks into the infection protocol we tested for the presence of *P. neurophilia* by randomly selecting one fish from each tank (n = 15 per group) by euthanizing the fish in an overdose of Tricaine methanesulfonate (1 g/L; MS-222; Sigma, St. Louis Missouri, USA), before dissecting out the whole brain. The brains were excised within 3 min and rapidly frozen on dry ice, then stored at −80 °C until further qPCR analysis for the presence of *P. neurophilia*. In addition, brain tissue from approximately 80% of experimental fish (tested in behavioural trials) was similarly stored and analysed for the presence of *P. neurophilia* after behavioural testing. Fish were not screened for the presence of other pathogens, as the SPF fish obtained from SARL were maintained in quarantine from the NMBU facility´s other zebrafish after arrival (in a separate room) in a new tank and then in a recirculating system that had not been used for fish husbandry prior to this study.

DNA extraction and qPCR. Brain tissue from infected and uninfected fish was transferred to 50 µL MilliQ water (Merck, Darmstadt, Germany). Samples were sonicated for 2 minutes at 55 W (QSonica Sonicators, Connecticut Newtown, USA) and immediately placed on ice. The sonicator probe was decontaminated with 100% ethanol and MilliQ water between samples. The DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) was used to extract DNA according to manufacturer's protocol, with the addition of an overnight proteinase K and lysis buffer digestion at 56 °C, following the protocol outlined in Sanders and Kent[39]. Samples were then eluted in 100 µL storage buffer (provided in the kit). The qPCR protocol for analysis of infection status was established by Sanders and Kent[39]. Briefly, all reactions were performed in 25 µL, with forward and reverse primer concentrations of 900 nm each, 250 nM hydrolysis probe, 1X TaqMan and 2 µL DNA sample. Forward primer, reverse primers and hydrolysis probe used were 5′-GTAATCGCGGGCTCACTAAG-3′, 5′-GCTCGCTCAGCCAAATAAAC-3′ and 5′-6-carboxyfluorescein (FAM)-ACACACCGCCCGTCGTTATCGAA-3′-Black Hole Quencher 1 (BHQ1) respectively. The qPCR was performed using the following program: 50 °C for 2 minutes, 95 °C for 10 minutes followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 minute on a LightCycler 96 instrument and analysed using the LightCycler 96 software (Roche, Basel, Switzerland).

Primers are species-specific for *P. neurophilia*, thus all expression indicates presence of the parasite, however Cq-values above 38 were too considered negative.

Behavioural testing. Following 10 weeks of experimental infections, zebrafish were tested in one of the three test arenas described below. Each fish was only tested once. Only fish with subclinical infections (*i.e.* fish without scoliosis or any signs of emaciation) were used for behavioural testing. For all tests, behavioural experiments were video recorded from above, with arenas shielded from surroundings by black plastic while trials were conducted. All behavioural trials used filtered and UV-sterilised water maintained at 28 °C. Water was changed between each trial. All trials were performed between 09:00 and 14:00. All fish were euthanised immediately after each test as described above. Fish were measured for weight and length, which was used to calculate Fulton's K condition factor (100*(weight/length3)).

Open field and mirror biting test. To assess anxiety, exploration and aggression, we used a combination of protocols for the open field test[35] and for the mirror biting test[37]. It is generally assumed that, similar to rodents, zebrafish show a natural aversion for brightly lit open spaces, but simultaneously have a natural drive for exploring novel environments[65]. Thus, in the open field test, freezing behaviour (moving < 0.1 cm/s) and avoidance of the centre of arena is interpreted as anxiety-like behaviour. Conversely, visits to and time spent in centre of arena is classically interpreted as boldness and willingness to explore. In the mirror biting test, aggression is analysed

by quantifying time tracing the mirror, frequency of mirror bites (i.e. biting or butting head at own mirror image) and latency to first attack (i.e., time it takes for the fish to conduct its first bite towards mirror). We tested 18 control and 18 *P. neurophilia*-infected fish. The test was performed in an apparatus measuring 30 × 30 × 10 cm (W × L × D), with black walls and a white bottom (Fig. 1A,B). The apparatus was filled with 4 L of water. Fish were video-recorded for 5 minutes after being placed in the area. Following the initial 5 min, a mirror was placed at one side of the arena, and fish were left to interact with their mirror image for a total of 6 minutes, while continuing to be video-recorded.

Light/dark preference. In zebrafish, scototaxis (*i.e.*, the avoidance of bright places) is a behavioural correlate for anxiety[66], with increased time in dark being associated with increased anxiety. To test whether *P. neurophilia* affects this trait in zebrafish, we performed a light/dark preference test, following the protocol by Araujo *et al.*[38]. A total of 25 *P. neurophilia*-infected and 25 control fish were tested. The apparatus (15 ×45 ×10 cm, W x L x D) was divided vertically into a black and a white half with removable doors in corresponding colours to their location (Fig. 1C). The apparatus was filled with 4 L of water. Fish were individually moved to the central compartment (*i.e.*, between the removable doors) for a 5-min acclimation period, after which the doors were removed. Fish were then video-recorded for 15 mins. Water was changed between each trial. Time spent in white, freezing behaviour in white and total number of crossings between the dark and light compartments were recorded and analysed.

Social preference. Zebrafish actively form shoals, a trait that is attributed to social behaviour[67]. Thus, to test whether *P. neurophilia* affects sociability in its host, we performed a social preference test using the protocol developed by Pham *et al.*[37]. The protocol was followed with minor changes; briefly, a Plexiglas arena (10 × 50 × 15 cm, W × L × H) was divided into five compartments with transparent dividers. The three middle compartments were separated by removable dividers (Fig. 1D). The apparatus was filled with 3 L water and five zebrafish (three females, two males) were placed in one of the end-compartments, while the other end-compartment remained empty. The target fish used in this test originated from the F1 generation, were size-matched to the tested individuals and were not infected with *P. neurophilia*. A total of 18 *P. neurophilia*-infected and 18 uninfected fish was tested. Fish were individually placed in the central compartment and allowed to acclimate for 5 mins, after which the transparent dividers were removed. The fish's behaviour was then video-recorded for 6 mins. In between tests, water was changed and the right/left location for the target fish was alternated in order to avoid lateral bias. Time spent in each compartment and number of crossings between compartments were quantified in order to establish a proxy for social preference in infected and non-infected zebrafish (following methodology by Miller & Gerlai[67]).

Video analysis. Videos were manually analysed by a researcher blinded to the knowledge of specific treatments in order to avoid any bias. Biting (mirror biting test), number of entries to a zone (social preference test, light/dark preference test) and time spent freezing (open field test) were quantified manually. All zebrafish behaviour was furthermore tracked and quantified using Ethovision XT 13 (Noldus, Wageningen, The Netherlands).

Statistics. We conducted all statistical analysis in the R Statistical Environment v3.2.4[68], using the packages "lme4", "MASS", "pscl", "multcomp", "MuMin", and "car". For all models, to check that assumptions concerning normality and homoscedasticity were met, residual and quantile-quantile plots were inspected visually. For data that did not meet these assumptions, alternative distributions were used, as outlined below. Each model´s complete statistical output and R^2 are included in the supplementary material (Table S1).

All measurements of size and condition (weight, length, Fulton´s K condition factor) as well as total distance moved (social preference test) were analysed using generalized linear models, with treatment (infected, uninfected), sex (male, female) and their interaction included as explanatory variables. For the proportion of time spent with conspecifics (social preference test), number of bites at the mirror image (mirror bite test), and time in the centre (open field test), a negative binomial generalized linear model was used (to address overdispersion and non-normal distribution in the data), with treatment, sex and their interaction included as explanatory variables. Freezing behaviour (in the mirror bite and open field tests) was analysed using a negative binomial generalized linear mixed-effects model (to address overdispersion and non-normal distribution in the data), with treatment, sex, test (mirror bite, open field) and all associated interactions included as fixed effects and individuals included as a random effect (due to repeated measures). Crossings between compartments and time spent in the dark (light/dark preference test) were analysed using zero-inflated count data regression models (to address the high proportion of zeros for these traits), with treatment, sex and their interaction included as explanatory variables. Significant interaction effects were followed by Tukey's multiple comparisons post-hoc tests in order to ascertain significant differences between all groups.

Data availability

The datasets generated during and analysed during the current study are available in the NMBU Open Reseach Data repository, [https://dataverse.no/dataset.xhtml?persistentId=doi:10.18710/OD7M8N].

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Author contributions

I.B.J. and Ø.Ø. conceived the project. H.E.M., I.B.J., M.A.V. and Ø.Ø. 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 and analysis of data. L.N. and H.E.M. conducted statistical analysis. H.E.M., I.B.J. and L.N. wrote the paper. All authors edited the paper.

Competing interests

The authors declare no competing interests.

Additional information

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Paper II

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

Running head: Microsporidia infection and brain transcriptome

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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 down-regulated 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 down-regulation 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.

Key words: RNA sequencing, microsporidium, immune system, immune evasion, health monitoring

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 et al., 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 was first raised in the mid 1900's (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 manipulateion. There is, indeed, a rapidly expanding availability of genomic resources for this species (Lieschke and Currie, 2007, Kinth et al., 2013, 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 (Crim and Riley, 2012, Collymore et al., 2016, Marancik et al., 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 symptoms)(Matthews et al., 2001, Kent and Bishop-Stewart, 2003) 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 1980's (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 et al., 2015a, Spagnoli et al., 2017). Moreover, it negatively affects growth (Ramsay et al., 2009a, Sanders et al., 2020) and general activity (Midttun et al., in press). 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 et al., 2012) - do not appear to provoke severe inflammation (Spagnoli et al., 2015b). 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 et al., 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 as well as 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 and 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 protozoan infections on host phenotype.

Materials and methods

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).

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 25L glass tank (40cm x 25cm x 25cm; L x W x 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 1L 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 x 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

1L 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 h light:10 h dark.

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 x 15.3 x 16.5 cm; L x W x 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 (40cm x 25cm x 25; L x W x 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, 100mL of water from each tank was substituted with 100mL 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.

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 were 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 program was used: 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 15s, 60°C for 1 minute 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.

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 100µL RNAlater (Thermo Fisher Scientific, Waltham Massachusetts, USA) and immediately placed on dry ice before being stored at -80°C until further analysis.

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.

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 2100 Bioanalyzer and quantification by qPCR. Libraries were sequenced as 150bp, paired-end reads on an Illumina Hiseq 2500 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.

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 et al., 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).

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 et al., 2014) was used for 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 cutoff of less than 0.05 false-discovery adjusted (Benjamini-Hochberg) p-value. A fold change cutoff 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 were estimated using the clusterProfiler package v3.12.0(Yu et al., 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.

Results

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 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.

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 39701 genes were identified. For *P. neurophilia* infected zebrafish the total reads per sample ranged from 20.01-23.88 million (mean=21.81 million), while for uninfected controls the reads ranged from 21.58-22.32 million (mean=22.07 million). Mapping rate percentage, i.e. 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).

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, $p < 0.05$). 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 down-regulated 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.

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 signaling 1a
1.49	$2.7*10^{-4}$	447809	T cell activation RhoGTPase 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	Tumor 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	Tumor 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

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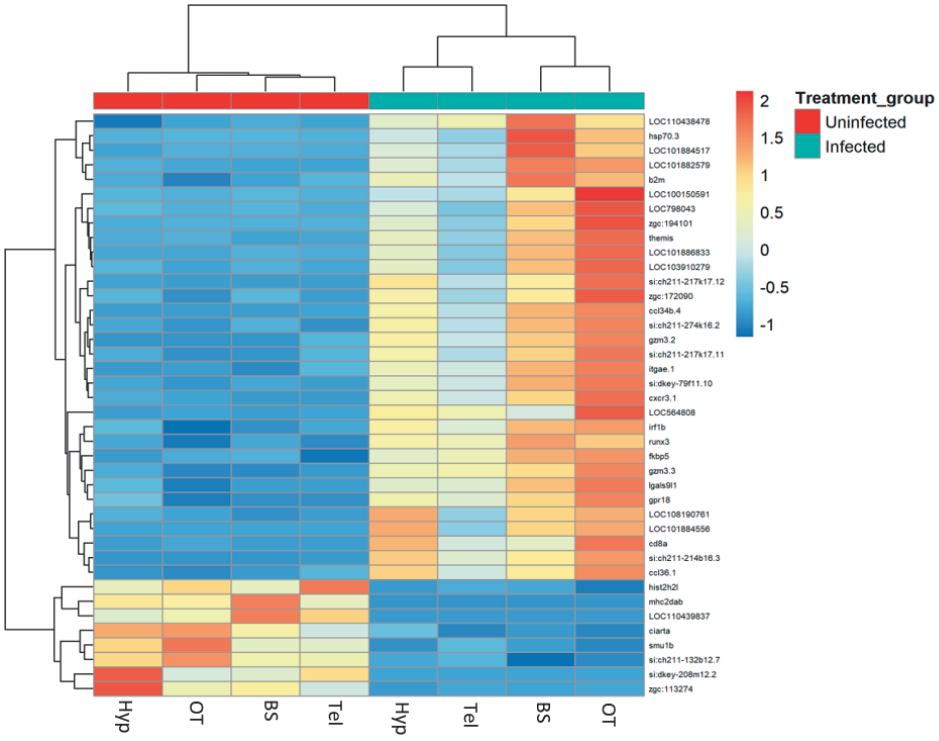


Figure 1. Heatmap of top 40 differentially expressed genes between zebrafish experimentally infected with the microsporidian parasite *Pseudoloma neurophilia* and uninfected controls. Brain parts from four control and four infected samples were compared, where Hyp = hypothalamus, BS = brain stem, OT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

KEGG over-representation analysis

Over-representation analysis identified four significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Table 2). Enriched pathways are grouping of genes participating in same cellular biological systems, containing an over-represented number of significantly DEGs in the analysed samples ($p < 0.05$) 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. 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 (Fig. 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).

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 ($p < 0.05$).

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
				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
				4.138	100322456	si:dkey-11f4.20
				1.511	557797	Integrin, beta 2
				1.705	793819	*CD40 ligand
				4.377	100006534	*cd8 beta
				5.903	677754	*CD8a molecule
				1.816	559154	Protein tyrosine phosphatase receptor type c
dre04060	Cytokine-cytokine receptor interaction	7	0.0085	3.213	108190761	*Tumor necrosis factor ligand superfamily member 14-like
				2.845	100135062	Colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage)
				3.956	798119	*TNF superfamily member 14
				3.675	405790	*Interferon gamma 1
				1.705	793819	*CD40 ligand
				2.336	654692	*Chemokine (C-X-C) receptor 3, tandem duplicate 1
				6.192	101887143	*CD27 molecule
dre05168	Herpes simplex virus 1 infection	6	0.0302	3.213	108190761	*Tumor necrosis factor ligand superfamily member 14-like
				3.956	798119	*TNF superfamily member 14
				1.366	30400	Beta-2-microglobulin
				3.675	405790	*Interferon gamma 1
				-8.324	30762	*Major histocompatibility complex class II DAB gene
				0.716	100034470	TAP binding protein (tapasin), tandem duplicate 2

