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RESEARCH ARTICLE

Mechanisms and Consequences of Infection-induced Phenotypes

Brain-infecting parasites leave lasting effects on behaviour even in resistant hosts

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Abstract

1. Parasites can have profound effects on intra- and interspecific interactions at the population and community levels through their influence on host behaviour, physiology and fitness. While host phenotypic changes are typically thought of in terms of established infections, parasite encounters may be sufficient to induce behavioural changes, even when no viable infections are established.
2. Here, we use the Japanese rice fish medaka *Oryzias latipes* and the brain-infecting microsporidan parasite *Pseudoloma neurophilia* to understand how parasite resistance influences behaviour.
3. Although a previous study suggested that medaka are a suitable host for *P. neurophilia*, an eight-week parasite exposure regime resulted in no detectable infection in our study. Both parasite-exposed and control (no parasite exposure) medaka were tested in behavioural assays that assessed boldness, activity and sociality. We detected considerable changes in medaka behaviour following parasite exposure, with parasite-exposed fish being more active, less bold and more social when compared to control fish.
4. These data indicate that parasite encounters may induce behavioural alterations even in non-susceptible hosts. In addition to established infection, individual differences in parasite exposure must also be considered in studies of host responses across ecological scales.

KEYWORDS
established infection, host resistance, host susceptibility, host–parasite interactions, infection history, infection-induced phenotypes, parasite exposure, *Pseudoloma neurophilia*

1 | INTRODUCTION

Parasites are now recognized as important players in ecological communities, as they contribute substantially to shaping both intra- and interspecific interactions. Beyond the direct consumptive effects of parasites on their host (i.e. increase in energy consumption by the host to fight the parasite), parasites may exert numerous indirect, nonconsumptive effects by eliciting a myriad of host trait responses.

These trait responses, defined as adaptive morphological, physiological and behavioural changes (Daversa et al., 2021), may affect individual fitness or cause indirect effects across ecological scales. In recent years, studies began focusing on host trait responses to parasitism that extend beyond the period of active infection.

Indeed, host trait responses to parasites may occur before, during or after infection, or even in the absence of successful infections (Buck et al., 2018). First, susceptible hosts may limit the risk of encountering parasites by altering their behaviour. For example, to avoid infection risk, animals may change their social interactions (e.g. avoiding infected animals), their territories (e.g. avoiding parasite infested locations) or their resources (e.g. using materials that may repel parasites), all of which may lead to repercussions at multiple trophic levels (Buck et al., 2018). For example, Nadler et al. (2021) showed that acute exposure to parasites results in an increase in activity and metabolic rate in both naïve and chronically infected hosts. Second, hosts can respond in ways to minimize the attacking parasite's damage, such as mounting an immune response that enhances tissue repair or even eradicates the parasite. Immune responses to infection are often accompanied by behavioural alterations. Sickness behaviours, for instance, are triggered by an immune response and are characterized by changes in activity, exploration and sociability (Lopes et al., 2021). This suite of behaviours is generally regarded as a host strategy to: (1) redirect energy resources towards the immune system to fight the infection and (2) decrease transmission risk to conspecifics (Eisenberger et al., 2017; Hennessy et al., 2014; Kirsten et al., 2018). Third, parasites may even elicit trait responses in nonsusceptible hosts. For example, the parasitoid *Aphidius colemani* elicits this kind of impact in a nontarget host, the pea aphid *Acyrthosiphon pisum*. While attacking its target host, green peach aphids *Myzus persicae*, the parasitoid elicits defensive action by the pea aphid, leading to a reduced population growth rate in this unsuitable host (Fill et al., 2012). However, few studies have examined whether parasite encounters can affect the phenotype of resistant hosts.