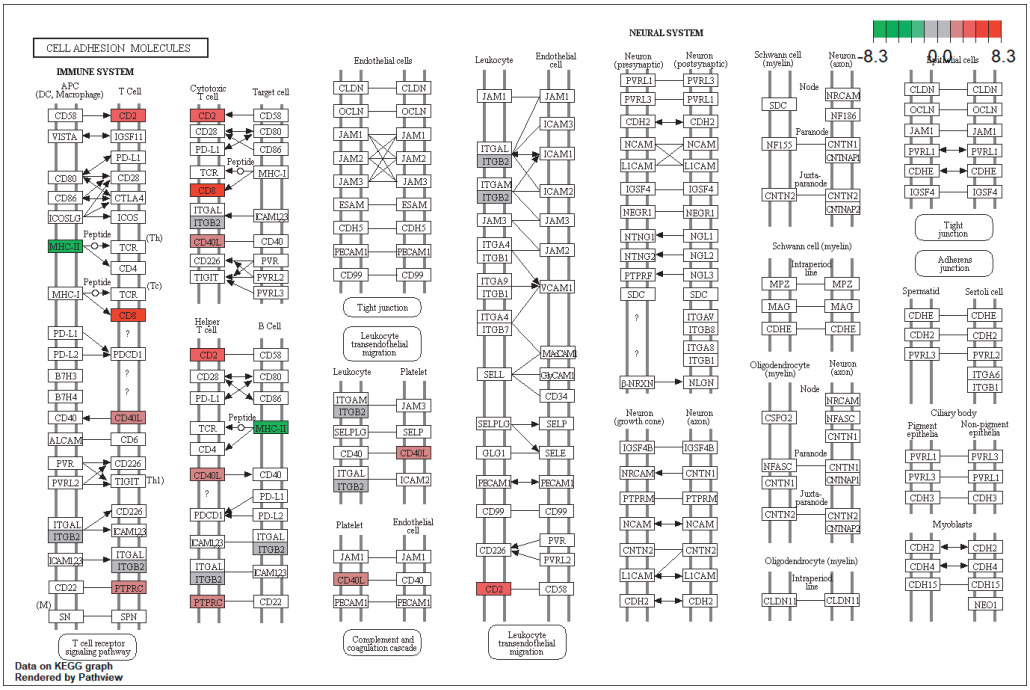
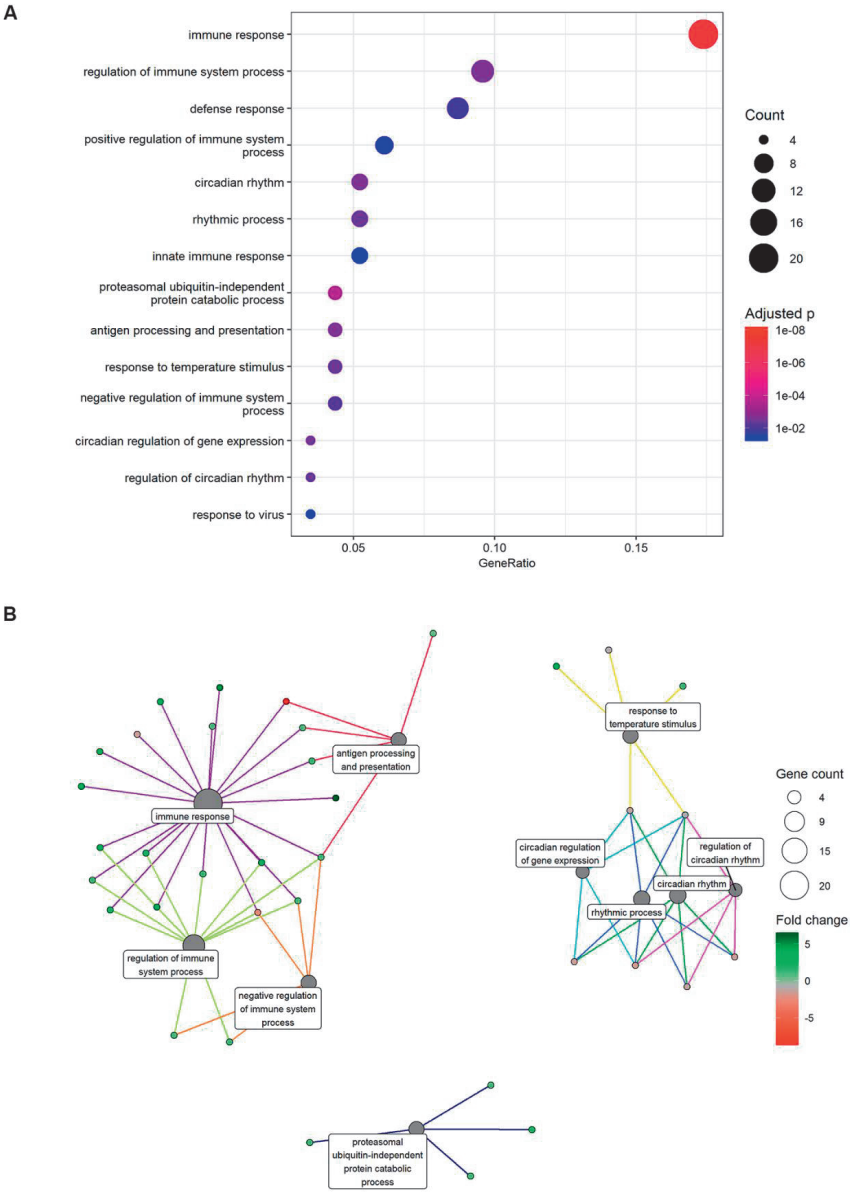


Figure 2: Kyoto Encyclopedia of Genes and Genomes (KEGG) map for the cellular pathway "cell adhesion molecules" containing significantly differentially expressed genes in brain tissue of laboratory zebrafish experimentally infected with the microsporidian parasite *Pseudoloma neurophilia* versus uninfected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

Gene ontology over-representation test

To identify biological processes affected by *P. neurophilia*, a Gene Ontology (GO) over-representation analysis was performed by 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 (Fig. 3a). Genes associated with immune responses were generally upregulated, such as *cd27*, interferon gamma 1 (*ifng1*), TNF superfamily member 14 (*tnfsf14*), *mhclzba*. Genes associated with circadian rhythm were mostly downregulated, for example the genes *period*

293 *circadian clock 1b* (*per1b*) and *nuclear receptor subfamily, group d, member 1* (*nr1d1*) (Fig.3b). All GO
294 terms and genes affected can be found in Table S3.


A
immune response
regulation of immune system process
defense response
positive regulation of immune system process
circadian rhythm
rhythmic process
innate immune response
proteasomal ubiquitin-independent protein catabolic process
antigen processing and presentation
response to temperature stimulus
negative regulation of immune system process
circadian regulation of gene expression
regulation of circadian rhythm
response to virus
0.05
0.10
0.15
GeneRatio
Count
4
8
12
16
20
Adjusted p
1e-08
1e-06
1e-04
1e-02
B
antigen processing and presentation
immune response
regulation of immune system process
negative regulation of immune system process
response to temperature stimulus
circadian regulation of gene expression
regulation of circadian rhythm
circadian rhythm
rhythmic process
proteasomal ubiquitin-independent protein catabolic process
Gene count
4
9
15
20
Fold change
5
0
-5

295

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.

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 and 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 et al., 2011). Because MHC II is important for antigen presentation and hence clearance of pathogens (Forsyth and 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.

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 and Jakobsen, 2000). CD8 has been found to play a similar crucial role in teleost species (Fischer et al., 2006, Somamoto et al., 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* (Yasuike et al., 2010, Kato et al., 2013), 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 et al., 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 proinflammatory mechanism used to eliminate or control invading pathogens is for T lymphocytes 1 cells (Th1) to secrete cytokines such as interferon gamma (IFNγ) or tumor necrosis factor alpha (TNFα) (Slifka and 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 proinflammatory immune response against *P. neurophilia*.

In contrast to the general up-regulation 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 et al., 2014). Interestingly, infection with the intracellular parasite *Toxoplasma gondii* downregulates MHC II in rodent hosts in order to evade the immune response (Lüder et al., 1998). A similar strategy appears to be employed by the Epstein Barr Virus, the poxvirus Vaccinia and the Hepatitis C virus (Forsyth and 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 and 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 et al., 2015b). Furthermore, interference with MHC II function could contribute to chronic infections despite activation of a strong Th1 immune response.

Circadian rhythm

Zebrafish infected with *P. neurophilia* displayed a down-regulation of multiple genes important for circadian rhythm as shown by the GO over-representation test. The circadian clock is a temporal 24-hour 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 and Merrow, 2016). Continued disturbance of the circadian clock has been associated with cancer, diabetes and autoimmunity (Bass and 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 et al., 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 et al., 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 (Lundkvist et al., 2010, Rijo-Ferreira et al., 2018, Carvalho Cabral et al., 2019).

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 and Øverli, 2012), resulting in conspicuous swimming behaviour (Lafferty and 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 and Schwarz, 2012). Considering that the parasite induce inflammation in the brain (Spagnoli et al., 2015b) and that previous studies (Spagnoli et al., 2015a, Spagnoli et al., 2017), as well as our own research (Midttun et al., in press) show that *P. neurophilia*-infected and 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 et al., 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.

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 et al., 2009b, Spagnoli et al., 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* show reduced

activity in several common zebrafish behavioural tests (Midttun et al., in press). Reduced activity in response to infection may reflect sickness behaviour which is mediated by host-induced upregulation of cytokines like TNFα and INFγ (Dantzer et al., 2008, Kirsten et al., 2018a, Kirsten et al., 2018b). Thus, increased expression of cytokines in the current study support 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.

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 proinflammatory Th1 response. In addition, the parasite down-regulates 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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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

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. Additionally, the dataset generated and analysed during the current study is available in supplementary data.

Competing interests

The authors declare no competing interests.

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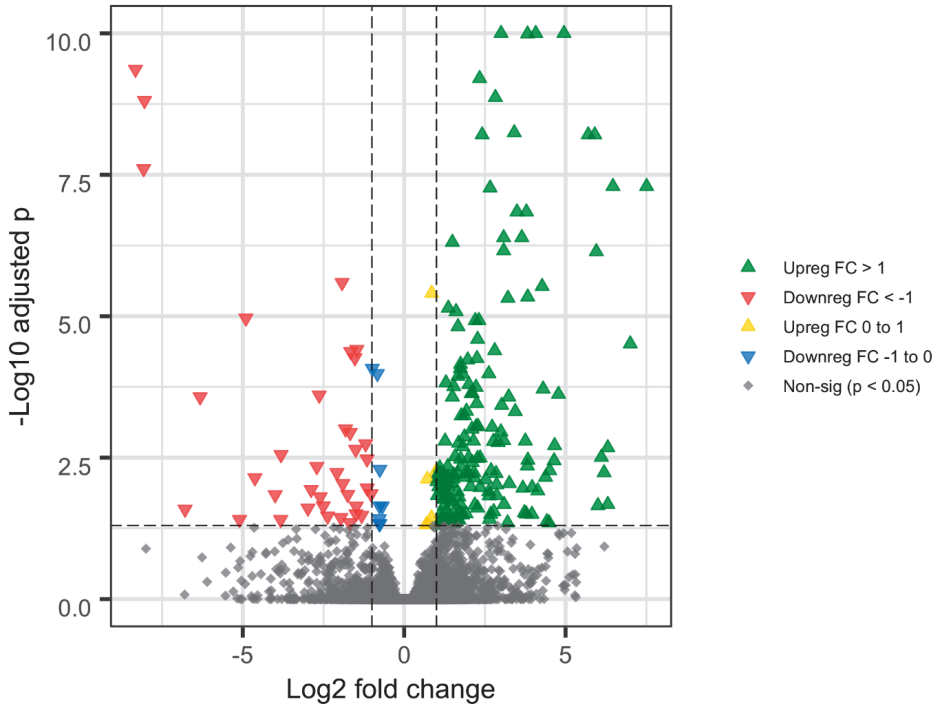
Supplementary data

Table S1: Summary of RNA sequencing mapping data of zebrafish infected with *Pseudoloma neurophilia* (I 1-4) and uninfected controls (C 1-4). Table shows distribution of reads and over-all mapping rate of each sample used for analysis.

Sample ID	**Total reads (10^6)**	**Uniquely mapped reads**		**Multi-mapped reads**		**Overall mapping rate (%)**
		Per sample	Percent	Per sample	Percent	
Control, Hypothalamus	21.75	15787146	73%	2210822	10%	89%
Control, Brain stem	22.32	15889261	71%	2518005	11%	88%
Control, Optic tectum	22.62	16396903	72%	2349529	10%	89%
Control, Telencephalon	21.58	15652746	73%	2136972	10%	89%
Infected, Hypothalamus	23.66	17019243	72%	2480451	10%	89%
Infected, Brain stem	20.01	14620075	73%	2232260	11%	90%
Infected, Optic tectum	22.69	16705039	74%	2383420	11%	90%
Infected, Telencephalon	20.88	15299436	73%	2079941	10%	89%



633

634 **Figure S1**: Volcano plot showing fold changes in gene expression in brain tissue of zebrafish
635 experimentally infected with *P. neurophilia* vs. uninfected controls. Out of 220 differently expressed
636 genes, 175 were upregulated (green and yellow) and 45 were downregulated (red and blue).

Table S2. Significantly differentially expressed genes in response to infection with the microsporidian parasite *Pseudoloma neurophilia* in zebrafish, compared to uninfected controls.

Gene ID	Log2FC	p-value	Adjusted p-value	Gene_symbol	Description
564346	4,07	2.8e-41	6.2e-37	si:ch211-214b16.3	si:ch211-214b16.3
556621	4,95	6.9e-39	7.5e-35	ccl34b.4	chemokine (C-C motif) ligand 34b, duplicate 4
100007523	3,00	6.1e-16	4.4e-12	si:dkey-79f11.10	si:dkey-79f11.10
30671	3,82	1.9e-14	1.0e-10	hsp70.3	heat shock cognate 70-kd protein, tandem duplicate 3
30762	-8,32	1.0e-13	4.3e-10	mhc2dab	major histocompatibility complex class II DAB gene
654692	2,34	1.7e-13	6.3e-10	cxcr3.1	chemokine (C-X-C motif) receptor 3, tandem duplicate 1

100537088	2,83	4.4e-13	1.4e-09	ccl36.1	chemokine (C-C motif) ligand 36, duplicate 1
110439837	-8,04	5.7e-13	1.5e-09	LOC110439837	uncharacterized LOC110439837
555433	3,41	2.4e-12	5.7e-09	themis	thymocyte selection associated
798684	5,70	3.4e-12	6.2e-09	zgc:194101	zgc:194101
100001138	2,41	3.1e-12	6.2e-09	gzm3.3	granzyme 3, tandem duplicate 3
677754	5,90	3.2e-12	6.2e-09	cd8a	CD8a molecule
100538282	-8,07	1.5e-11	2.5e-08	si:dkey-208m12.2	si:dkey-208m12.2
559555	7,51	3.3e-11	5.1e-08	si:ch211-217k17.12	si:ch211-217k17.12
564808	6,46	3.5e-11	5.1e-08	LOC564808	uncharacterized LOC564808
101882579	2,66	4.0e-11	5.4e-08	LOC101882579	sterile alpha motif domain-containing protein 9-like
100001065	3,79	1.2e-10	1.4e-07	gzm3.2	granzyme 3, tandem duplicate 2
101884517	3,49	1.2e-10	1.4e-07	LOC101884517	uncharacterized LOC101884517
101886833	3,08	3.7e-10	4.1e-07	LOC101886833	C-X-C motif chemokine 11-like
100002907	3,64	3.7e-10	4.1e-07	si:ch211-274k16.2	si:ch211-274k16.2
337597	1,49	4.8e-10	4.9e-07	lgals9l1	lectin, galactoside-binding, soluble, 9 (galectin 9)-like 1
798849	3,08	7.0e-10	7.0e-07	si:ch211-217k17.11	si:ch211-217k17.11
100150591	5,95	7.6e-10	7.2e-07	LOC100150591	immunoglobulin kappa variable 1-9-like
386920	-1,93	2.8e-09	2.6e-06	hist2h2l	histone 2, H2, like
103910279	4,27	3.4e-09	3.0e-06	LOC103910279	Ig mu chain C region membrane-bound form-like
368924	0,85	4.7e-09	3.9e-06	fkbp5	FKBP prolyl isomerase 5
798043	3,83	5.7e-09	4.6e-06	LOC798043	immunoglobulin C1-set domain
108190761	3,21	6.2e-09	4.8e-06	LOC108190761	tumor necrosis factor ligand superfamily member 14-like
30400	1,37	9.6e-09	7.2e-06	b2m	beta-2-microglobulin
792160	1,61	1.1e-08	8.3e-06	irf1b	interferon regulatory factor 1b
552925	-4,90	1.5e-08	1.1e-05	zgc:113274	zgc:113274
565611	2,31	1.7e-08	1.2e-05	zgc:172090	zgc:172090
100333951	2,20	1.8e-08	1.2e-05	itgae.1	integrin, alpha E, tandem duplicate 1
110438478	1,67	2.4e-08	1.5e-05	LOC110438478	obg-like ATPase 1
556711	2,27	4.1e-08	2.6e-05	gpr18	G protein-coupled receptor 18
101884556	7,00	5.1e-08	3.1e-05	LOC101884556	interferon-induced very large GTPase 1-like

564009	-1,47	6.6e-08	3.9e-05	ciarta	circadian associated repressor of transcription a
58127	2,80	7.1e-08	4.1e-05	runx3	RUNX family transcription factor 3
570074	-1,66	7.6e-08	4.2e-05	smu1b	SMU1 DNA replication regulator and spliceosomal factor b
564531	-1,53	1.0e-07	5.4e-05	si:ch211-132b12.7	si:ch211-132b12.7
100331542	2,25	1.1e-07	5.6e-05	si:dkey-260g12.1	si:dkey-260g12.1
64280	1,97	1.1e-07	5.9e-05	psmb13a	proteasome subunit beta 13a
798492	1,75	1.4e-07	7.1e-05	rasal3	RAS protein activator like 3
101882060	1,74	1.7e-07	8.4e-05	LOC101882060	protein NLRC3-like
171477	-0,99	1.7e-07	8.4e-05	rbp5	retinol binding protein 1a, cellular
393155	-0,83	2.2e-07	1.0e-04	osgn1	oxidative stress induced growth inhibitor 1
561000	2,62	2.2e-07	1.0e-04	LOC561000	tumor necrosis factor receptor superfamily member 5-like
793246	1,81	2.3e-07	1.1e-04	si:ch211-132p1.3	si:ch211-132p1.3
110439622	1,67	2.6e-07	1.2e-04	LOC110439622	protein NLRC3-like
30647	1,29	3.4e-07	1.5e-04	psme2	proteasome activator subunit 2
101883796	2,00	3.8e-07	1.6e-04	LOC101883796	adhesive plaque matrix protein-like
64279	1,54	4.2e-07	1.7e-04	psmb12	proteasome subunit beta 12
30666	2,23	4.4e-07	1.8e-04	psmb8a	proteasome subunit beta 8A
558217	4,30	4.8e-07	1.9e-04	fbn2a	fibrillin 2a
108179126	2,08	6.0e-07	2.3e-04	LOC108179126	GTPase IMAP family member 8-like
108183929	2,11	5.9e-07	2.3e-04	LOC108183929	gastrula zinc finger protein XlCGF8.2DB-like
101884895	4,78	6.3e-07	2.4e-04	igl4v8	immunoglobulin light 4 variable 8
794824	-2,63	6.6e-07	2.5e-04	lgals17	galectin 17
101886839	-6,32	7.5e-07	2.7e-04	LOC101886839	uncharacterized LOC101886839
447809	1,49	7.3e-07	2.7e-04	tagapb	T cell activation RhoGTPase activating protein b
108179354	3,24	7.5e-07	2.7e-04	LOC108179354	uncharacterized LOC108179354
108190699	2,25	1.0e-06	3.5e-04	LOC108190699	transposon Tf2-1 polyprotein
795805	3,02	1.1e-06	3.7e-04	si:dkey-222h21.9	si:dkey-222h21.9
796649	1,93	1.4e-06	4.8e-04	si:ch211-114l13.9	si:ch211-114l13.9
101886501	3,44	1.4e-06	4.8e-04	LOC101886501	uncharacterized LOC101886501
393651	1,77	1.8e-06	5.8e-04	grap2b	GRB2 related adaptor protein 2b

100330830	1,86	1.8e-06	5.8e-04	nbeal2	neurobeachin-like 2
100538332	2,29	2.8e-06	8.9e-04	LOC100538332	uncharacterized LOC100538332
100004948	2,23	2.8e-06	8.9e-04	si:ch211-153b23.7	si:ch211-153b23.7
103909973	2,72	2.9e-06	9.1e-04	LOC103909973	interferon-induced protein 44
101883850	-1,82	3.3e-06	9.9e-04	LOC101883850	5-hydroxytryptamine receptor 3C-like
565810	2,10	3.3e-06	9.9e-04	prkd4	protein kinase D4
101884253	3,00	3.7e-06	1.1e-03	LOC101884253	Fc receptor-like protein 4
494487	-1,67	3.9e-06	1.1e-03	nr1d1	nuclear receptor subfamily 1, group d, member 1
103910066	1,87	4.7e-06	1.4e-03	LOC103910066	B- and T-lymphocyte attenuator-like
101886584	3,08	5.5e-06	1.6e-03	si:ch211-66k16.2	si:ch211-66k16.2
567656	3,75	5.7e-06	1.6e-03	LOC567656	C-X-C motif chemokine 11-6-like
30665	1,27	5.8e-06	1.6e-03	psmb9a	proteasome subunit beta 9a
100006475	2,14	5.8e-06	1.6e-03	zgc:171500	zgc:171500
323739	2,76	6.0e-06	1.6e-03	selenou1a	selenoprotein U 1a
108179300	2,91	6.4e-06	1.7e-03	LOC108179300	uncharacterized LOC108179300
557062	1,69	6.6e-06	1.8e-03	fmnl1a	formin-like 1a
100535864	-1,19	6.9e-06	1.8e-03	cipca	CLOCK-interacting pacemaker a
60652	4,66	7.5e-06	1.9e-03	nitr4a	novel immune-type receptor 4a
559154	1,82	7.9e-06	2.0e-03	ptprc	protein tyrosine phosphatase receptor type C
101885359	6,31	8.3e-06	2.1e-03	si:cabz01030277.1	si:cabz01030277.1
449794	-1,50	9.1e-06	2.3e-03	h2afx1	H2A histone family member X1
100149148	-3,82	1.1e-05	2.8e-03	myha	myosin, heavy chain a
568891	1,58	1.2e-05	3.0e-03	si:ch211-79k12.1	si:ch211-79k12.1
60647	6,11	1.3e-05	3.1e-03	nitr2b	novel immune-type receptor 2b
100332077	2,00	1.3e-05	3.2e-03	cabz01076234.2	cabz01076234.2
100001210	2,35	1.3e-05	3.2e-03	gzm3.4	granzyme 3, tandem duplicate 4
393607	2,27	1.4e-05	3.3e-03	apbb1ip	amyloid beta (A4) precursor protein-binding, family B, member 1 interacting protein
100334847	1,75	1.4e-05	3.3e-03	LOC100334847	uncharacterized LOC100334847
558788	-1,15	1.5e-05	3.3e-03	si:ch211-233m11.1	si:ch211-233m11.1

101884219	3,83	1.5e-05	3.5e-03	crtam	cytotoxic and regulatory T-cell molecule
100330485	4,64	1.6e-05	3.6e-03	LOC100330485	uncharacterized LOC100330485
567964	2,11	1.7e-05	3.9e-03	si:ch211-114l13.10	si:ch211-114l13.10
564884	3,81	2.0e-05	4.4e-03	si:ch211-271e10.3	si:ch211-271e10.3
103910799	1,35	2.1e-05	4.6e-03	LOC103910799	gastrula zinc finger protein XlCGF57.1-like
555303	-2,71	2.1e-05	4.6e-03	si:dkeyp-118h9.7	si:dkeyp-118h9.7
368901	1,76	2.1e-05	4.6e-03	si:ch211-214p16.1	si:ch211-214p16.1
436849	1,10	2.3e-05	4.8e-03	rida	reactive intermediate imine deaminase A homolog
406293	-0,76	2.4e-05	5.1e-03	tcp11l2	t-complex 11, testis-specific-like 2
795887	2,69	2.6e-05	5.4e-03	LOC795887	interferon-induced protein 44
100148259	4,51	2.6e-05	5.4e-03	sh2d1ab	SH2 domain containing 1A duplicate b
791453	0,98	2.8e-05	5.7e-03	mhc1zba	major histocompatibility complex class I ZBA
368967	2,16	2.9e-05	5.8e-03	trac	T cell receptor alpha constant
325675	-2,08	3.0e-05	5.8e-03	col1a1b	collagen, type I, alpha 1b
101887143	6,19	2.9e-05	5.8e-03	cd27	CD27 molecule
101886774	1,93	3.0e-05	5.8e-03	LOC101886774	prolyl endopeptidase-like
100135062	2,85	3.2e-05	6.0e-03	csf2rb	colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage)
570229	1,65	3.2e-05	6.0e-03	si:ch211-250k18.8p	si:ch211-250k18.8, pseudogene
335573	1,11	3.2e-05	6.0e-03	ptpn6	protein tyrosine phosphatase non-receptor type 6
558211	1,06	3.1e-05	6.0e-03	dennd4a	DENN/MADD domain containing 4A
794894	2,00	3.2e-05	6.1e-03	si:dkey-222h21.2	si:dkey-222h21.2
30645	1,20	3.4e-05	6.3e-03	cd74b	CD74 molecule, major histocompatibility complex, class II invariant chain b
100126123	1,53	3.4e-05	6.4e-03	hsp70.1	heat shock cognate 70-kd protein, tandem duplicate 1
569162	1,37	3.8e-05	7.0e-03	si:dkey-33i11.1	si:dkey-33i11.1
100034482	1,85	3.9e-05	7.0e-03	si:dkey-242h9.3	si:dkey-242h9.3
100006534	4,38	3.9e-05	7.0e-03	cd8b	cd8 beta
101886666	2,17	4.1e-05	7.2e-03	pik3r6b	phosphoinositide-3-kinase, regulatory subunit 6b
791449	1,33	4.1e-05	7.2e-03	zgc:113363	zgc:113363
100537095	-4,63	4.1e-05	7.2e-03	aipl1	aryl hydrocarbon receptor interacting protein-like 1

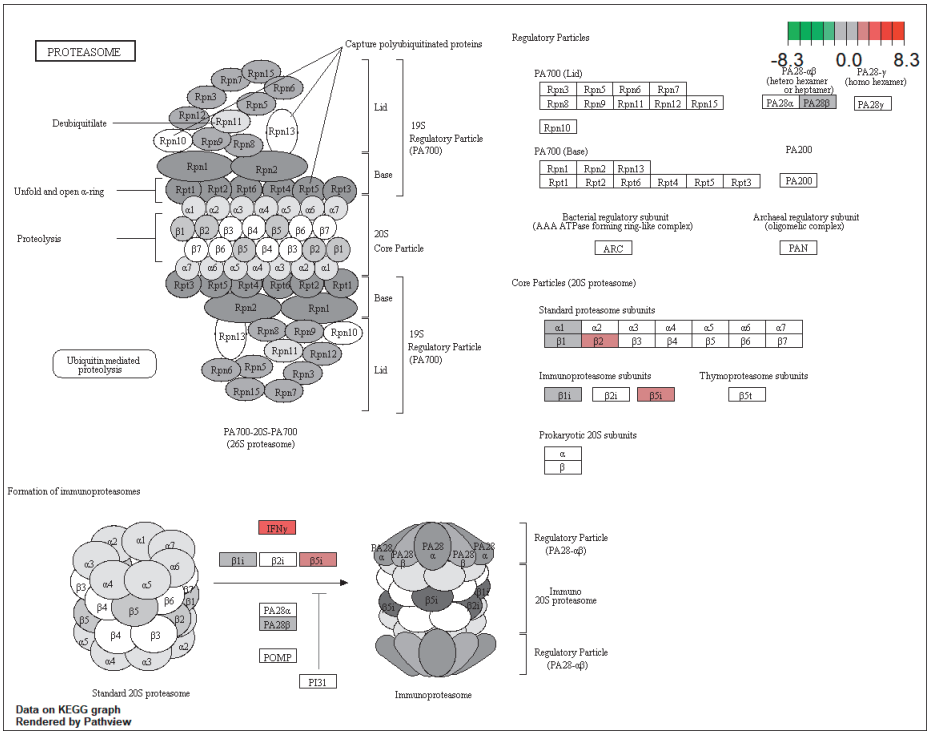
110439470	1,02	4.2e-05	7.4e-03	LOC110439470	uncharacterized LOC110439470
100535428	1,08	4.3e-05	7.4e-03	LOC100535428	NACHT, LRR and PYD domains-containing protein 3-like
557797	1,51	4.4e-05	7.5e-03	itgb2	integrin, beta 2
110439915	1,14	4.5e-05	7.6e-03	LOC110439915	uncharacterized LOC110439915
100034470	0,72	4.5e-05	7.6e-03	tapbp.2	TAP binding protein (tapasin), tandem duplicate 2
360145	2,53	4.6e-05	7.7e-03	mxc	myxovirus (influenza virus) resistance C
562542	1,02	5.0e-05	8.3e-03	LOC562542	uncharacterized LOC562542
563036	1,45	5.4e-05	8.9e-03	LOC563036	uncharacterized LOC563036
393830	3,26	5.5e-05	9.1e-03	tor1l3	torsin family 1 like 3
100334422	1,36	5.6e-05	9.1e-03	LOC100334422	E3 ubiquitin-protein ligase RNF12-B-like
110439533	-1,90	5.7e-05	9.1e-03	LOC110439533	endochitinase A1-like
336478	2,86	5.9e-05	9.5e-03	si:dkey-92i15.4	si:dkey-92i15.4
83917	1,24	6.3e-05	9.9e-03	psma6l	proteasome subunit alpha 6, like
555805	1,34	6.5e-05	1.0e-02	vaspa	vasodilator stimulated phosphoprotein a
100003547	1,07	6.6e-05	1.0e-02	arhgap15	Rho GTPase activating protein 15
492817	2,05	6.6e-05	1.0e-02	vps53	VPS53 subunit of GARP complex
492587	3,96	6.9e-05	1.1e-02	si:dkeyp-87d8.8	si:dkeyp-87d8.8
100003654	2,35	6.9e-05	1.1e-02	si:dkey-58f10.11	si:dkey-58f10.11
405790	3,68	7.2e-05	1.1e-02	ifng1	interferon gamma 1
563771	-1,15	7.2e-05	1.1e-02	bhlhe41	basic helix-loop-helix family, member e41
108183501	1,19	7.5e-05	1.1e-02	LOC108183501	E3 ubiquitin-protein ligase TRIM39
387299	-2,88	7.8e-05	1.2e-02	p2rx8	purinergic receptor P2X, ligand-gated ion channel, 8
100322456	4,14	8.2e-05	1.2e-02	si:dkey-11f4.20	si:dkey-11f4.20
797491	1,65	8.6e-05	1.3e-02	itgb7	integrin, beta 7
798906	2,62	8.6e-05	1.3e-02	itk	IL2 inducible T cell kinase
100001718	1,17	8.8e-05	1.3e-02	si:ch211-108p6.4	si:ch211-108p6.4
100126101	-1,03	9.6e-05	1.4e-02	zgc:171220	zgc:171220
553776	1,34	9.8e-05	1.4e-02	calcoco2	calcium binding and coiled-coil domain 2
100334291	-1,75	1.0e-04	1.4e-02	muc5d	mucin 5d
541372	-4,00	1.0e-04	1.4e-02	phf23a	PHD finger protein 23a
100538290	2,87	1.1e-04	1.5e-02	si:ch1073-15f19.2	si:ch1073-15f19.2
100141355	1,03	1.1e-04	1.5e-02	zgc:171497	zgc:171497

100007467	2,63	1.1e-04	1.5e-02	LOC100007467	interaptin-like
568448	1,28	1.1e-04	1.5e-02	aco1	aconitase 1, soluble
561370	1,37	1.1e-04	1.6e-02	jak3	Janus kinase 3 (a protein tyrosine kinase, leukocyte)
393801	-2,60	1.2e-04	1.6e-02	gnao1b	guanine nucleotide binding protein (G protein), alpha activating activity polypeptide O, b
100144561	1,69	1.2e-04	1.6e-02	prkcha	protein kinase C, eta, a
393342	1,23	1.3e-04	1.7e-02	zgc:64051	zgc:64051
100003708	1,22	1.3e-04	1.7e-02	si:ch211-226m16.2	si:ch211-226m16.2
110438472	1,48	1.3e-04	1.8e-02	LOC110438472	uncharacterized LOC110438472
565345	1,47	1.5e-04	1.9e-02	myo1f	myosin IF
100150349	1,74	1.6e-04	2.1e-02	si:dkey-27h10.2	si:dkey-27h10.2
678553	6,31	1.6e-04	2.1e-02	zgc:136683	zgc:136683
100329726	3,09	1.6e-04	2.1e-02	xcr1b.3	chemokine (C motif) receptor 1b, duplicate 3
101883440	2,19	1.7e-04	2.2e-02	LOC101883440	PWWP domain-containing protein MUM1L1-like
30721	6,00	1.7e-04	2.2e-02	pdx1	pancreatic and duodenal homeobox 1
554157	1,24	1.8e-04	2.3e-02	ctss2.1	cathepsin S, ortholog2, tandem duplicate 1
415169	-2,51	1.8e-04	2.3e-02	rps13	ribosomal protein S13
406204	-0,76	1.8e-04	2.3e-02	per1b	period circadian clock 1b
322372	-0,68	1.8e-04	2.3e-02	fah	fumarylacetoacetate hydrolase (fumarylacetoacetase)
100150732	-1,49	1.8e-04	2.3e-02	si:ch211-141e20.2	si:ch211-141e20.2
100004976	2,27	2.0e-04	2.5e-02	si:dkey-58f10.10	si:dkey-58f10.10
360143	-2,98	2.0e-04	2.5e-02	mxb	myxovirus (influenza) resistance B
393706	-6,79	2.1e-04	2.6e-02	ppa1a	pyrophosphatase (inorganic) 1a
445073	1,32	2.3e-04	2.7e-02	socs1a	suppressor of cytokine signaling 1a
794999	2,77	2.4e-04	2.9e-02	gzmk	granzyme K
793819	1,71	2.5e-04	3.0e-02	cd40lg	CD40 ligand
101886409	3,72	2.5e-04	3.0e-02	si:dkey-76b14.2	si:dkey-76b14.2
799591	1,70	2.6e-04	3.1e-02	zc2hc1c	zinc finger, C2HC-type containing 1C
641415	1,52	2.6e-04	3.1e-02	zgc:123107	zgc:123107
799901	1,92	2.6e-04	3.1e-02	reep1	receptor accessory protein 1
406752	1,13	2.6e-04	3.1e-02	pitpnbl	phosphatidylinositol transfer protein, beta, like
798704	3,78	2.7e-04	3.1e-02	si:ch211-217k17.10	si:ch211-217k17.10