Parasite resistance is a measure of a host's ability to reduce or prevent parasite establishment (Daversa et al., 2021; Rohr et al., 2010). Indeed, like in the case of active infections, immune responses are often mounted following parasite encounters even if they do not result in the establishment of an infection (Buck et al., 2018). Given the wealth of evidence linking host immunity and behaviour, mere parasite encounters could potentially involve host responses related to immune activation and subsequent behavioural alterations. We do not yet know if parasite encounters can elicit trait responses in nonsusceptible hosts not related to active parasite avoidance. Such responses could represent an additional dimension to the current trait response framework and aid our understanding of host–parasite interactions across ecological scales (Buck et al., 2018; Daversa et al., 2021). Notably, there is evidence that parasitic wasps induce transgenerational epigenetic changes in their *Drosophilla melanogaster* hosts, which illustrates the long-term effects of parasite exposure and infection (Montanari & Royet, 2021).

Here, we use the Japanese rice fish medaka *Oryzias latipes* and the common brain-infecting microsporidian parasite *Pseudoloma neurophilia* to understand how parasite resistance may influence behavioural trait responses. Medaka is a small freshwater fish native to Far East Asia that is popular in both the aquarium trade and as an alternative to zebrafish *Danio rerio* as a model species in animal research (Kinoshita et al., 2009; Wittbrodt et al., 2002). In its natural habitat, medaka harbour a variety of parasites (Wittbrodt et al., 2002). Published evidence about the susceptibility of lab-reared medaka to parasites varies. While one study suggested that medaka are suitable hosts for *P. neurophilia* (Sanders et al., 2016), surveys of medaka facilities have not detected *P. neurophilia* in sampled fish, while it has become a common problematic pathogen in zebrafish facilities globally (Legendre et al., 2016). In its suitable zebrafish host, chronic *P. neurophilia* infection is associated with a cerebral pro-inflammatory immune response (Midttun, Vindas, Whatmore, et al., 2020), anorexia, reduced activity and increased freezing behaviour (Midttun, Vindas, Nadler, et al., 2020). In a recent publication, it was proposed that the immune and behavioural responses associated with *P. neurophilia* infection can be attributed to sickness behaviour induced by the host's anti-parasite response (Midttun, Vindas, Whatmore, et al., 2020). However, whether similar trait responses are initiated following parasite encounters that do not result in successful infection remains unknown.

To examine the effects of host resistance on various behavioural traits in medaka, we adopted a previously used zebrafish—*P. neurophilia*—infection protocol for 8 weeks. Approximately 2 weeks after this parasite exposure regime concluded, we subjected both parasite-exposed and control (no parasite exposure) fish to open field and mirror test behavioural assays. These tests are commonly used to assess behavioural traits, including boldness, exploratio, and sociability (Godwin et al., 2012; Pham et al., 2012), which were previously found to be altered by *P. neurophilia* infection in zebrafish (Midttun, Vindas, Nadler, et al., 2020). We hypothesized that parasite exposure could induce behavioural trait responses in resistant medaka, similar to those observed with established infections. That is, the immune response initiated by medaka to fight the parasite would likely lead to similar behavioural changes as zebrafish, even if, unlike in zebrafish, the medaka are able to eradicate the parasite.

2 | MATERIALS AND METHODS

2.1 | Ethics

Our work was approved by the Norwegian Animal Research Authority (NARA), following the Norwegian laws and regulations controlling experiments and procedures on live animals (permit number 11241 granted 2017).

2.2 | Fish husbandry

All experiments were performed at the Norwegian University of Life Sciences, campus Adamstuen (Oslo, Norway). A total of 413 medaka