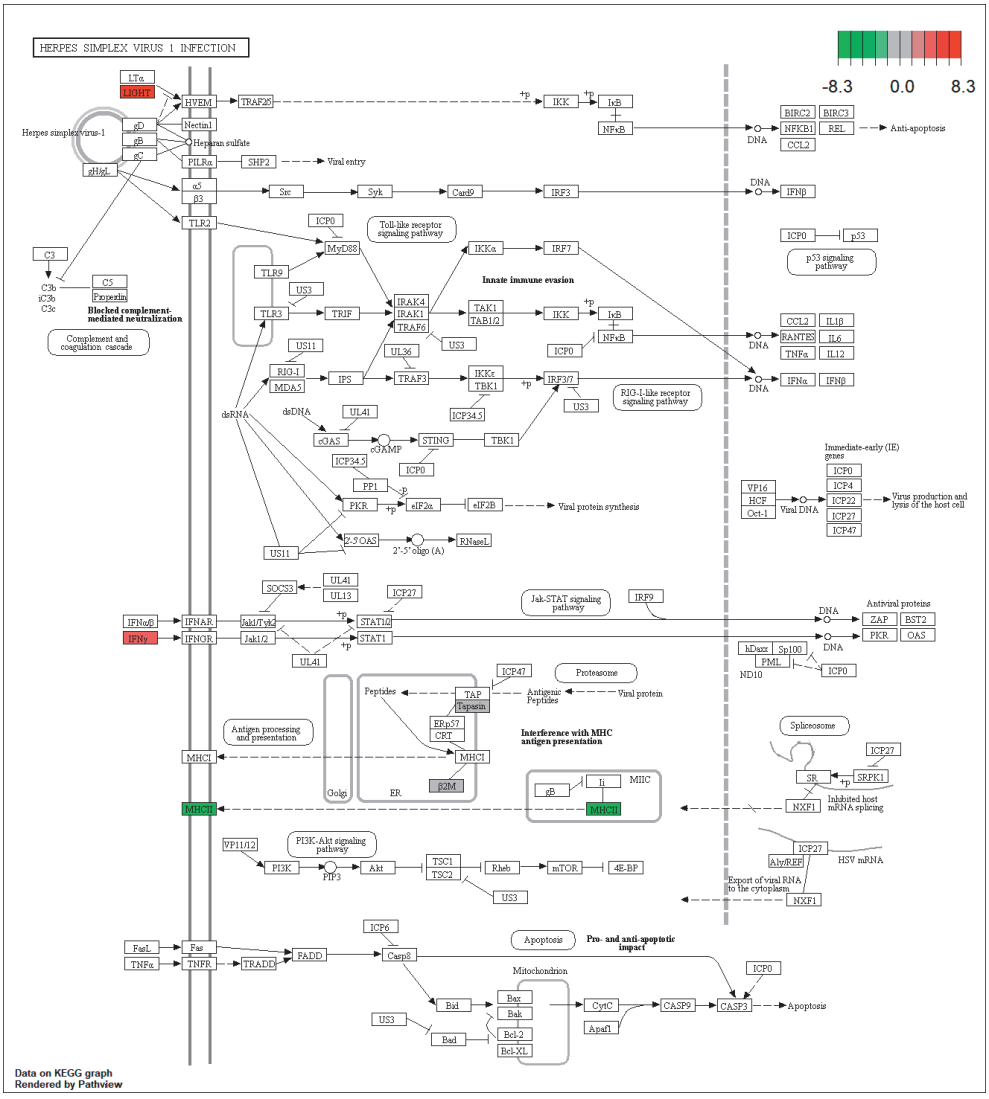
568850	1,80	2.7e-04	3.1e-02	ftr79	finTRIM family, member 79
100331748	-1,49	2.7e-04	3.1e-02	si:cabz01081777.1	si:cabz01081777.1
798697	2,68	2.8e-04	3.2e-02	gfi1ab	growth factor independent 1A transcription repressor b
798119	3,96	2.8e-04	3.2e-02	tnfsf14	TNF superfamily member 14
100037359	1,81	2.9e-04	3.3e-02	rgs13	regulator of G protein signaling 13
100004014	-1,32	3.0e-04	3.3e-02	thoc6	THO complex 6
405868	-2,37	3.1e-04	3.4e-02	lrrc59	leucine rich repeat containing 59
100150889	0,85	3.3e-04	3.6e-02	tapbpl	TAP binding protein like
799350	-1,96	3.3e-04	3.6e-02	si:ch211-191i18.4	si:ch211-191i18.4
494108	-0,77	3.5e-04	3.8e-02	ptgr1	prostaglandin reductase 1
393285	1,15	3.5e-04	3.8e-02	irs2a	insulin receptor substrate 2a
555849	1,36	3.5e-04	3.8e-02	arhgap45b	Rho GTPase activating protein 45b
791524	1,33	3.6e-04	3.9e-02	grna	granulin a
566600	2,64	3.6e-04	3.9e-02	si:ch211-284e13.9	si:ch211-284e13.9
492336	1,63	3.6e-04	3.9e-02	anxa3b	annexin A3b
553708	-5,10	3.7e-04	3.9e-02	pde6ga	phosphodiesterase 6G, cGMP-specific, rod, gamma, paralog a
337166	-3,83	3.7e-04	3.9e-02	si:dkey-40g16.6	si:dkey-40g16.6
103911103	1,70	3.8e-04	4.0e-02	LOC103911103	uncharacterized LOC103911103
559103	1,14	3.9e-04	4.1e-02	inpp5d	inositol polyphosphate-5-phosphatase D
553797	4,42	4.1e-04	4.3e-02	wdr78	WD repeat domain 78
794765	1,47	4.1e-04	4.3e-02	si:dkey-222h21.1	si:dkey-222h21.1
100329767	3,20	4.3e-04	4.5e-02	cabz01093075.1	cabz01093075.1
100002266	4,49	4.3e-04	4.5e-02	si:ch211-236p5.3	si:ch211-236p5.3
101884057	-1,69	4.3e-04	4.5e-02	LOC101884057	uncharacterized LOC101884057
101882795	1,44	4.4e-04	4.5e-02	LOC101882795	uncharacterized LOC101882795
368519	1,33	4.5e-04	4.6e-02	stat4	signal transducer and activator of transcription 4
140427	-0,77	4.6e-04	4.6e-02	hsf2	heat shock transcription factor 2
565801	1,18	4.6e-04	4.7e-02	gmip	GEM interacting protein
394039	0,73	4.7e-04	4.7e-02	zgc:66475	zgc:66475
571408	1,53	4.6e-04	4.7e-02	dock2	dedicator of cytokinesis 2
571148	-0,76	4.7e-04	4.7e-02	ankha	ANKH inorganic pyrophosphate transport regulator a
561460	0,68	4.9e-04	4.9e-02	zgc:172302	zgc:172302

10014134 1	1,11	5.0e-04	5.0e-02	arhgap17b	Rho GTPase activating protein 17b

A)



B)


HERPES SIMPLEX VIRUS 1 INFECTION
-8.3
0.0
8.3
Herpes simplex virus-1
Heparan sulfate
Viral entry
Toll-like receptor signaling pathway
Innate immune evasion
Blocked complement-mediated neutralization
Complement and coagulation cascade
p53 signaling pathway
RIG-I-like receptor signaling pathway
Immediate-early (IE) genes
Viral protein synthesis
Virus production and lysis of the host cell
Jak-STAT signaling pathway
Antiviral proteins
Proteasome
Antigen processing and presentation
Interference with MHC antigen presentation
Spliceosome
Inhibited host mRNA splicing
PI3K-Akt signaling pathway
HSV mRNA
Export of viral RNA to the cytoplasm
Apoptosis
Pro- and anti-apoptotic impact
Mitochondrion
Anti-apoptosis
Apoptosis
Data on KEGG graph
Rendered by Pathview

C)

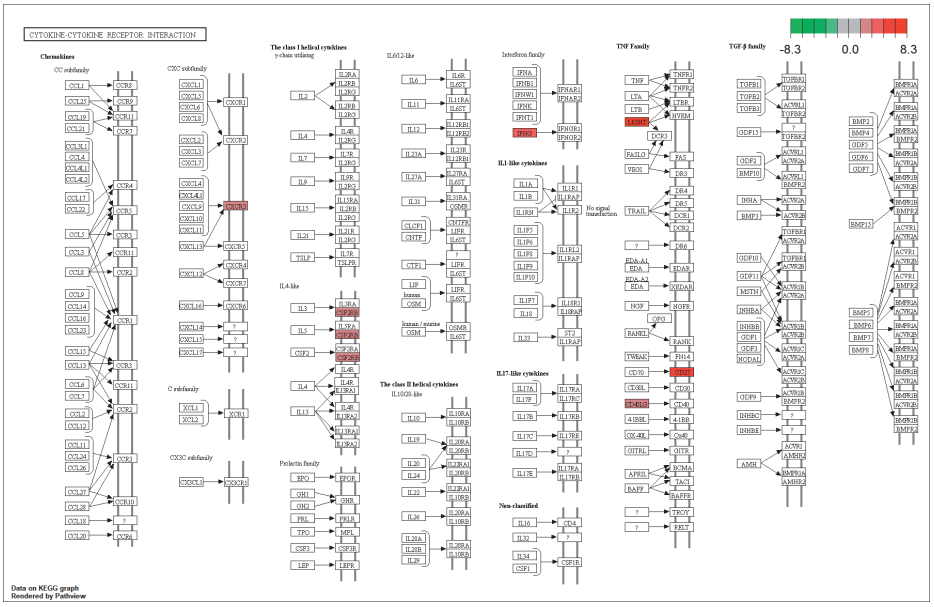


Figure S2: Enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways between zebrafish infected with the microsporidium *Pseudoloma neurophilia* and uninfected controls. (A) Proteasome, (B) Herpes simplex virus I, (C) Cytokine-cytokine receptor interaction. Differentially expressed genes are coloured by log fold, where red indicates upregulation while green indicates downregulation.

Table S3: Gene Ontology (GO) over-representation test of biological processes affected by the microsporidium *Pseudoloma neurophilia* in zebrafish, compared to uninfected controls.

GO term ID	Log2FC	Gene_symbol	Entrez_ID	Gene_description
GO.0006955 Immune Response	2,230	si:ch211-153b23.7	100004948	si:ch211-153b23.7
	3,001	si:dkey-79f11.10	100007523	si:dkey-79f11.10
	2,825	ccl36.1	100537088	chemokine (C-C motif) ligand 36, duplicate 1
	6,192	cd27	101887143	CD27 molecule
	1,366	b2m	30400	beta-2-microglobulin
	1,195	cd74b	30645	CD74 molecule, major histocompatibility complex, class II invariant chain b
	-8,324	mhc2dab	30762	major histocompatibility complex class II DAB gene
	1,111	ptpn6	335573	protein tyrosine phosphatase non-receptor type 6

	3,675	ifng1	405790	interferon gamma 1
	1,318	socs1a	445073	suppressor of cytokine signaling 1a
	-1,502	h2afx1	449794	H2A histone family member X1
	3,409	themis	555433	thymocyte selection associated
	4,949	ccl34b.4	556621	chemokine (C-C motif) ligand 34b, duplicate 4
	1,522	zgc:123107	641415	zgc:123107
	2,336	cxcr3.1	654692	chemokine (C-X-C motif) receptor 3, tandem duplicate 1
	1,607	irf1b	792160	interferon regulatory factor 1b
	1,705	cd40lg	793819	CD40 ligand
	-2,633	lgals17	794824	galectin 17
	3,956	tnfsf14	798119	TNF superfamily member 14
	2,620	itk	798906	IL2 inducible T cell kinase
GO.0010499 Proteasomal Ubiquitin-independent Protein Catabolic Process	1,266	psmb9a	30665	proteasome subunit beta 9a
	2,226	psmb8a	30666	proteasome subunit beta 8A
	1,535	psmb12	64279	proteasome subunit beta 12
	1,968	psmb13a	64280	proteasome subunit beta 13a
	1,237	psma6l	83917	proteasome subunit alpha 6, like
GO.0007623 Circadian Rhythm	-1,195	cipca	100535864	CLOCK-interacting pacemaker a
	-0,764	per1b	406204	period circadian clock 1b
	-1,673	nr1d1	494487	nuclear receptor subfamily 1, group d, member 1
	-1,146	bhlhe41	563771	basic helix-loop-helix family, member e41
	-1,468	ciarta	564009	circadian associated repressor of transcription a
	-1,527	si:ch211-132b12.7	564531	si:ch211-132b12.7
GO.0002682 Regulation of Immune System Process	2,230	si:ch211-153b23.7	100004948	si:ch211-153b23.7
	1,366	b2m	30400	beta-2-microglobulin
	1,492	lgals9l1	337597	lectin, galactoside-binding, soluble, 9 (galectin 9)-like 1
	1,318	socs1a	445073	suppressor of cytokine signaling 1a
	3,409	themis	555433	thymocyte selection associated
	1,144	inpp5d	559103	inositol polyphosphate-5-phosphatase D
	2,336	cxcr3.1	654692	chemokine (C-X-C motif) receptor 3, tandem duplicate 1
	1,607	irf1b	792160	interferon regulatory factor 1b
	1,705	cd40lg	793819	CD40 ligand
	-2,633	lgals17	794824	galectin 17
	2,620	itk	798906	IL2 inducible T cell kinase
GO.0019882 Antigen Processing	0,847	tapbpl	100150889	TAP binding protein like
	1,366	b2m	30400	beta-2-microglobulin

and Presentation	1,195	cd74b	30645	CD74 molecule, major histocompatibility complex, class II invariant chain b
	-8,324	mhc2dab	30762	major histocompatibility complex class II DAB gene
	1,522	zgc:123107	641415	zgc:123107
GO.0032922 Circadian Regulation of Gene Expression	-0,764	per1b	406204	period circadian clock 1b
	-1,673	nr1d1	494487	nuclear receptor subfamily 1, group d, member 1
	-1,146	bhlhe41	563771	basic helix-loop-helix family, member e41
	-1,468	ciarta	564009	circadian associated repressor of transcription a
GO.0048511 Rhytmic Process	-1,195	cipca	100535864	CLOCK-interacting pacemaker a
	-0,764	per1b	406204	period circadian clock 1b
	-1,673	nr1d1	494487	nuclear receptor subfamily 1, group d, member 1
	-1,146	bhlhe41	563771	basic helix-loop-helix family, member e41
	-1,468	ciarta	564009	circadian associated repressor of transcription a
	-1,527	si:ch211-132b12.7	564531	si:ch211-132b12.7
GO.0009266 Response to Temperature Stimulus	1,531	hsp70.1	100126123	heat shock cognate 70-kd protein, tandem duplicate 1
	-0,771	hsf2	140427	heat shock transcription factor 2
	3,821	hsp70.3	30671	heat shock cognate 70-kd protein, tandem duplicate 3
	-0,764	per1b	406204	period circadian clock 1b
	-1,146	bhlhe41	563771	basic helix-loop-helix family, member e41
GO.0042752 Regulation of Circadian Rhythm	-1,195	cipca	100535864	CLOCK-interacting pacemaker a
	-0,764	per1b	406204	period circadian clock 1b
	-1,673	nr1d1	494487	nuclear receptor subfamily 1, group d, member 1
	-1,527	si:ch211-132b12.7	564531	si:ch211-132b12.7
GO.0002683 Negative Regulation of Immune System Process	1,366	b2m	30400	beta-2-microglobulin
	1,492	lgals9l1	337597	lectin, galactoside-binding, soluble, 9 (galectin 9)-like 1
	1,318	socs1a	445073	suppressor of cytokine signaling 1a
	1,144	inpp5d	559103	inositol polyphosphate-5-phosphatase D
	-2,633	lgals17	794824	galectin 17
GO.0006952 Defense Response	2,230	si:ch211-153b23.7	100004948	si:ch211-153b23.7
	1,366	b2m	30400	beta-2-microglobulin
	1,111	ptpn6	335573	protein tyrosine phosphatase non-receptor type 6
	1,333	stat4	368519	signal transducer and activator of transcription 4
	3,675	ifng1	405790	interferon gamma 1
	1,318	socs1a	445073	suppressor of cytokine signaling 1a
	-1,502	h2afx1	449794	H2A histone family member X1
	4,949	ccl34b.4	556621	chemokine (C-C motif) ligand 34b, duplicate 4
	2,336	cxcr3.1	654692	chemokine (C-X-C motif) receptor 3, tandem duplicate 1

	1,607	irf1b	792160	interferon regulatory factor 1b
GO.0002684 Positive Regulation of Immune System Process	2,230	si:ch211-153b23.7	100004948	si:ch211-153b23.7
	1,366	b2m	30400	beta-2-microglobulin
	1,318	socs1a	445073	suppressor of cytokine signaling 1a
	3,409	themis	555433	thymocyte selection associated
	1,705	cd40lg	793819	CD40 ligand
	-2,633	lgals17	794824	galectin 17
	2,620	itk	798906	IL2 inducible T cell kinase
GO.0009615 Response to Virus	-2,981	mxb	360143	myxovirus (influenza) resistance B
	2,526	mxc	360145	myxovirus (influenza virus) resistance C
	3,675	ifng1	405790	interferon gamma 1
	1,607	irf1b	792160	interferon regulatory factor 1b
GO.0045087 Innate Immune Response	2,230	si:ch211-153b23.7	100004948	si:ch211-153b23.7
	1,366	b2m	30400	beta-2-microglobulin
	1,111	ptpn6	335573	protein tyrosine phosphatase non-receptor type 6
	1,318	socs1a	445073	suppressor of cytokine signaling 1a
	-1,502	h2afx1	449794	H2A histone family member X1
	4,949	ccl34b.4	556621	chemokine (C-C motif) ligand 34b, duplicate 4

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Paper III

Metabolic and neurophysiological effects of a microsporidian parasite depend on the host´s previous infection status

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Abstract: Hosts incur energetic and often fitness-related costs from harbouring parasites. However, these costs may not arise solely from established infections, with mounting evidence indicating that hosts produce metabolic and physiological responses to parasite exposure associated with stress, tissue damage, and immunity. While studies often focus exclusively on the long-term costs of host infection, we know little about how the costs of established infection compare to those from parasite exposure, and whether these responses shift depending on the host´s infection status. Using the brain-infecting microsporidian parasite *Pseudoloma neurophilia* and its fish host (the zebrafish, *Danio rerio*), we measured how acute parasite exposure alters metabolic rate through time in naïve fish versus fish with an established infection, and whether these effects are accompanied by changes in behaviour (i.e., activity) or brain monoamine neurotransmitter signalling (i.e., serotonergic and dopaminergic activity). While established infection moderately increased metabolic needs and reduced aerobic capacity, both previously naïve and long-term infected zebrafish responded to parasite exposure with a spike in metabolic rate at three days post-exposure, which was mitigated by six days post-exposure. Further, fish with an established infection increased activity with each subsequent testing day regardless of exposure treatment, suggesting interactive effects of stress and long-term *P. neurophilia*-infection on behaviour. Previously naïve fish exhibited the strongest changes in brain dopaminergic and serotonergic signalling following acute parasite exposure, indicating that initial parasite exposure may generate an extensive and prolonged neural response that is mitigated during subsequent infection events. Our results show that host responses to infectious parasite stages vary at multiple levels of biological organization, depending on their previous exposure history and current infection status, highlighting a previously overlooked driver of individual variability in host responses to parasites.

Keywords: host-parasite relationship, metabolism, monoamine neurotransmitters, model species, sub-clinical infection

1. Introduction

Hosts incur metabolic costs from harbouring parasites, through the direct energy drain from infection and mechanisms associated with infection resistance (Dallas et al., 2016). However, hosts may also experience additional metabolic costs at the time of parasite exposure, even before parasitic infection is actually established. While many studies have quantified the physiological and energetic costs associated with established infection, we still have a limited understanding of the short-term effects of acute parasite exposure and how these impacts vary during repeated exposure events.