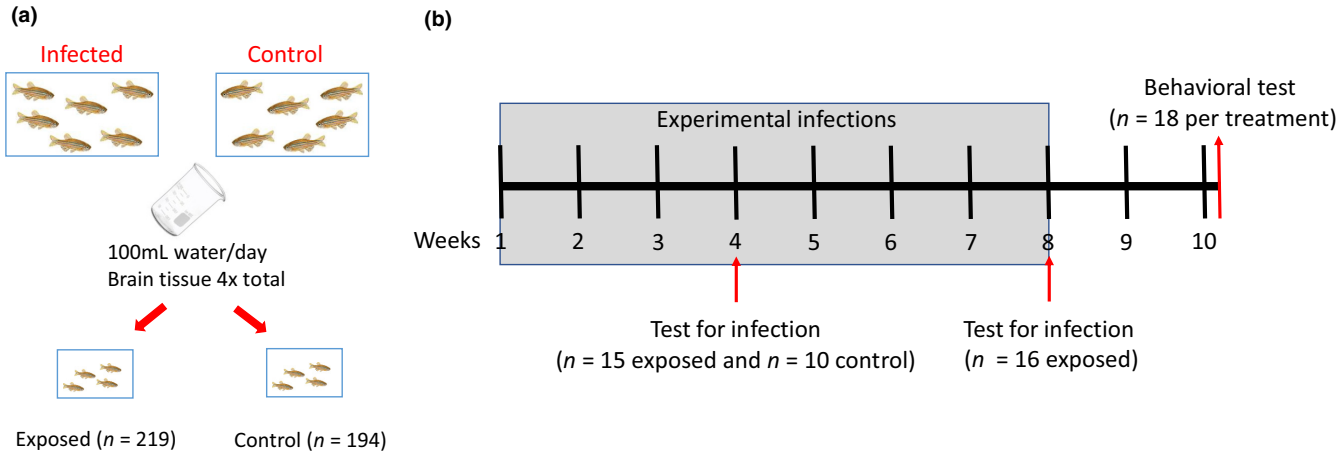

FIGURE 1 Diagram showing the infection in parasite exposed (*Pseudoloma neurophilia*) and control (no parasite exposure) medaka *Oryzias latipes* treatment protocols (a) and the timeline for the experimental design (b).

were obtained from the medaka rearing facility at Adamstuen, NMBU and were split across the control (n = 194) and parasite-exposed (n = 219) treatments. A subset of each experimental group was used for the study described here. Due to logistical reasons, we were only able to use females in this study. At 6 months post-hatch, fish were moved from the medaka rearing facility to a quarantine room where they were maintained in 3 L tanks (23 cm × 15.3 cm × 16.5 cm) in groups composed of 13–14 fish, which matched the group size and density in their holding tanks during rearing. The tanks were filled with filtered, UV-treated water and provided continuous aeration. A full water change was completed three times weekly to further maintain high standards of water quality. Water was maintained at 28°C, pH 7.5 and conductivity 800 µS following husbandry practices recommended for medaka (Kinoshita et al., 2009). All fish were fed a dry feed once daily (Gemma Micro, Skretting, Stavanger, Norway). The light: dark cycle was kept at 14 h light:10 h dark for the duration of the study.

2.3 | Infection protocol

We assigned medaka to treatment groups systematically so that groups were composed of unrelated individuals. Concurrently, *P. neurophilia*-infected zebrafish (confirmed via qPCR procedure described below) were maintained in a 25 L tank (40 cm × 25 cm × 25 cm; L × W × H).

Experimental infections were conducted over an 8-week period. During this time, in each medaka holding tank in the parasite-exposed treatment, 100 ml of water was replaced each day with 100 ml of water from the tank containing *P. neurophilia*-infected zebrafish, as infectious spores are known to be found in the water with infected zebrafish (Sanders et al., 2016). In addition, medaka were fed central nervous system (CNS) tissue from infected zebrafish weekly during this 8-week period (for eight CNS exposures total), according to the infection protocol outlined in (Peneyra et al., 2018). Briefly, macerated CNS from infected fish was mixed with medaka food and subsequently fed to the study fish (an exposure rate of 1 zebrafish CNS per 21 medaka individuals). Similarly, control fish received water from tanks containing uninfected medaka and CNS tissue (at a rate of 1 medaka CNS per 21 medaka individuals) from uninfected medaka fish. The presence of *P. neurophilia* was tested at both four (n = 15 exposed and n = 10 non-exposed) and 8 weeks (n = 16 exposed) into the infection protocol. This sampling was done by randomly selecting one fish from each tank and euthanizing in an overdose of tricaine methanesulfonate (1 g/L; MS-222; Sigma), before dissecting out the whole brain and spinal cord. The CNS were excised within 3 min and rapidly frozen on dry ice, then stored at −80°C until further analysis for the presence of *P. neurophilia*. In addition, the presence of *P. neurophilia* was also tested on whole body tissue for 16 exposed individuals following the completion of the 8-week infection protocol (see Figure 1 for an illustration of the infection protocol and time-line for the experiment).