Acute parasite exposure can lead to increased metabolic needs, as initial encounters with parasites elicit a range of responses associated with stress, tissue damage, and immunity. Studies have detected several physiological indicators of stress following parasite exposure, including elevated ventilation, respiration, and heart rates (Nadler et al., in review; Laitinen et al., 1996; Voutilainen et al., 2008). For example, in the *Drosophila hydei* fly and its ectoparasitic mite *Macrocheles muscaedomesticae*, Luong et al. (2017) found that CO_2 production (an indicator of respiration) more than doubles when flies are exposed to infectious mites. New parasite infections may also be more harmful in some cases than established infections, due to tissue damage caused by the parasite migrating to its preferred site of infection. One example includes fathead minnows (*Pimephales promelas*) exposed to the liver trematode *Ornithodiplostomum* sp., who experience cell damage during the earliest days following new infections (Stumbo et al., 2012). To prevent new infections, hosts can use mechanisms of ~~develop~~ innate immunity upon first exposure to a novel parasite, and may develop specific, inducible immune defences to increase protection upon subsequent encounters (Jones, 2001; Sadd and Schmid-Hempel, 2006). In the social bumblebee *Bombus terrestris*, for instance, hosts re-exposed to homologous bacterial pathogens exhibit higher survival, enhanced protection from infection, and increased specificity of their immune response for prolonged periods of time following their initial exposure. All of these responses (i.e., stress, tissue damage, immunity) are known to produce spikes in metabolic needs that may equal or even exceed those arising from established parasite infections, which are known to vary widely within and among host-parasite systems in the literature (reviewed in Robar et al., 2011). In the few studies to investigate changes in host energy

88 expenditure in response to acute versus established parasite infection, metabolic costs
89 associated with acute parasite exposure appeared to be mitigated in the long-term (Nadler et al.,
90 in review; Luong et al., 2017; Voutilainen et al., 2008). Thus, contradictory results in the literature
91 arising within and among host-parasite systems could stem in part from variability in the time
92 since last parasite exposure.

93 The brain plays multiple important roles in regulating whole-organism energy metabolism, by
94 regulating energy homeostasis, modulating hormones involved in cellular glucose uptake and
95 mobilizing energy reserves during periods of high energy demand (Rittschof et al., 2015). The
96 phylogenetically ancient monoamine neurotransmitters, such as serotonin (5-HT) and dopamine
97 (DA) in particular, control a range of central and peripheral systems, including behavioural and
98 physiological traits that should be central to the energetic response to parasite exposure and
99 established infection. Among these functions are production of stress hormones, release of other
100 neurotransmitters, response to sensory cues, feeding motivation, locomotor function,
101 respiration, and immunoregulatory function (Andrews et al., 2015; Bacque-Cazenave et al., 2020;
102 Dellu-Hagedorn et al., 2018; Herr et al., 2017; Matt and Gaskill, 2020; Song et al., 2015; Wu et al.,
103 2019). Some studies have investigated how host serotonergic and dopaminergic signalling
104 responds to established infection and found variable effects depending on the host-parasite
105 system. For instance, while the *Schistocephalus* tape worm increases brain serotonergic activity
106 in its stickleback host (*Gasterosteus aculeatus*) (Øverli et al., 2001), the trematode *Euhaplorchis*
107 *californiensis* decreases serotonergic activity in its killifish host (Helland-Riise et al., in revision;
108 Shaw et al., 2009; Shaw and Øverli, 2012). Likewise, dopaminergic signalling also exhibits
109 variation among different host-parasite systems, with, for example, dopaminergic activity
110 increasing in *Toxoplasma gondii*-infected rodents (Prandovszky et al., 2011) and decreasing in
111 aquatic isopods following acanthocephalan infection (Kopp et al., 2016). In all of these studies,
112 effects of parasites on monoaminergic activity were investigated in response to established
113 infections, and the time since last parasite exposure varied greatly. However, no one has yet
114 tested a time series post-exposure to determine when metabolic costs are mitigated, whether
115 additional physiological processes (such as monoaminergic-signalling changes) remain on-going

once metabolic rate stabilizes, and/or if these responses to parasite exposure shift following established infection.

In this study, we focused on the impacts of acute parasite exposure and established infection on host metabolism, behaviour, and neurotransmitter signalling for the zebrafish (*Danio rerio*) and its microsporidian parasite *Pseudoloma neurophilia.* The use of zebrafish in biomedical studies has boomed since the 1990s (Rosenthal and Ashburner, 2002), but efforts to standardize health screening and pathogen detection among zebrafish facilities remain ongoing (Borges et al., 2016; Collymore et al., 2016; Kent and Varga, 2012; Marancik et al., 2019). By far the most commonly detected pathogen in these facilities is the microsporidian parasite *P. neurophilia*. Indeed, the Zebrafish International Resource Centre (ZIRC) recently reported detection of this parasite in as much as 74% of the zebrafish facilities that sent samples for routine pathogen testing (Murray et al., 2011). While severe infections can produce detectable external symptoms (e.g., spine curvature, skinny disease), *P. neurophilia* frequently produce sub-clinical infections lacking discernible symptoms (Kent et al., 2012), except for moderate, potentially sex-specific reductions in body condition (Sanders et al., 2020) and size (length and mass; Midttun et al., in press). Some behavioural modifications have been recorded, including reduced locomotor activity, increased shoal cohesion, and limited habituation to threat stimuli in zebrafish with an established, but sub-clinical, infection (Midttun et a., in press; Spagnoli et al., 2017; Spagnoli et al., 2015). Further, this parasite is capable of infecting a range of other fish species. Simple cohabitation between infected laboratory zebrafish and other aquarium fishes (e.g., medaka, goldfish, neon tetra, fathead minnow) can lead to parasite transmission (Sanders et al., 2016), suggesting that the potential impacts of this parasite on experimental studies with common aquarium fishes are far reaching.

Microsporidians like *P. neurophilia* are unicellular, intracellular parasites that are typified by production of a resistant spore stage, which can live in the environment for lengthy periods of time (Vavra and Larsson, 2014). In zebrafish, mature *P. neurophilia* infections predominantly aggregate in the central nervous system (CNS), particularly the spinal cord and hindbrain tissue (Matthews et al., 2001), but spores can also be found throughout the body particularly in the skeletal muscle (West et al., 2014). Although vertical transmission is possible (Sanders et al.,

2013), new hosts are primarily exposed to infectious spores through ingestion (Sanders et al., 2014), either through exposure to water-borne spores or cannibalism of dead conspecifics (Murray et al., 2011; Sanders et al., 2012). Following exposure, the parasite penetrates through the intestinal lumen, and travels through the pancreas and kidney, reaching the spinal cord approximately three to four days after exposure, with mature spores first detectable in the brain after approximately six days (Cali et al., 2012; Sanders et al., 2014). Given the frequency that zebrafish may encounter *P. neurophilia* in aquarium facilities, acute parasite exposure could produce a greater net energetic impact on hosts than established infection, if it elicits a metabolic response.

Here, we quantified zebrafish metabolism, behaviour, and monoaminergic signalling to address the following linked questions concerning *P. neurophilia*:

1) Does established infection alter aerobic metabolic rate? As moderate reductions in body size and condition have been observed in zebrafish with even sub-clinical infections (Midttun et al., in press; Sanders et al., 2020), zebrafish hosts may exhibit elevated metabolic needs and/or reduced aerobic capacity to support processes such as condition and growth.
2) Do zebrafish respond metabolically or behaviourally in the days following parasite exposure? Since *P. neurophilia* takes approximately six days to reach its endpoint in the brain (Sanders et al., 2014), we hypothesized that any metabolic and behavioural response to parasite exposure would peak at this time point.
3) Do established infection or parasite exposure alter brain monoaminergic signalling? Given the important role that dopaminergic and serotonergic signalling play in stress, immunity, and energy allocation (Andrews et al., 2015; Song et al., 2015), brain monoaminergic activity may modulate the metabolic response to parasite exposure and established infection.

Taken together, we compared metabolic, behavioural and monoaminergic responses to acute parasite exposure (in a time-series post-exposure) versus established infection. By exposing both individuals naïve to the parasite as well as individuals with an established infection to *P.*

neurophilia spores, we were also able to determine if established infection alters these physiological and behavioural responses to repeated parasite exposure (Fig. 1).

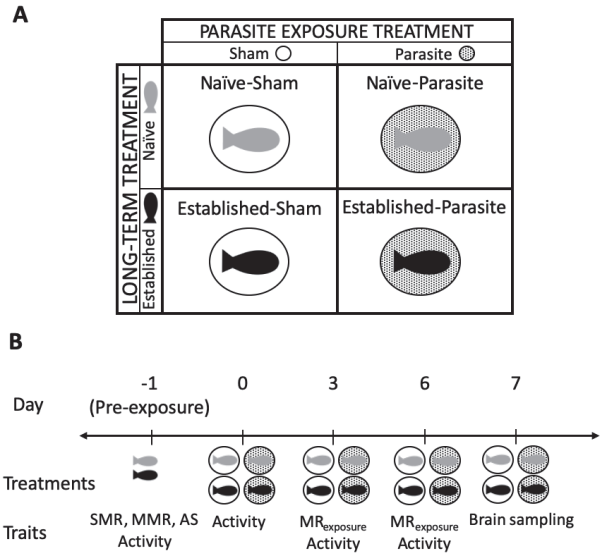


Figure 1. Illustrations of experimental treatment groups and experiment timeline. A) Experimental treatment groups were developed through a two-by-two experimental design, crossing "long-term treatment" (*naïve* or *established* infection with the microsporidian parasite *Pseudoloma neurophilia*) with "parasite exposure treatment" (*sham*- or *parasite*-exposure). B) The experimental portion of the study occurred over eight days. The figure illustrates the *treatments* compared each testing *day*, as well as the *traits* measured on each respective day, including standard metabolic rate (SMR), maximum metabolic rate (MMR), aerobic scope (AS), post-exposure metabolic rate ($MR_{exposure}$), activity (measured as mean number of 180° turns per min), and brain sampling (through which dopaminergic and serotonergic signaling in the brain was analyzed).

2. Material & Methods

This study involved laboratory-rearing of fish from gametes collected from specific pathogen free (SPF) zebrafish. These fish were exposed for 10 weeks to different "long-term treatments" (fish

gained an “established” infection through repeated exposure to CNS tissue from *P. neurophilia*-positive fish, vs. “naïve” fish that were repeatedly sham-exposed to CNS tissue from uninfected fish). In this study, we compared the metabolic, behavioural, and neurophysiological responses of naïve and established-infection zebrafish following an “acute parasite exposure treatment”. That is, fish were exposed to either “sham” (i.e., exposed to CNS tissue from uninfected fish) or “parasite” (i.e., exposed to CNS tissue from infected fish) treatments. This two-by-two experimental design resulted in four treatment groups that will from here on be abbreviated as: naïve-sham, naïve-parasite, established-sham and established-parasite. Metabolic rate and activity were measured pre-exposure as well as three- and six-days post-exposure to determine the effect of acute parasite exposure in the naïve versus established-infection zebrafish. In addition, individuals were sampled to assay serotonergic and dopaminergic activity at seven days post-exposure to determine how long-term treatment and acute parasite exposure influenced neurotransmitter signalling, and their link to changes in metabolic rate and activity. Figure 1 illustrates the study timeline, treatments used, and traits measured. Methodological details are provided below.

(a) *Zebrafish rearing*

All zebrafish used in this experiment were reared at the Norwegian University of Life Sciences Zebrafish Facility (Oslo, Norway) from ten adult zebrafish (AB strain) that were obtained from a *P. neurophilia* an SPF facility (Sinnhuber Aquatic Research Laboratory (SARL), Oregon State University, USA). Upon arrival, fish were quarantined for two months. During quarantine, fish were maintained in a 25L glass tank (40cm x 25cm x 25cm; L x W x H) with filtered and UV-treated water (28°C, pH 7.4-7.6, conductivity 500µS, 14:10 light:dark cycle; (Westerfield, 2007)) and fed a diet composed of live brine shrimp (Ocean Nutrition, Essen, Belgium) and flake food (Special Diets Services (SDS), Witham, United Kingdom). Following quarantine, fish were transferred to a recirculating system (Techniplast, Buguggiate, Italy), where males and females were kept separately. Once weekly, fish were transferred to standard 1-L breeding tanks (Techniplast, Buguggiate, Italy). Here, females and males were separated by dividers overnight. Dividers were removed the following morning and fish were allowed to spawn for up to four hours before being

transferred back to their respective holding tanks. Following spawning, eggs were collected, rinsed with autoclaved water, and counted. Eggs were maintained in petri dishes (95 x 15 mm; Heger, Rjukan, Norway) at 28°C for five days post-fertilization (dpf) at a density of 50 eggs/30mL water. Water was changed and dead eggs were removed daily. Five dpf zebrafish larvae were transferred to 1-L plastic beakers (VWR, Radnor Pennsylvania, USA) at a density of 1 larvae/6mL of UV-treated and filtered water (changed daily). Larvae were fed freeze-dried rotifers and small-grain flaked food (SDS). Juvenile zebrafish were transferred to the aforementioned recirculating system at 21 dpf, where they were kept at density of 5 fish/L using the husbandry conditions previously described.

(b) *Long-term infection procedure*

At approximately five months post-hatch, zebrafish (n = 252) were transferred to an infection holding room and randomly assigned to plastic holding tanks (23 x 15.3 x 16.5 cm, L x W x H; Exo Terra, Montreal, Canada; n = 15 tanks per long-term treatment) at a density of 5 fish/L and a male:female ratio of 1:1 per tank. Individual tanks were maintained as closed systems to prevent cross-contamination of parasites from fish with an established infection to those naïve to the parasite. Tanks were aerated (Eheim, Stuttgart, Germany) and maintained at 26-28°C, with 50% water changes three times weekly and 100% water changes biweekly. In preparation for the infection procedures, two larger, additional groups (n = 80 fish per group) of zebrafish were transferred to the infection room (housed in two replicate, 25 L glass tanks; 40 cm x 25 cm x 25; L x W x H) to serve as donor fish for sham and parasite exposure treatments. One group was confirmed positive for *P. neurophilia* infection (using the procedure described below) and the second group was composed of uninfected SPF fish. During this period, all fish were fed to satiation daily with flake food (SDS).

Experimental infections were executed following the procedure outlined in Midttun et al. (in press). Briefly, over a 10-week period, experimental zebrafish holding tanks were exposed to 100 mL of tank water from either the sham donor (i.e., the naïve treatment group) or the *P. neurophilia*-positive donor holding tanks (i.e., the established-infection treatment group) (Spagnoli et al., 2017). Additionally, the established-infection treatment group was exposed

directly to infectious spores at four times points (separated by ≥ two weeks). Spores were obtained by euthanising *P. neurophilia*-positive donor fish in an overdose of buffered (pH = 7) Tricaine methanesulfonate (1g L^{-1}; MS-222; Sigma, St. Louis Missouri, USA) and dissecting tissue from their CNS (i.e., brain and spinal cord). The CNS tissue was macerated by passing the samples through sterile needles with decreasing gauge size (18, 23, 26 G) (Braun Medical, Sempach, Switzerland). Macerated CNS tissue was mixed with brine shrimp and fed to zebrafish (at an exposure rate of 1 fish´s CNS tissue per 20 fish) (Peneyra et al., 2018). The naïve treatment group received CNS tissue obtained from SPF donor fish. Throughout the study, the established infection treatment exhibited a 10% mortality rate. Two fish (2% of total) from the naïve treatment group were euthanized due to a swim bladder disorder.

(c) *DNA extraction and qPCR*

To confirm the prevalence of *P. neurophilia* infection after 10 weeks, 50% of the fish from each treatment group were tested using qPCR. Briefly, zebrafish were euthanized and brains were individually transferred to 50 µL MilliQ water (Merck, Darmstadt, Germany). Samples were sonicated at 55W for 2 mins (QSonica Sonicators, Connecticut Newtown, USA) on ice. Between each sample, the sonicator probe was decontaminated in 100% ethanol and rinsed in Milli-Q water. To extract DNA, the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) was used according to manufacturer's protocol with minor changes. Samples were digested overnight in proteinase K and lysis buffer at 56°C, as suggested by Sanders and Kent (2011). Samples were eluted in 100 µL storage buffer (provided in the kit) and kept at -20°C until further analysis.

The qPCR protocol by Sanders and Kent (2011) was followed. Reactions were performed in 25 µL, where forward and reverse primer concentrations were 900nm each, 250nM hydrolysis probe, 1X TaqMan and 2 µL DNA sample. Forward primer, reverse primers and hydrolysis probe used were 5'-GTAATCGCGGGCTCACTAAG-3', 5'-GCTCGCTCAGCCAAATAAAC-3' and 5'- 6-carboxyfluorescein (FAM)-ACACACCGCCCGTCGTTATCGAA – 3'-Black Hole Quencher 1 (BHQ1), respectively. The following program was used: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 60°C for 1 min on a LightCycler 96 instrument and analysed using the LightCycler 96 software (Roche, Basel, Switzerland). Primers are designed to be species-specific

for *P. neurophilia*, meaning all expression indicates the presence of the parasite (with a cutoff Cq-value of 38, which is suitable for diagnostic testing, (Purcell et al., 2011)). These analyses indicated a 100% infection prevalence in the established-infection treatment, and 0% infection prevalence in the naïve treatment.

(d) *Intermittent-flow respirometry and acute parasite exposure*

We used intermittent-flow respirometry to measure oxygen uptake of zebrafish as a proxy for aerobic metabolism, which is a technique in which dissolved oxygen levels inside of a sealed and intermittently-flushed respirometry chamber are monitored continuously to calculate the rate of oxygen uptake ($\dot{M}O_2$) (Svendsen et al., 2016a). The $\dot{M}O_2$ is a suitable representation of aerobic metabolic rate, as oxygen is used in the breakdown of stored energy to fuel essential processes (e.g., maintenance, locomotor activity, digestion, growth) (Nelson, 2016).

Prior to the start of this study, naïve (n = 16) and established-infection (n = 14) fish were moved from the infection room to an experimental holding room, where the respirometry trials were conducted. Fish were held for the remainder of the experiment in groups composed of three to four fish each (23 x 15.3 x 6 cm, L x W x H; Exo Terra, Montreal, Canada; density: 1 fish per 250 mL; n = 8 holding tanks). All fish were tagged two weeks prior to the start of experimentation with visible implant elastomer (Northwest Marine Technology, Tumwater, WA, USA) so that they were individually identifiable throughout the course of the experiment.

In this study, each respirometer included a glass cylindrical tube (5 cm length, 33 cm inner diameter) with acrylic end caps, oxygen-impermeable tubing in a closed loop to a recirculating peristaltic pump (Watson Marlow 205S, United Kingdom; mean total water volume = 122 mL, range: 107-144 mL), and a flushing pump (Eheim600, Germany; outflow split among four chambers and set to turn on/off in cycles of 5-min flushing and 13-min measuring, which maintained oxygen saturation levels above 80% air saturation (Hughes, 1973)). A total of eight respirometers were measured in parallel each day, split in replicate pairs across four holding tanks (27 cm L x 20 cm W x 6 cm H). Fish metabolic rate was measured across four sequential testing groups, with metabolic rate for six to eight fish measured concurrently in each group. Flushing pumps were placed in two adjacent water sumps (47 cm L x 28 cm W x 40 cm), where

holding tanks overflowed during flushing cycles. This configuration allowed us to maintain two isolated water systems daily, to prevent cross-contamination of parasites from fish with an established infection to naïve fish. Each sump was fitted with a 10W UV sterilizer (Deltec Gmbh, Delmenhorst, Germany) to minimize bacterial respiration in the system and a bar heater to maintain temperature (25°C ± 1°C). Two four-channel FireStingO2 fibre-optic oxygen meters (Pyroscience, Germany) connected to a PC were used to record dissolved oxygen concentration in each respirometry chamber (frequency = 0.5 Hz), with the oxygen-sensing optode placed into the recirculation loop to ensure that the flow was sufficient for a rapid sensor response time (Svendsen et al., 2016b). Slopes (*s*) of oxygen decline were calculated from plots of oxygen concentration versus time using ordinary least squares linear regression (LabChart v.6) and converted to $\dot{M}O_2$. Background bacterial respiration was quantified both before and after trials in each chamber for three measurement periods (5-mins flushing, 13-mins measuring), from which background $\dot{M}O_2$ was estimated using as an exponential increase in oxygen depletion through time (Rodgers et al., 2016). This exponential growth curve was estimated from 24-hour measurements of empty respirometry chambers. Estimated background $\dot{M}O_2$ was subtracted from all measures of fish $\dot{M}O_2$. To minimize accumulation of bacteria in the system, all respirometer components were cleansed thoroughly with soap, bleach, and hot water following each use.

We focused on four measures of metabolic rate including: i) standard metabolic rate (SMR, the metabolic rate of a resting, fasting and undisturbed individual), ii) maximum metabolic rate (MMR, the upper constraint on an individual's oxygen-consuming physiological activities), iii) aerobic scope (AS, the capacity to support activities beyond basic maintenance, calculated as the difference between MMR and SMR) (Chabot et al., 2016b; Farrell, 2016; Killen et al., 2017) and iv) metabolic rate following acute parasite exposure ($MR_{exposure}$). Prior to all respirometry testing, food was withheld for 24 hours to ensure that fish were in a post-absorptive state. First, prior to experimental parasite exposure, MMR and SMR were measured in naïve and established-infection zebrafish. MMR was measured using the chase protocol, in which fish are chased to exhaustion (i.e., when they no longer respond to chase with burst swimming) in a round bucket (28 cm D x 13 cm H) followed by 1-min of air exposure in a mesh net (to fully deplete all

endogenous oxygen stores) before being placed in their respective respirometer. $\dot{M}O_2$ was then measured continuously until oxygen saturation levels reached 80% air saturation (4 – 25 minutes; 11.9 $\pm$ 1.1 minutes, mean $\pm$ s.e.). These oxygen decline slopes were then measured in 3-min intervals, with the greatest $\dot{M}O_2$ measured during this period taken as MMR (Killen et al., 2017). Following the chase protocol, fish remained undisturbed in the respirometers for 25-26 hours to recover from exercise and reach SMR, a time period deemed sufficient in past studies in zebrafish (e.g., Yuan et al., 2018). SMR was calculated as the lowest 10th percentile of all $\dot{M}O_2$ measurements for each fish during this period (Chabot et al., 2016b; Killen, 2014).

Following measurement of MMR and SMR, the metabolic response of zebrafish to *P. neurophilia* exposure was quantified. Immediately following measurement of SMR, naïve and established-infection fish were either sham-exposed (n = 8 naïve-sham, n = 7 established-sham) or parasite-exposed (n = 8 naïve-parasite, n = 7 established-parasite) through CNS tissue injected into the respirometry chamber. These tissue samples were prepared as described above (see (b) above) but were not mixed with zebrafish food. However, we used a higher ratio of CNS tissue per fish during each exposure to help ensure that the parasite-exposed fish (i.e., naïve-parasite and established-parasite treatment groups) sustained sufficient parasite encounters. For this experimental portion of the study, the available uninfected donor fish were approximately 50% smaller by body mass than the available *P. neurophilia*-positive donor fish, so CNS tissue from 1.5x more donor fish were used for sham-exposures than parasite-exposures (at a rate of 0.75 and 0.50 donor fish per exposure for sham- and parasite-exposures, respectively). Homogenized CNS tissue was injected into the respirometry chamber using a syringe immediately following the end of a flushing cycle, through a three-way valve in the tubing from the flush pump to the chamber. The tubing from the syringe to the chamber was flushed by injecting an additional 15 mL of water. A preliminary test using food-dye confirmed that this sequence of steps successfully deposited the CNS tissue into the chamber. This procedure was repeated twice for each fish (for a total exposure of 1.5 and 1.0 CNS tissue samples per zebrafish in sham- and parasite-exposures, respectively). Following CNS exposure, fish were left in the respirometer for an additional 15 – 17 hours overnight, at which point they were returned to their respective holding tanks. As the CNS-tissue exposures produced specific dynamic action (i.e., an increase in metabolic rate

associated with digestion; (Chabot et al., 2016a)), a resting metabolic rate could not be calculated for fish in the period following tissue exposure, so $MR_{exposure}$ was not analysed at this time point of the experiment. As it takes approximately five to six days for *P. neurophilia* spores to reach the spinal cord and brain (Cali et al., 2012; Sanders et al., 2014), we measured $MR_{exposure}$ at three- and six-days post-exposure (referred to as $MR_{exposure\text{-}3}$ and $MR_{exposure\text{-}6}$, respectively) to estimate the host´s metabolic changes associated with sequential development of early infection by *P. neurophilia.* Fish were placed into the respirometer and left undisturbed for a 17 – 20 hours measurement period. Between each measure of $MR_{exposure}$, all fish were housed in their respective holding tanks and fed to satiation with flake food (except for the 24-hour period prior to each measurement, when food was withheld to ensure fish were in a post-absorptive state prior to measurements of $\dot{M}O_2$). To estimate $MR_{exposure\text{-}3}$ and $MR_{exposure\text{-}6}$, we used the same methodology employed to estimate SMR (the lowest 10^{th} percentile of all $\dot{M}O_2$ measurements during the measurement period). One established-parasite fish died in its holding tank at five days post-exposure, and was not included in analyses of metabolic rate or monoamine analyses (as described below).

During measurements of $\dot{M}O_2$ (i.e., SMR, $MR_{exposure}$), we also continuously recorded each fish's behaviour using a webcam (H264 Webcam Software) to quantify activity. Activity was measured hourly during daylight hours, starting two hours after the fish were placed in the respirometers and lasting until the lights turned off at 2100 (5 – 9 measurements per individual each day, equivalent to 25 to 45 minutes of behavioural measurements per individual each day). To quantify activity, we counted the frequency of 180° turns over a five-min period, beginning two min after the cessation of a flushing cycle (see methods in Nadler et al., 2016a; Nadler et al., 2016b), from which the overall mean number of turns per min was calculated.

Immediately after each individual was taken out of the respirometer following $MR_{exposure\text{-}6}$ (at 7 days post-exposure), they were euthanized using an overdose of buffered (pH = 7) MS-222 (1 g L^{-1}; Sigma, St. Louis, USA). The brain was then rapidly dissected into four distinct brain regions, including the telencephalon, optic tectum, hypothalamus, and brainstem (Øverli et al., 1999), stored in 100 μL of a sodium acetate buffer (pH 5.0) containing 2,3-dihydroxybenzoic acid (DHBA,

internal standard; Sigma, St. Louis, USA) (Summers et al., 2005), and frozen on dry ice. Brain region samples were stored at -80°C until further analyses.

(e) *Monoamine analysis*

All brain samples were thawed on ice and homogenized using an ultrasonic disintegrator (QSonica Sonicators, Connecticut Newtown, USA), then centrifuged at 10,000 *g* for 10 mins at 4°C. The supernatant was analysed for the monoamine neurotransmitters serotonin (5-hydroxytraptamine, 5-HT) and dopamine (DA) as well as their catabolites (5-hydroxyindoleacetic acid, 5-HIAA, and 3,4-dihydroxyphenylacetic acid, DOPAC, respectively) using high performance liquid chromatography with electrochemical detection (HPLC-ED), following the methodology outlined in Bakke et al. (2010). Briefly, this system used a mobile phase containing 10.35 g L^{-1} NaH_2PO_4 (Sigma-Aldrich, Switzerland), 0.10 g L^{-1} octyl sulfate sodium salt (Biochemika, Fluka, Switzerland), and 0.004 g L^{-1} EDTA (Sigma-Aldrich, Switzerland) in deionized water containing 7% acetonitrile (Rathburn Chemicals, Ltd Walkerburn, Scotland), brought to pH 3.1 with phosphoric acid. Using an autoinjector (Midas, Holland Spark, The Netherlands), 50 μL of sample was injected into the HPLC-ED system and pumped at a rate of 1.3 mL/min (ESA 583 HPLC pump) through a reverse phase column (4 x 150 mm, C18, ReproSil-Pur 120 C18 5 μm Dr Maisch,). Electrochemical detection was achieved using an ESA Coulochem II detector (ESA, Bedford, MA, USA) with two electrodes at -40 mV and +320 mV. Monoamine and catabolite concentrations were calculated using standards of known concentration for all compounds. The protein content of each sample was assessed by dissolving the tissue pellet in 300 μL of 0.4M NaOH buffer and analysing using the Bradford protein assay (Vindas et al., 2014). The results are presented as ng 5-HT, 5-HIAA, DA, and DOPAC per mg^{-1} protein. In addition, the data is presented as the ratio of the catabolite to transmitter (i.e., 5-HIAA/5-HT and DOPAC/DA), as these ratios indicate an estimate of monoaminergic turnover and activity and is therefore a more direct indicator of changes in serotonergic and dopaminergic signalling (Shannon et al., 1986; Winberg and Nilsson, 1993).

(f) *Statistical analysis*

We conducted all statistical analysis in the R Statistical Environment (v3.2.4, R Development Core Team, 2016), using the packages "lme4", "emmeans", "MuMin", "car", and "lmtest". For all models, we checked that model assumptions were met by visually inspecting residual and quantile-quantile plots. Each model´s R^2 (including the marginal and conditional R^2 for mixed-effects models, indicated as R^2m and R^2c respectively) are included in the supplementary material. For all analyses of repeated-measures response variables (i.e., activity, $MR_{exposure}$, monoaminergic signalling across all brain regions), individual was included as a random effect. For all models, a likelihood ratio test (LRT) was used to determine if random effects denoting holding tank and/or testing group increased the explanatory power of the model. Those models in which the best-fit model includes these random effects are detailed below.

Aerobic metabolic rate (SMR, MMR, and AS) was analysed using linear mixed-effects models (LMM) with long-term treatment (naïve, established), and body mass (in g) as fixed effects. For SMR, activity was also included as a fixed effect and holding tank was included as a random effect in the model. Models for MMR and AS included testing group as a random effect.

Activity (measured as mean number of 180° turns per min) was analysed using a LMM, with long-term treatment, parasite exposure treatment (sham- or parasite-exposure), and day relative to parasite exposure as fixed effects (pre-exposure, day 0, day 3, day 6), and individual as a random effect.

To determine the role of parasite exposure in metabolic rate, $MR_{exposure}$ was assessed using a LMM, with long-term treatment, parasite exposure treatment, day relative to parasite exposure, activity, and body mass included as fixed effect predictors, and individual as a random effect. $MR_{exposure}$ was log-transformed to meet the assumptions of the LMM.

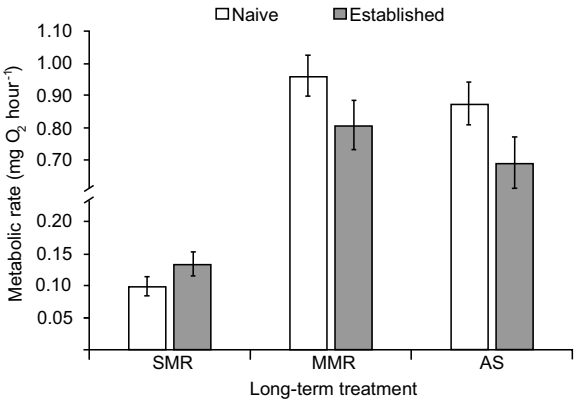
Monoaminergic signalling was analysed in two ways. First, we examined signalling across all brain regions, using a LMM for each measure of monoaminergic signalling (DOPAC/DA, 5-HIAA/5-HT, DOPAC, DA, 5-HIAA, 5-HT), with long-term treatment, parasite exposure treatment, and brain region as fixed effects, and individual as a random effect. For DOPAC, DA, and DOPAC/DA, holding tank was also included as a random effect. To meet the assumptions of homoscedasticity and normality of these tests, all traits (except 5-HT) were log-transformed. In addition, more targeted

analyses were conducted, in order to assess signalling in the brain region where each monoamine has the highest aggregation of nuclei. Dopaminergic nuclei primarily aggregate in the hypothalamus (Wulliman et al., 1996), so generalized linear models (GLM) were used to determine the role of long-term treatment and parasite exposure treatment in DOPAC, DA, and DOPAC/DA in the hypothalamus. As serotonergic nuclei primarily aggregate in the brainstem (Wulliman et al., 1996), additional GLMs were used to assess the role of long-term treatment and parasite exposure treatment in 5-HIAA, 5-HT, and 5-HIAA/5-HT in the brainstem.

Significant interactive effects discovered for all GLMs and LMMs described above were further investigated among treatments using multiple comparisons post-hoc tests with a false discovery rate (FDR) multiple testing correction to p-values. Complete model output for all statistic tests are detailed in the supplementary material.

3. Results

Long-term treatment had marginal but non-significant effects on SMR (LMM: $F_{1,6} = 2.10$, $p = 0.20$) and MMR (LMM: $F_{1,23} = 2.60$, $p = 0.12$), with SMR 35% higher and MMR 18% lower on average in established-infection compared to naïve fish (Figure 2, Table S1). This resulted in a weakly significant reduction in AS of 24% in established-infection fish (LMM: $F_{1,24} = 4.36$, $p = 0.05$; Figure 2, Table S1), suggesting *P. neurophilia* infection may moderately increase energetic needs and reduce aerobic capacity. A marginally significant trend was also revealed indicating that SMR increased with activity in both naïve and established-infection groups (LMM: $F_{1,16} = 4.73$, $p = 0.05$, Table S1).



471

472 **Figure 2.** Effect of long-term treatment (naïve vs. established infection with the microsporidian
473 parasite *Pseudoloma neurophilia*) on standard metabolic rate (SMR), maximum metabolic rate
474 (MMR) and aerobic scope (AS) of the zebrafish (*Danio rerio*; n = 30). Bars represent the estimated
475 marginal means $\pm$ s.e. (derived from the generalized linear model), controlling for body mass and
476 its interaction with long-term treatment. Moderate but non-significant effects of established
477 infection were observed for SMR and MMR ($p > 0.05$). Aerobic scope was significantly lower in
478 established-infection fish ($p = 0.048$).

479

480 Activity increased in the established treatment group (LMM: $F_{1,26} = 5.96$, $p = 0.02$), depending
481 on day post-exposure (LMM, Infection*Day interaction: $F_{3,77} = 5.06$, $p = 0.003$; Figure 3, Table S2),
482 regardless of whether fish were in the sham- or parasite-exposure treatment (LMM: $F_{1,26} = 0.73$,
483 $p = 0.40$). Post-hoc analyses indicated that naïve and established fish differed significantly at six
484 days post-exposure (FDR-corrected multiple comparison post-hoc test, naïve-day 6 vs.
485 established-day 6: $p = 0.0004$). Fish from the established treatment executed more than twice as
486 many 180° turns per minute on average than naïve fish on day 6, suggesting that fish with an
487 established *P. neurophilia* infection may increase activity in response to repeated handling stress.
488 While naïve and established fish did not differ significantly on day 3 ($p > 0.05$ for day-3 post-hoc
489 comparisons), qualitatively, we observed a marginal increase in activity on this testing day as
490 well, with established fish executing approximately 50% more turns on this testing day,

suggesting an upward trend in activity on each subsequent testing day. On day 0, we observed a moderate, but non-significant ($p > 0.05$ for all FDR-corrected postdoc comparisons between pre-exposure and day 0) reduction in activity in both long-term treatments, potentially because fish were not removed from the respirometer between the pre-exposure and day 0 measurements and thus may have been more comprehensively acclimated to the respirometer.