2.4 | DNA extraction and qPCR

The brain tissue from control and parasite-exposed fish were individually transferred to 50 µl MilliQ water (Merck). Samples were sonicated for 2 min at 55 W (QSonica Sonicators) 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) 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 & Kent, 2011). Samples were then eluted in 100 µl storage buffer (provided in the kit). We followed the qPCR protocol for analysis of infection status established by Sanders and Kent (2011). 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′-GC TCGCTCAGCCAAATAAAC-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 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). Positive controls (DNA samples from *P neurophilia* infected zebrafish brains) and no template controls were included for each plate. Samples were considered positive when cycle quantification (Cq) values were below 38; that is, higher Cq values or the absence of a Cq value indicate that no infection is detected (Sanders & Kent, 2011).

2.5 | Open field and mirror test

All fish were tested in open field and mirror tests 16 days after their final parasite exposure. These tests are commonly used to assess boldness, exploration and sociality (Godwin et al., 2012; Pham et al., 2012). Similar to rodents, fish exhibit a natural aversion for brightly lit open spaces, but simultaneously have an innate tendency to explore novel environments (Stewart et al., 2012). Thus, in the open field test, freezing behaviour (i.e. time spent immobile and 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, sociality is normally assessed as either aggressive interactions with the mirror image or time spent swimming close to the mirror. In our study, we were not able to distinguish between the two (due to poor camera resolution) and we therefore report it as a general increase or decrease in sociality. We tested 18 control and 18 *P.* parasite-exposed fish. The test was performed in an apparatus measuring 30×30×10 cm (W×L×D), with black walls and a white bottom (Figure 1a,b). The apparatus was filled with 4 L of water. Fish were video recorded from above the arena for 5 min immediately after being placed in the arena. 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 min, while continuing to be video recorded. The arena was divided into two zones: (1) no more than 3 cm from the mirror (approximately one body length) and (2) the remaining arena. The arena was filled with filtered and UV-sterilized water maintained at 28°C and the water was changed between each trial. All trials were performed between 08:30 and 16:30. Behaviour was tracked and analysed using Ethovision XT 13 (Noldus).

2.6 | Statistical analysis

All statistical analyses were done using RStudio software v. 4.0.4 (R Development Core Team, http://www.rproject.org) and the statistical package 'NLME' was used for the assessment of linear mixed effect models (LME). Total distance moved and percent of time immobile were analysed with treatment (control vs. parasite exposed), test (open field vs. mirror test), and their interaction as fixed effects and individual as a random effect (to account for the repeated measures design). The best-fit model was selected based on a comparison of all possible model combinations, with the final model being the one with the lowest Akaike information criterion for low sample numbers (AICc) score; that is, the model with the best data fit when weighted against model complexity. Interactive effects between treatment and test were assessed using Tukey–Kramer honestly significant difference post hoc test. The percent of time spent in the centre of the open field test and the percent of time spent in front of the mirror were analysed with treatment (control vs. parasite exposed) as the fixed effect and fish as the random effect. Visual inspection of the qqnorm and residual plots to check the assumptions of normality and homoscedascity confirmed that these models conformed to these assumptions. The freezing data for both the open field and the mirror tests were log transformed to achieve normality. Significance was assigned at $p<0.05$ and data are presented as mean±SEM. Data points, AICc scores and the Rscript used for the statistical analysis are included in Supporting Information.

3 | RESULTS

3.1 | Infection status

To verify infection status, we tested brain tissue from infected and uninfected fish for the presence of *P. neurophilia* by qPCR. All tested fish at both weeks 4 and 8 (including samples with CNS only and whole bodies) in the parasite-exposed and control groups tested negative for the parasite; that is, no samples show melting curves, and therefore, no Cq value is measured, which indicates no parasite infection.