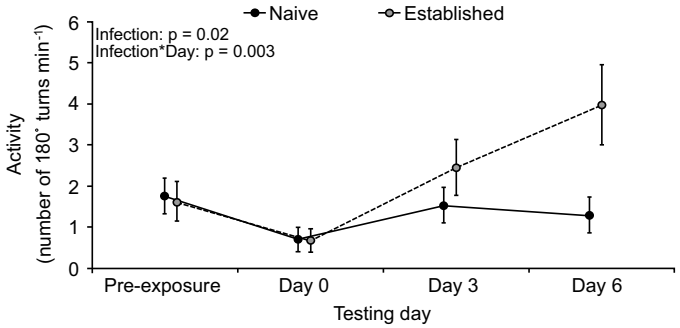
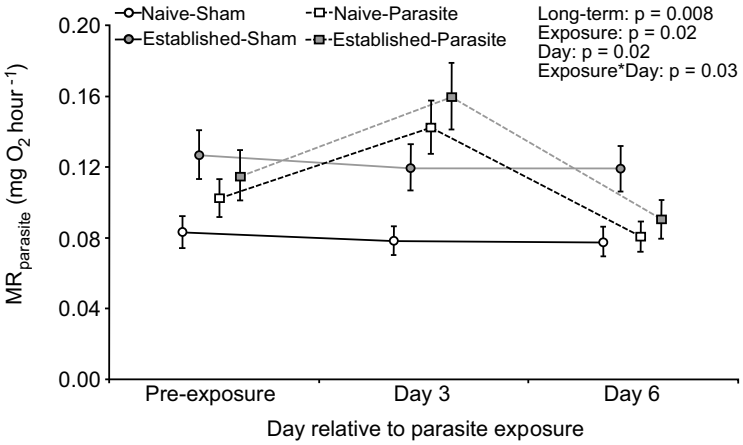


Figure 4. Effect of long-term treatment (naïve vs. established infection with the microsporidian parasite *Pseudoloma neurophilia*) and acute parasite exposure (sham- vs. parasite-exposure) on standard metabolic rate ("pre-exposure"), three days post-exposure ("Day 3") and 6 days post-exposure ("Day 6") in zebrafish hosts (*Danio rerio*; n = 30). Points represent estimated marginal means $\pm$ s.e. from linear mixed-effects model analysis, from which p-values were determined.

$MR_{exposure}$ varied with both long-term treatment and parasite exposure treatment (Table S3). Fish with an established infection exhibited higher $MR_{exposure}$ overall (LMM: $F_{1,12} = 10.13$, $p = 0.008$). However, $MR_{exposure}$ was approximately 40% higher on day 3 for all parasite-exposed fish (both the naïve-parasite and established-parasite treatment groups), compared to the naïve-sham and established-sham treatment groups (LMM, Exposure*Day interaction: $F_{2,26} = 4.25$, $p = 0.03$; $p < 0.05$ for all FDR-corrected post-hoc comparisons with day 3 parasite-exposed fish; Figure 4). No differences were detected among the treatments on day 6 ($p > 0.05$ for all FDR-corrected post-hoc comparisons). This suggests that an energetically-costly physiological response to acute parasite exposure at three days post-exposure was mitigated by six days post-exposure.



512

513 **Figure 4.** Effect of long-term treatment (naïve vs. established infection with the microsporidian
514 parasite *Pseudoloma neurophilia*) and acute parasite exposure (sham- vs. parasite-exposure) on
515 standard metabolic rate ("pre-exposure"), three days post-exposure ("Day 3") and 6 days post-
516 exposure ("Day 6") in zebrafish hosts (*Danio rerio*; n = 30). Points represent estimated marginal
517 means ± s.e. from linear mixed-effects model analysis, from which p-values were determined.

518

519 Analyses of brain monoaminergic activity across brain regions indicated significant three-way
520 interactions among long-term treatment, parasite exposure treatment, and brain region for 5-
521 HT, 5-HIAA, and DOPAC ($p < 0.05$), with marginal trends observed for 5-HIAA/5-HT (LMM: $F_{3,75} =$
522 2.55, $p = 0.06$) and DA (LMM: $F_{3,75} = 2.44$, $p = 0.07$) (Table 1, Table S4). These findings suggest
523 widespread effects of long-term treatment and parasite exposure that varied by brain region.
524 For our more targeted analyses of dopaminergic signalling in the hypothalamus (i.e., the brain
525 region in which dopaminergic nuclei are most highly aggregated), both DOPAC (GLM: $F_{1,27} = 4.49$
526 $p = 0.04$, Figure 5A, Table S5A) and DA (GLM: $F_{1,27} = 6.35$, $p = 0.02$, Figure 5B, Table S5A) decreased
527 significantly with long-term infection (by 28% and 47% for DA and DOPAC, respectively, on
528 average). For DOPAC, this effect appears to be driven largely by the naïve-parasite treatment
529 group, though post-hoc tests revealed no significant differences with that group likely due to its
530 high level of variability (FDR-corrected multiple comparison post-hoc test: $p > 0.05$ for all

comparisons with the naïve-parasite group). For DA, a significant interaction was observed between long-term treatment and parasite exposure treatment (GLM: $F_{1,25} = 5.11$, $p = 0.03$). That is, DA decreased with acute-parasite exposure in naïve fish and increased with exposure in established-infection fish (FDR-corrected multiple comparison post-hoc test, naïve-sham vs. established-sham: $p = 0.004$, $p > 0.05$ for all other comparisons). We observed no significant effects of long-term treatment, parasite exposure treatment, or their interaction on DOPAC/DA ($p > 0.05$), though we did observe non-significant, marginal increases in this ratio in the naïve-parasite treatment group (Figure 5C, Table S5A).

For our more targeted analyses of serotonergic signalling in the brainstem (i.e., the brain region in which serotonergic nuclei are most highly aggregated), 5-HT increased significantly with long-term infection (by 24% on average; GLM: $F_{1,27} = 8.08$, $p = 0.009$, Figure 5E, Table S5B), while 5-HIAA did not change with either long-term treatment or parasite exposure ($p > 0.05$, Table S5B). For 5-HIAA/5-HT, a significant long-term treatment*parasite exposure treatment interaction was revealed (GLM: $F_{1,25} = 7.75$, $p = 0.01$, Figure 5F, Table S5B), as serotonergic activity was significantly higher in the naïve-parasite group than all other groups (FDR-corrected multiple comparison post-hoc test: $p = 0.003$, $p = 0.02$, and $p = 0.003$ for comparisons of the naïve-parasite treatment with the naïve-sham, established-sham, and established-parasite treatments, respectively). This difference in 5-HIAA/5-HT in the naïve-parasite treatment group appears to be driven by a non-significant decline in 5-HT and increase in 5-HIAA compared to the naïve-sham treatment group.

Table 1. Summary table for dopaminergic and serotonergic signalling in all sampled brain regions (mean ± s.e., in ng mg^{-1} protein), including the concentrations of dopamine (DA), its catabolite 3,4-dihydroxyphenylacetic acid (DOPAC), and their ratio (DOPAC/DA) as well as the concentration of serotonin (5-HT), its catabolite 5-hydroxyindoleacetic acid (5-HIAA), and their ratio (5-HIAA/5-HT).

Brain Region	Treatment	[DOPAC/DA]	[5-HIAA/5-HT]	[DOPAC]	[5-HIAA]	[DA]	[5-HT]
Brainstem	Naïve-Sham	0.94 ± 0.03	0.96 ± 0.03	3.00 ± 0.15	4.32 ± 0.13	3.93 ± 0.20	4.53 ± 0.35
	Naïve-Parasite	0.21 ± 0.37	0.65 ± 0.05	0.99 ± 1.06	3.57 ± 0.45	4.75 ± 0.38	5.56 ± 0.39
	Established-Sham	0.18 ± 0.07	0.68 ± 0.03	0.76 ± 0.49	4.35 ± 0.51	4.53 ± 0.53	6.46 ± 0.54
	Established-Parasite	0.20 ± 0.06	0.70 ± 0.15	1.16 ± 0.23	4.31 ± 0.82	5.11 ± 0.40	6.09 ± 0.41
Hypothalamus	Naïve-Sham	0.03 ± 0.00	0.25 ± 0.01	0.43 ± 0.07	4.40 ± 0.55	14.26 ± 1.95	17.70 ± 2.48
	Naïve-Parasite	0.29 ± 0.15	0.43 ± 0.04	2.02 ± 0.89	5.20 ± 0.45	10.37 ± 1.27	12.68 ± 1.40
	Established-Sham	0.05 ± 0.02	0.35 ± 0.03	0.39 ± 0.17	2.93 ± 0.26	7.57 ± 0.52	8.52 ± 0.70
	Established-Parasite	0.03 ± 0.01	0.33 ± 0.03	0.30 ± 0.06	4.49 ± 0.77	10.11 ± 1.23	13.89 ± 2.13
Optic tectum	Naïve-Sham	0.33 ± 0.04	0.58 ± 0.05	0.39 ± 0.06	1.27 ± 0.11	1.13 ± 0.10	2.26 ± 0.22
	Naïve-Parasite	2.28 ± 0.91	0.97 ± 0.11	1.62 ± 0.60	1.76 ± 0.18	0.96 ± 0.12	1.90 ± 0.19
	Established-Sham	0.51 ± 0.16	0.86 ± 0.12	0.66 ± 0.25	1.84 ± 0.21	1.25 ± 0.12	2.21 ± 0.13
	Established-Parasite	0.31 ± 0.09	0.79 ± 0.08	0.36 ± 0.14	1.34 ± 0.15	1.12 ± 0.18	1.75 ± 0.19
Telencephalon	Naïve-Sham	0.14 ± 0.01	0.82 ± 0.05	0.35 ± 0.04	2.87 ± 0.20	2.55 ± 0.23	3.48 ± 0.18
	Naïve-Parasite	0.76 ± 0.34	1.53 ± 0.22	1.39 ± 0.52	4.42 ± 0.51	2.30 ± 0.27	3.09 ± 0.34
	Established-Sham	0.22 ± 0.04	1.55 ± 0.14	0.64 ± 0.13	4.20 ± 0.62	2.96 ± 0.25	2.83 ± 0.45
	Established-Parasite	0.12 ± 0.02	0.99 ± 0.13	0.30 ± 0.06	3.82 ± 0.56	2.54 ± 0.39	2.85 ± 0.23

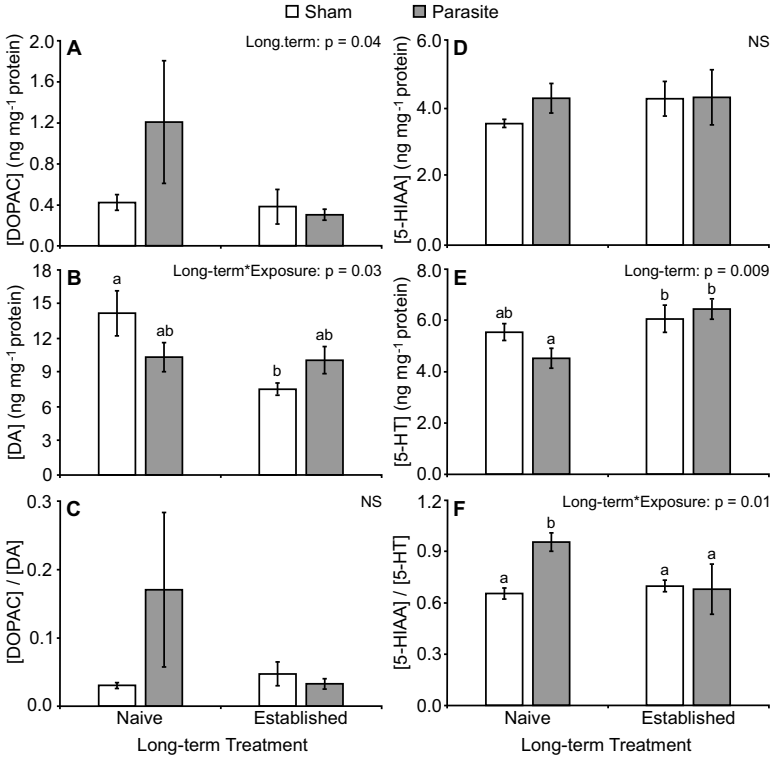


Figure 5. Effect of long-term treatment (naïve vs. established infection with the microsporidian parasite *Pseudoloma neurophilia*) and acute parasite exposure (sham- vs. parasite-exposure) on monoaminergic activity in zebrafish hosts (*Danio rerio*; n = 30). Dopaminergic activity (in the hypothalamus) is calculated as the ratio of 3,4-dihydroxyphenylacetic acid (DOPAC, the principal dopamine catabolite) to dopamine (DA). Serotonergic activity (in the brainstem) is calculated as the ratio of 5-hydroxyindoleacetic acid (5-HIAA, the principal serotonin catabolite) to serotonin (5-HT). Bars represent estimated mean ± s.e. P-values were determined using generalized linear model analysis and letters above bars represent significant differences ($p < 0.05$) detected using FDR-corrected multiple comparisons post-hoc tests.

4. Discussion

These findings demonstrate that the physiological and behavioural processes stimulated in response to parasite exposure vary depending on previous *P. neurophilia* infection status in laboratory zebrafish. While fish with an established parasite infection modulated aerobic metabolic capacity and behaviour, acute parasite exposure substantially altered metabolic rate, but only within the first three days post-exposure, with metabolic rate returning to pre-exposure levels by day 6. For monoaminergic signalling, interactive effects of long-term treatment and parasite exposure appear to drive the response, with dopaminergic and serotonergic signalling shifting in previously naïve fish experiencing a novel parasite exposure (i.e., naïve-parasite treatment). These results highlight the role of infection history in modulating the metabolic, behavioural and neurophysiological response to parasite exposure, and underscore the importance of evaluating multiple levels of biological organization in a time-series post-exposure to capture the range of compensatory responses employed.

The metabolic response to parasite exposure (i.e., $MR_{exposure}$) spiked at three days post-exposure, regardless of long-term treatment, with metabolic rate approximately 40% higher than sham-exposed fish. Studies in other host-parasite systems have previously observed elevated metabolic rate in the short-term (i.e., minutes and hours) following parasite exposure (Nadler et al., in review; Luong et al., 2017; Voutilainen et al., 2008). However, the parasites in these other studies (including killifish-trematode, fly-ectoparasite, and salmonid-trematode host-parasite systems) are easier to detect by sensory cues (e.g., visual, olfactory, or tactile cues), and produce some skin tissue damage upon attachment/penetration to a new host (Luong et al., 2017; Voutilainen et al., 2008). Therefore, these types of parasites could stimulate more rapid mechanisms for parasite avoidance compared to what would be expected by *P. neurophilia*. Here, we show for the first time that similar changes in metabolic rate can be stimulated in the days following parasite exposure in a microscopic, microsporidian parasite, potentially due to a pro-inflammatory response to the parasite as it migrates from its point of entry to its infection endpoint in the CNS (Chen et al., 2018).

Indeed, molecular studies indicate that genes associated with a pro-inflammatory immune response are upregulated in fish with a *P. neurophilia* established infection (Midttun et al., in

598 press). A pro-inflammatory response could therefore contribute to both the higher overall
599 $MR_{exposure}$ across all testing days with established infection as well as the spike in $MR_{exposure}$ at
600 three days post parasite-exposure. Notably, in another microsporidian system, spores of
601 *Encephalitozoon* spp. increased production of the cytokines TFN-α, INF-γ and IL-10 in human
602 macrophages, which are important in defence against intracellular pathogens (Franzen et al.,
603 2005). Extrapolating these results to our experiment, it is possible that similar mechanisms may
604 have contributed to the trends observed here. Previous histological studies in zebrafish primarily
605 detected the parasite in organs outside of the CNS at three days post-exposure, including the
606 intestinal lumen, pancreas, kidney, liver, and pharynx (Cali et al., 2012; Sanders et al., 2014). As
607 such, we hypothesize that the energetically-costly response detected here stems from
608 physiological processes occurring in these organs and seem to be mitigated by six days post-
609 exposure, as metabolic rate returned to pre-exposure levels by this time point. However, as
610 histological analyses over a similar time scale were not conducted here, we cannot confirm the
611 locality of the parasite on each testing day, and hence it is possible that the progression of the
612 infection through the body was either faster or slower than previous work. As such, studies
613 examining the infection´s progression through time in conjunction with investigations into the
614 mechanisms driving the spike in metabolic rate at particular time points post-exposure would
615 constitute worthy avenues for future work.
616 Established infection caused a marked two-fold increase in activity in zebrafish, regardless of
617 exposure treatment, but only in the final days of respirometry testing. This result was surprising
618 for a number of reasons. First, we would have expected changes in activity to mirror the trends
619 observed for metabolic rate through time, which peaked on day-3 in parasite-exposed fish, as
620 described above. However, activity levels were generally low in all treatments in part due to the
621 limited space available in the respirometer for movement, peaking in established-infection
622 zebrafish on day 6 at a frequency of four 180° turns per minute. This low frequency of turns is
623 unlikely to amount to strenuous exercise, and as such may not have had a strong enough effect
624 on $MR_{exposure}$ to outweigh the effects of parasite exposure and long-term treatment. For
625 comparison, a tropical coral-reef fish species executed more than twice as many turns on average
626 under routine conditions (~10 turns/min), when using a comparable methodology to quantify

activity (Nadler et al., 2016b). Second, Midttun et al. (in press) reported an overall reduction in locomotor activity in *P. neurophilia*-infected zebrafish in a range of neurobehavioral assays (e.g. light-dark preference test, open field test and social preference test). Thus, our findings suggest that *P. neurophilia* may induce context-specific changes in behaviour due to some combination of potentially non-exclusive mechanisms, for example, responses to multiple stressors, mechanisms to compensate for infection, manipulation by parasites to increase survival, or neuromodulatory effects of glucocorticoid stress hormones (reviewed in Defolie et al., 2019). Here, zebrafish of all treatments would have experienced a combination of minor stressors related to the respirometry protocol over the week-long experimental period (Martins et al., 2011; Nadler et al., 2016a), including handling stress each time they were transferred to the respirometer as well as isolation and confinement stress while in the respirometer. The combination of these stressors appears to promote moderate increases in activity, but only in combination with *P. neurophilia* infection. This result is in line with previous behavioural studies in this host-parasite system (Spagnoli et al., 2015), which reported that *P. neurophilia*-infected zebrafish exhibit reduced startle-response habituation to a threat stimulus through time, executing high-velocity startle responses even following repeated stimulation. This interactive effect of stress and *P. neurophilia* infection could have important consequences for individual fitness, with Ramsay et al. (2009) reporting increased rate of stress-induced mortality with *P. neurophilia* infection relative to naïve zebrafish. Taken together, our findings, in conjunction with past studies, indicate a complex interplay among behaviour, stress, and *P. neurophilia* infection that could considerably alter an individual´s behavioural and physiological phenotypes.