3.2 | Behavioural response to parasite exposure

The final model did not include an interaction effect between treatment and test for the total distance moved. Both treatment and test had a significant impact on total distanced moved (Treatment: $\chi^2_{(1)} = 18.3$, $p<0.001$, Test: $\chi^2_{(1)} = 15.6$, $p<0.001$; Figure 2a). Specifically, parasite-exposed fish covered approximately 30% more distance than control fish in both the open field and the mirror tests ($p = 0.001$ for both). In addition, both groups had an approximately 17% higher distance covered during the mirror test when compared to the open field test ($p = 0.003$ for both).

The final model for time spent immobile did not include an interaction effect between treatment and test, but both treatment and test significantly altered the amount of time spent immobile (Treatment: $\chi^2_{(1)} = 10.1$, $p = 0.001$, Test: $\chi^2_{(1)} = 6.08$, $p<0.01$; Figure 2b). Specifically, control fish spent approximately 58% and 88% more time immobile than parasite-exposed fish in the open field and the mirror tests ($p = 0.02$ for both), respectively. However, the post-hoc analysis showed no differences in time spent immobile across tests. Despite this general increase in activity in response to

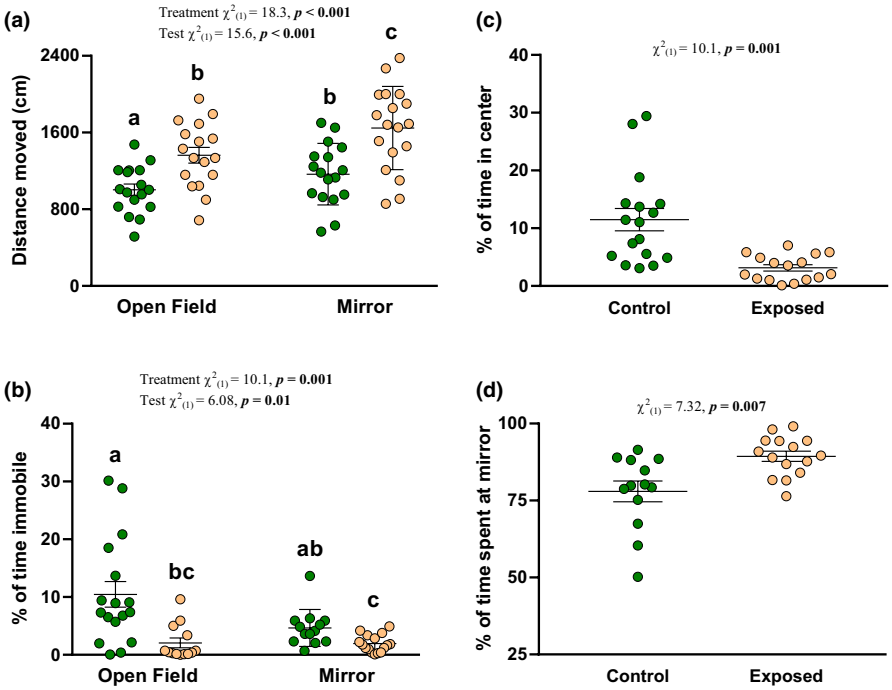

FIGURE 2 Behavioural effects of *Pseudoloma neurophilia* in parasite-exposed and control (no parasite exposure) medaka *Oryzias latipes*. Distance moved (average ± SEM) in the open field and mirror test (a). Percent time immobile (% ± SEM) (i.e. freezing) in the open field and mirror tests (b). Percent time (mean ± SEM) spent in the centre of the open field test (c) and percent time (mean ± SEM) spent near the mirror in the mirror test (d). Statistics based on linear mixed effects models are provided in each panel with small letters representing Tukey HSD post-hoc significant differences between groups.

parasite exposure, exposed fish spent approximately 90% less time in the centre of the arena ($\chi^2_{(1)} = 10.1$, $p = 0.001$; Figure 2c) but 29% more time near the mirror ($\chi^2_{(1)} = 7.32$, $p = 0.007$; Figure 2d).

Please refer to the Data file provided in Supporting Information for raw values for behavioural outputs.

4 | DISCUSSION

In this study, we provide evidence that parasite exposure even in the absence of successful infection can induce considerable behavioural changes in fish hosts. Parasite exposure increased activity, social interactions and centre avoidance in resistant medaka. To our knowledge, these findings represent the first demonstration of behavioural trait responses in fish to parasite resistance and illustrates that both past and present encounters with parasites must be considered to the variety of factors explaining intraspecific variability in animal behaviour.

To understand the parasite-induced phenotypic alteration in resistant hosts, we compared our results with how the same parasite-induced host phenotypic alterations in a susceptible host (i.e. zebrafish) from our previous study (Midttun, Vindas, Nadler, et al., 2020). Of note, the *P. neurophilia* infection regime used in this study was previously shown to successfully infect zebrafish; that is, zebrafish show positive infection status 6 weeks post first experimental infection and reached an 80% infection rate after 10 weeks (Midttun, Vindas, Nadler, et al., 2020). Although, we cannot exclude the possibility that the parasite succeeded in establishing an early infection that was later eliminated by the medaka, negative testing of individuals at 4- and 8-weeks post exposure limits the likelihood of this explanation. Importantly, we found that the parasite-induced changes in activity in resistant medaka were diametrically opposite from those observed in susceptible zebrafish; that is, whereas successful *P. neurophilia* infection in zebrafish was associated with a general reduction in locomotion (i.e. reduction in distance moved and increased immobility) (Midttun, Vindas, Nadler, et al., 2020), exposure to this parasite in medaka resulted in a general increase in activity based on the same behavioural outputs.

Interestingly, whereas *P. neurophilia* infection had no profound effect on social interaction in zebrafish (Midttun, Vindas, Nadler, et al., 2020), exposed medaka spent more time interacting with their own mirror image, indicating either increased social preference or increased aggression (Pham et al., 2012). Notably, we conducted the mirror test immediately after the open field test and thus, it is possible that the increased distance moved seen in the mirror compared to the open field test is due to fish having had a longer acclimation time to the arena. However, the differences between treatments were consistent throughout the tests, which suggests that parasite exposure is the main factor driving differences across contexts.

The only behavioural response that infected zebrafish and resistant medaka appeared to have in common was a tendency to avoid the centre of the test arena (i.e. thigmotaxis). In general, it is difficult to interpret animal behaviour in laboratory tests, and perhaps especially in lab-reared fish with little to no prior experience with natural aversive stimuli. Thus, care should be taken when attempting to link the observed behavioural outputs to emotional states (e.g. anxiety) or motivational behaviours (e.g. exploration and sociability). However, by examining individual behaviours across a range of contexts (i.e. using different behavioural tests and outputs) behavioural patterns indicative of such states and behaviours may emerge. For example, stationary behaviour (immobility) in zebrafish tend to coincide with both reduced locomotion and thigmotaxis and vice versa (Baker et al., 2018). Such a suite of correlated behaviours (i.e. behavioural syndrome; Bell, 2007) was indeed induced in zebrafish

infected with *P. neurophilia* (Midttun, Vindas, Nadler, et al., 2020), where immobility co-occurred with reduced locomotion and increased thigmotaxis. If attempting to interpret this behavioural profile, a combination of these behaviours is a typical measure of suppressed exploratory behaviour or even anxiety both in fish and rodent models (Blaser et al., 2010). It is therefore curious that these behaviours do not correlate in medaka exposed to *P. neurophilia*. On the contrary, parasite exposure increased locomotor activity as well as centre avoidance. From an ecological perspective, it could be speculated that the observed increase in locomotion represent increased exploration, but that the fish minimize risk by avoiding the centre of the arena.