Long-term treatment had moderate effects on aerobic metabolic capacity, with significant differences between naïve and established-infection observed for AS. These moderate changes in metabolic rate, in conjunction with effects on $MR_{exposure}$ following parasite exposure, could contribute to the reduced body size and condition observed with established infection in past studies (Midttun et al., in press; Sanders et al., 2020). Established infection with *P. neurophilia* may also be energetically costly due to sporadic bursts in inflammation. Indeed, Apicomplexan parasitophorous vacuoles that are highly similar to *P. neurophilia* parasite clusters are known to intermittently rupture, causing a pro-inflammatory response to address damage to the

surrounding tissue (Rodriguez-Tovar et al., 2011). However, given the relatively weak statistical connection between aerobic metabolic traits (i.e., SMR, MMR, AS) and long-term treatment, it is likely that the metabolic costs associated with novel or renewed parasite exposure may exceed those incurred from established infection. Our findings therefore highlight the necessity for further studies differentiating the physiological processes stimulated by parasite exposure versus those dictated by established infection.

Monoaminergic signalling shifted primarily in previously naïve fish experiencing their first parasite exposure. Evidence from a broad range of taxa, from fish to mammals, suggests that monoaminergic signalling changes in response to stress and immune challenges (Delrue et al., 1994; Haukenes et al., 2011). These changes could consequently drive a suite of behavioural effects in the short-term due to the importance of these brain signalling systems in behavioural phenotypes, including social behaviour (Scerbina et al., 2012), aggression (Teles et al., 2013), learning (Messias et al., 2016), and activity (Mok and Munro, 1998), among others (reviewed in Summers and Winberg, 2006; Winberg and Nilsson, 1993; Winberg and Thörnqvist, 2016). Serotonergic activity increased significantly in naïve-parasite fish, driven by moderate, but non-significant reductions in available 5-HT stores and increases in 5-HIAA catabolite levels. Similarly, dopaminergic activity exhibited a non-significant but moderate increase in fish from the naïve-parasite treatment, resulting in a spike in DOPAC concentration and a reduction in available DA (though neither of these changes were statistically different from the naïve-sham treatment). Both of these results indicate that naïve-parasite fish are using a higher fraction of their available neurotransmitter to maintain each monoamine´s respective rate of neurotransmission, a condition typically detected in animals recovering from a highly stressful challenge (Griffiths et al., 2012; Song et al., 2015). The fact that much of the effects of novel acute parasite exposure are mitigated upon renewed exposure (i.e., as seen in the established-parasite treatment) may work to enhance the parasite´s fitness. Depleting neurotransmitter stores are known to reduce sociability in fish (Andrews et al., 2015), which would limit the host´s capacity for direct transmission of *P. neurophilia* to new hosts. While the effects of parasite exposure and long-term treatment on monoaminergic activity differ from those observed for metabolic rate, it is also important to note that sampling of zebrafish brains for this study occurred following

measurement of $MR_{exposure-6}$, on the seventh day post-exposure. As $MR_{exposure}$ peaked three days post-exposure, further studies on dopaminergic and serotonergic activity at this key time point may uncover more explicit links to the metabolic response.

In summary, we examined how the microsporidian parasite, *P. neurophilia*, alters the energetics and brain signalling of its host, zebrafish. Both previously naïve zebrafish and zebrafish with an established infection exhibited spikes in metabolic rate at three days post-acute parasite exposure, indicating that this response is driven at least in part by mechanisms of innate immunity (Rodriguez-Tovar et al., 2011). However, previously infected fish exhibited context-specific increases in activity following repeated experimental testing that were absent in naïve fish, suggesting that stress induced behavioural changes in fish with an established *P. neurophilia.* Established infection also had moderate impacts on aerobic metabolic rate, which could contribute to the previously reported effects of long-term *P. neurophilia*-infection on body size and condition (Midttun et al., in press; Sanders et al., 2020). Despite these diverging effects of long-term treatment and parasite exposure on metabolic rate and behaviour, brain monoaminergic signalling was impacted primarily due to an interaction of these effects, with the most substantial changes in dopaminergic and serotonergic activity observed in previously naïve fish following novel parasite exposure. As established-parasite treatment fish did not exhibit similar alterations in monoaminergic activity, this result suggests that zebrafish develop mechanisms to prevent these stress-induced changes in neurophysiology upon subsequent encounters with *P. neurophilia*-spores, potentially through mechanisms associated with adaptive immunity (Rodriguez-Tovar et al., 2011). Our results reveal that host responses to infectious parasite stages vary at multiple levels of biological organization, including behaviour, whole-organism metabolic rate and brain signalling. Yet, importantly, these responses depend on the host´s own unique parasite exposure history and current infection status, highlighting a previously overlooked driver of individual variability in host responses to parasites.

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

Data accessibility. All data and code are available through the NMBU Open Research Data repository.

Authors´ contribution. L.E.N., H.L.E.M., and I.B.J. designed this research study. H.L.E.M. and I.B.J. designed the long-term infection procedure. H.L.E.M. performed the fish rearing and long-term infection procedures. S.S.K. provided equipment and advice on protocols for respirometry studies. L.N., H.L.E.M., and I.B.J. conducted the respirometry trials and acute parasite exposures. L.N., H.L.E.M., M.A.V. and I.B.J. sampled the fish brain tissue. M.A.V. and Ø.Ø. contributed to designing the procedures for HPLC-ED analyses of brain tissue. L.E.N. performed the HPLC-ED analyses, analysed the respirometry data and quantified behaviour from videos. S.S.K. assisted with analysis and interpretation of the respirometry data. L.N. wrote the initial manuscript. All authors critically revised the manuscript.

Competing interests. We declare that we have no competing interests.

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Supplementary tables

Table S1. Effect tests from linear mixed-effects model analysis of zebrafish (*Danio rerio*) metabolic traits in response to body mass (BM), long-term treatment (naïve vs. established parasite infection with the microsporida *Pseudoloma neurophilia*), and their interaction. Traits measured include standard metabolic rate (SMR), maximum metabolic rate (MMR) and aerobic scope (AS) (n = 29 individuals). For SMR, activity (and relevant interactions) was also included in as a fixed effect in the model and each fish´s holding tank was included as a random effect. For MMR and AS, each fish´s testing group was included as a random effect. The final column indicates the models´ marginal (R^2m) and conditional (R^2c) R^2 values.

Trait	**Factor**	**d.f.**	**F-value**	**p-value**	**R^2m, R^2c**
SMR	Body mass (BM)	1,16	24.07	0.0002	0.38, 0.66
	Long-term	1,6	2.10	0.20	
	Activity	1,16	4.73	0.05	
	BM*Long-term	1,21	0.02	0.88	
	BM*Activity	1,19	0.55	0.47	
	Long-term*Activity	1,17	0.19	0.67	
	BM*Long-term*Activity	1,18	0.18	0.67	
MMR	BM	1,23	7.51	0.01	0.36, 0.53
	Long-term	1,23	2.60	0.12	
	BM*Long-term	1,22	0.26	0.61	
AS	BM	1,23	3.80	0.06	0.34, 0.55
	Long-term	1,24	4.36	0.05	
	BM*Long-term	1,23	0.14	0.70	

Table S2. Effect test (using linear mixed-effects model analysis) assessing variation in activity (measured as mean number of 180° turns per min) with long-term treatment (naïve vs. established parasite infection with the microsporida *Pseudoloma neurophilia*) and acute parasite exposure (sham- vs. parasite-exposure) in zebrafish hosts (*Danio rerio*; n = 30). Due to the repeated-measures nature of the activity data, individual was included as a random effect. The final column indicates the model´s marginal (R^2m) and conditional (R^2c) R^2 values.

Trait	**Factor**	**d.f.**	**F-value**	**p-value**	**R^2m, R^2c**
Activity	Long-term	1,26	5.96	0.02	0.28, 0.35
	Exposure	1,26	0.73	0.40	
	Day	3,77	7.07	0.0003	
	Long-term*Exposure	1,26	0.08	0.78	
	Long-term*Day	3,77	5.06	0.003	
	Exposure*Day	3,77	0.24	0.87	
	Long-term*Exposure*Day	3,77	0.89	0.45	

Table S3. Effect test from linear mixed-effects model analysis of zebrafish (*Danio rerio*) metabolic rate follow parasite exposure ($MR_{exposure}$) in response to body mass (BM), long-term treatment (naïve vs. established parasite infection with the microsporida *Pseudoloma neurophilia*), parasite exposure treatment (sham- vs. parasite exposure), day post-exposure ("Day"), activity (measured as mean number of 180° turns per min), and all relevant interactions (n = 29 individuals). Due to the repeated-measures nature of the activity data, individual was included as a random effect. The final column indicates the model´s marginal (R^2m) and conditional (R^2c) R^2 values.

Trait	**Factor**	**d.f.**	**F-value**	**p-value**	**R^2m, R^2c**
$MR_{exposure}$	Body mass (BM)	1,13	44.64	< 0.0001	0.60, 0.61
	Long-term	1,12	10.13	0.008	
	Exposure	1,14	6.56	0.02	
	Day	2,23	4.46	0.02	
	Activity	1,39	4.05	0.05	
	BM*Long-term	1,20	0.37	0.55	
	BM*Exposure	1,15	0.66	0.43	
	Long-term*Exposure	1,14	6.35	0.02	
	BM*Day	2,26	0.62	0.54	
	Infection*Day	2,24	0.48	0.62	
	Exposure*Day	2,26	4.25	0.03	
	BM*Activity	1,39	1.14	0.29	
	Long-term*Activity	1,37	0.08	0.77	
	Exposure*Activity	1,38	0.03	0.86	
	Day*Activity	2,36	0.42	0.66	
	BM*Long-term*Exposure	1,23	0.24	0.63	
	BM*Long-term*Day	2,34	0.77	0.47	
	BM*Exposure*Day	2,28	0.23	0.79	
	Long-term*Exposure*Day	2,26	1.67	0.21	
	BM*Long-term*Activity	1,39	1.23	0.27	
	BM*Exposure*Activity	1,39	2.18	0.15	
	Long-term*Exposure*Activity	1,39	4.58	0.04	
	BM*Day*Activity	2,37	1.58	0.22	
	Long-term*Day*Activity	2,37	1.15	0.33	
	Exposure*Day*Activity	2,36	0.63	0.54	
	BM*Long-term*Exposure*Day	2,34	4.13	0.02	
	BM*Long-term*Exposure*Activity	1,39	1.57	0.22	
	BM*Long-term*Day*Activity	2,38	0.96	0.39	
	BM*Exposure*Day*Activity	2,39	0.18	0.83	
	Long-term*Exposure*Day*Activity	2,38	0.70	0.50	
	BM*Long-term*Exposure*Day*Activity	2,38	0.24	0.79	

Table S4. Effect tests (using linear mixed-effects model analysis) assessing variation in whole brain monoaminergic activity (including serotonin, its catabolite, 5-hydroxyindole acetic acid, and their ratio, as well as dopamine and its catabolite, 3,4-Dihydroxyphenylacetic acid, and their ratio) with long-term treatment (naïve vs. established parasite infection with the microsporida *Pseudoloma neurophilia*), parasite exposure treatment (sham- vs. parasite exposure), brain region (telencephalon, optic tectum, hypothalamus, brainstem), and all relevant interactions. For all traits, individual was included as a random effects due to the repeated measures nature of the data. For all traits associated with dopaminergic signalling, holding tank was also included as a random effect. The final column indicates the model´s marginal (R^2m) and conditional (R^2c) R^2 values.

Trait	**Factor**	**d.f.**	**F-value**	**p-value**	**R^2m, R^2c**
Serotonin (5-HT)	Long-term	1,25	2.24	0.15	0.77, 0.78
	Exposure	1,25	0.21	0.65	
	Brain region	3,75	116.08	< 0.0001	
	Long-term*Exposure	1,25	10.11	0.004	
	Long-term*Brain region	3,75	6.28	0.0007	
	Exposure*Brain region	3,75	0.11	0.95	
	Long-term*Exposure*Brain Region	3,75	6.46	0.0006	
5-hydroxyindole acetic acid (5-HIAA)	Long-term	1,25	0.03	0.87	0.69, 0.83
	Exposure	1,25	2.74	0.11	
	Brain region	3,75	134.62	< 0.0001	
	Long-term*Exposure	1,25	2.56	0.12	
	Long-term*Brain region	3,75	4.98	0.003	
	Exposure*Brain region	3,75	1.62	0.19	
	Long-term*Exposure*Brain Region	3,75	4.77	0.004	
5-HIAA/5-HT	Long-term	1,25	0.13	0.72	0.79, 0.87
	Exposure	1,25	7.18	0.01	
	Brain region	3,75	188.03	< 0.0001	
	Long-term*Exposure	1,25	21.34	< 0.0001	
	Long-term*Brain region	3,75	2.89	0.04	
	Exposure*Brain region	3,75	0.83	0.48	
	Long-term*Exposure*Brain Region	3,75	2.55	0.06	
Dopamine (DA)	Long-term	1,5	0.01	0.93	0.87, 0.93
	Exposure	1,22	0.47	0.50	
	Brain region	3,75	452.39	< 0.0001	
	Long-term*Exposure	1,7	0.52	0.50	
	Long-term*Brain region	3,75	5.67	0.001	
	Exposure*Brain region	3,75	0.38	0.77	
	Long-term*Exposure*Brain Region	3,75	2.44	0.07	
3,4-Dihydroxyphenylacetic acid (DOPAC)	Long-term	1,5	1.09	0.35	0.30, 0.79
	Exposure	1,18	0.45	0.51	

	Brain region	3,75	22.57	< 0.0001	
	Long-term*Exposure	1,7	1.18	0.31	
	Long-term*Brain region	3,75	0.71	0.55	
	Exposure*Brain region	3,75	0.15	0.93	
	Long-term*Exposure*Brain Region	3,75	3.60	0.02	
DOPAC/DA	Long-term	1,5	0.94	0.38	0.56, 0.91
	Exposure	1,18	0.67	0.42	
	Brain region	3,75	180.66	< 0.0001	
	Long-term*Exposure	1,7	1.43	0.27	
	Long-term*Brain region	3,75	1.46	0.23	
	Exposure*Brain region	3,75	0.19	0.90	
	Long-term*Exposure*Brain Region	3,75	0.12	0.12	

Table S5. Effect tests assessing variation in (A) brainstem serotonergic signalling (serotonin, its catabolite 5-hydroxyindole acetic acid, and their ratio) and (B) hypothalamus dopaminergic signalling (dopamine, its catabolite 3,4-Dihydroxyphenylacetic acid, and their ratio) with long-term infection treatment (naïve vs. established parasite infection with the microsporida *Pseudoloma neurophilia*), parasite exposure treatment (sham- vs. parasite-exposure) and their interaction for the zebrafish (*Danio rerio*). The brain regions were chosen for this targeted analysis as they represent the areas of greatest aggregation of relevant nuclei. The final column indicates the models' R^2 values.

(A)

Trait (Hypothalamus)	**Factor**	**d.f.**	**F-value**	**p-value**	**R^2**
Dopamine (DA)	Long-term	1,27	6.35	0.02	0.30
	Exposure	1,26	0.52	0.48	
	Long-term*Exposure	1,25	5.11	0.03	
3,4-Dihydroxyphenylacetic acid (DOPAC)	Long-term	1,27	4.49	0.04	0.18
	Exposure	1,26	1.26	0.27	
	Long-term*Exposure	1,25	0.38	0.38	
DOPAC/DA	Long-term	1,27	1.07	0.31	0.14
	Exposure	1,26	1.45	0.24	
	Long-term*Exposure	1,25	2.10	0.16	

(B)

Trait (Brainstem)	**Factor**	**d.f.**	**F-value**	**p-value**	**R^2**
Serotonin (5-HT)	Long-term	1,27	8.08	0.009	0.29
	Exposure	1,26	0.89	0.35	
	Long-term*Exposure	1,25	2.66	0.12	
5-hydroxyindole acetic acid (5-HIAA)	Long-term	1,27	0.60	0.44	0.06
	Exposure	1,26	0.79	0.38	
	Long-term*Exposure	1,25	0.53	0.47	

5-HIAA/5-HT	Long-term	1,27	3.79	0.06	0.35
	Exposure	1,26	3.78	0.06	
	Long-term*Exposure	1,25	7.75	0.01	

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