Even though we found no signs of an established infection in the medaka, it is likely that repeated parasite exposures resulted in immune activation during the infection regime, to either expel parasites trying to establish an infection or, to keep parasites entirely out of the host by increasing defence mechanisms. Immune defence comes at a cost that the fish may have to subsequently compensate for by increasing their energy intake. Thus, the increased activity observed in the current study could represent increased exploration for the purpose of foraging (Barber et al., 2000; Garrido et al., 2016). In this context, behavioural alterations in resistant versus susceptible hosts may be mediated by innate/adaptive anti-parasite immune responses. If so, variation in immune responses between hosts that developed an infection versus those that did not could provide clues about the proximate mechanisms underlying phenotypic alterations following parasite exposure. That is, following sustained infection, reduced locomotor activity may stem from a shift in energy allocation away from general activity towards tissue repair, homeostasis and/or immunity (Dallas et al., 2016). Indeed, reduced activity and exploration in the zebrafish host coincided with a comprehensive enrichment of cerebral pro-inflammatory immune pathways (Midttun, Vindas, Whatmore, et al., 2020). More specifically, groups of differentially expressed genes (DEGs) participating in the same immune pathways were over-represented in infected zebrafish compared to uninfected controls (Midttun, Vindas, Whatmore, et al., 2020). Future studies on the resistant medaka should focus on elucidating their immunological response towards acute and prolonged parasite exposures, to establish the cost of fighting/avoiding infection.

Nonetheless, changes in behavioural outputs related to activity, exploration and social interactions may have far-reaching implications, scaling from individuals to ecosystem-level processes. In ecological studies, risk of parasitism is primarily studied for known susceptible host species. For example, a high infection pressure may be predicted to have profound effects on the community ecology of a host species (Daversa et al., 2021; Doherty & Ruehle, 2020; Mierzejewski et al., 2019; Poulin, 1999). Parasite prevalence is assayed through examination of the host and its tissues, so parasite encounters that do not develop into an infection may easily be missed. However, empirical evidence indicates that parasite presence leads to an increase in physiological defence mechanisms in hosts, particularly when parasite encounters are increased by increasing number of conspecifics, which leads to higher positive density-dependent transmission rates (Cotter et al., 2004; Friesen et al., 2022; Silva et al., 2016). Similarly, our data indicate that the presence of parasites may have profound effects on behavioural outputs even in resistant study populations and should be considered in studies on how interactions between parasites and hosts influence ecological landscapes. Notably, it is important to point out that the medaka in our study were subjected to the behavioural assays 16 days after parasite exposure. This result suggests that previous parasite exposures in resistant individuals may lead to long-term changes in behaviour, even after parasites are no longer present in the environment.

In summary, we show that parasites may induce considerable behavioural changes in resistant hosts. The parasite studied here primarily targets the CNS in susceptible hosts and the behavioural changes observed in the current study may stem from direct interactions between the invading parasite and the immune system successfully eradicating it. Whether parasites targeting other tissues and organs systems may induce comparable changes in the behaviour of resistant hosts remains unknown. Thus, future work should investigate how these effects translate to other kinds of host–parasite systems in wild habitats to better understand how parasite encounters and resistance may impact community, population, and even ecosystem-level processes.

AUTHOR CONTRIBUTIONS

Ida B. Johansen, Øyvind Øverli, Marco A. Vindas, Helene L. E. Midttun and Lauren E. Nadler designed the experiment. Lauren E. Nadler and Helene L. E. Midttun conducted the study. Helene L. E. Midttun conducted behavioural analysis. Marco A. Vindas conducted data analysis. Romain Fontaine and Finn-Arne Weltzien helped provide fish resources. All authors helped in drafting the manuscript. All authors gave the final approval for publication.

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CONFLICT OF INTEREST

Lauren Nadler is a Guest Editor for Functional Ecology's SF on 'Mechanisms and consequences of infection-induced phenotypes' but took no part in the peer review and decision-making processes for this paper. The authors declare no competing or financial interests.

DATA AVAILABILITY STATEMENT

All relevant data are within the paper, published as Supporting Information and available at from the Dryad Digital Repository https://doi.org/10.5061/dryad.s4mw6m99j (Vindas et al., 2022).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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