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Brain-Encysting Trematodes Increase the Frequency but Reduce the Repeatability of Surfacing Behaviour in Mottled Triplefin

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ABSTRACT

Trophically transmitted parasites play a significant role in shaping food webs, especially in aquatic environments, due to their complex life cycles and transmission through predation. Helminths that use this transmission route, such as trematodes and cestodes, can manipulate host behaviour to increase the likelihood of predation by their next host. Recent research suggests that parasites may not only influence single behavioural traits but also multiple traits simultaneously, affecting both the expression and consistency of behaviours that potentially facilitate parasite transmission. To further test this hypothesis, our study examines the impact of the recently discovered brain-encysting trematode *Cardiocephaloides ovicorpus* on the behaviour and repeatability of personality traits in the mottled triplefin (*Forsterygion capito*) in Otago Harbour, New Zealand. Naturally infected fish were tested for seven behavioural traits, including exploration, observation, predator avoidance, boldness, surfacing, activity (tank crossing), and aggressiveness, across two trial days. Of the behavioural traits investigated, surfacing was the only one that showed a significant positive correlation with *C. ovicorpus* infection. Furthermore, our statistical model suggested a decrease in repeatability for this trait as a function of *C. ovicorpus* infection. It is possible that *C. ovicorpus* specifically targets the brain region responsible for controlling surfacing, as the increase in surfacing may expose fish to a higher risk of detection by avian predators, the parasite's definitive hosts. Additionally, a decrease in behavioural repeatability may increase the likelihood of mismatches between behavioural responses and stimuli, such as predator cues, further elevating predation risk. Future research could aim to uncover the complete life cycle of *C. ovicorpus* by identifying the unknown first intermediate host, enabling experimental infections of fish to determine the causal relationship between behavioural variation and parasite infection.

1 | Introduction

Parasites are a common component of ecosystems worldwide and contribute to food web structure, particularly in aquatic environments (Born-Torrijos et al. 2016; Fredensborg and Longoria 2012; Lafferty et al. 2008). This is largely due to the life history of many parasites, which have complex life cycles involving two or more host species and are often transmitted from one host to the next through predation, classifying them as trophically transmitted parasites (Dunne et al. 2013; Fredensborg and Longoria 2012; Poulin 2010). Parasitic helminths, including acanthocephalans, cestodes, trematodes, and nematodes, are good

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examples of trophically transmitted parasites. Many helminths are able to manipulate the phenotype of their intermediate host, including behaviour, to enhance the predation rate of the definitive host on the intermediate host, thereby increasing the success of parasite transmission (Fredensborg and Longoria 2012; Lafferty and Shaw 2013; Ruehle and Poulin 2021). These changes are not necessarily dramatic but are sufficient to increase the predation rate of the definitive host on the intermediate host (Poulin 2010). For example, the trematodes *Curtuteria australis* and *Acanthoparyphium* sp. (Babirat et al. 2004), which infect the foot muscle of New Zealand cockles (*Austrovenus stutchburyi*), reduce the cockles' ability to burrow into sediment and cause them to remain on the surface during low tide. This increases the parasites' transmission rate because surfaced cockles are more likely to be found and eaten by oystercatchers (*Haematopus* spp.), the definitive hosts of these parasites, consequently completing the parasites' life cycle (Babirat et al. 2004; Leung et al. 2009; Mouritsen and Poulin 2009).

There is growing evidence suggesting that multiple behavioural traits vary among individuals within a single population, while each individual consistently expresses these traits across different contexts and/or time, forming distinct animal personalities within the population (Bell and Sih 2007; Dingemanse et al. 2010; Kekäläinen et al. 2013; Thomson et al. 2020). The personality framework typically includes five aspects of behaviour: boldness, activity, exploration, aggressiveness, and sociality (Réale et al. 2007). Consequently, research on parasite-induced behavioural manipulation has shifted from examining one specific behaviour to simultaneously quantifying multiple traits in infected hosts to determine whether parasites contribute to the variation in animal personality within a population (Coats et al. 2010; Poulin 2010; Thomas et al. 2010). This shift is driven by the hypothesis that alteration in these personality traits, their consistency over time, or even the association between behaviours could be advantageous for parasite transmission (Coats et al. 2010; Kortet et al. 2010; Poulin 2010, 2013). For instance, increased aggressiveness and boldness in infected individuals could elevate their predation risk, thereby enhancing the parasite's transmission success to the next host (Hammond-Tooke et al. 2012; Dubois and Binning 2022).

Additionally, parasite infection may influence the consistency of behaviour (Coats et al. 2010; Kortet et al. 2010; Gradito et al. 2024; Poulin 2013). Lower repeatability of individual behaviour increases variance in host behaviour, which could benefit trophically transmitted parasites by raising the likelihood of host predation due to a mismatch between stimulus and response (Gradito et al. 2024; Poulin 2013). The mismatch may result in fish responding with behaviours at the least appropriate end of the spectrum for a particular stimulus more frequently, which can increase their risk of predation. For example, in common bullies (*Gobiomorphus cotidianus*), the repeatability of activity, aggressiveness, and boldness significantly decreased when the fish were exposed to predator (eel) cues compared to normal conditions following infection by trematodes (Hammond-Tooke et al. 2012). As a result, the fish may exhibit inappropriate levels of activity, boldness, or aggression in response to predator cues more frequently, increasing their risk of being detected and eaten by eels. However, this effect may be difficult to detect: one needs to focus on *individuals* because the *average* expression of behavioural traits in the population is generally consistent over time (Kortet et al. 2010; Poulin 2013). Despite its potential key role in transmission success, the effect of parasites on behavioural repeatability is an area that remains largely unexplored (Gradito et al. 2024).

Recently, a trophically transmitted trematode, *Cardiocephaloides ovicorpus* (Dubois and Angel 1972), was discovered in Otago Harbour (Presswell and Bennett 2021). This trematode encysts on the outer surface of the brain of two littoral fish species from the family Tripterygiidae: common triplefin (*Forsterygion lapillum*) and mottled triplefin (*Forsterygion capito*), both of which serve as the parasite's second intermediate host, following an unknown gastropod as the first intermediate host of the parasite (Bennett et al. 2023). The parasite's known definitive hosts are diving birds, including little pied cormorant (*Microcarbo melanoleucos*) and the Otago shag (*Leucocarbo chalconotus*), with transmission from fish to bird occurring via predation (Bennett et al. 2023; Presswell and Bennett 2021). However, it is also very likely that *C. ovicorpus* also utilizes other predatory birds such as herons as definitive hosts, given that bird-infecting trematodes are mostly generalists capable of infecting a wide range of bird species (Bennett et al. 2023). This discovery presents an opportunity to explore the effects of parasite infection on the behaviour of triplefins that may increase trophic parasite transmission. This is because previously studied brain-encysting trematodes, such as *Euhaplorchis* spp. and *Cardiocephaloides longicollis*, have been shown to induce significant changes in conspicuous behaviours, particularly in the swimming patterns of fish, making them more likely to be seen and eaten by definitive avian hosts (Fredensborg and Longoria 2012; Born-Torrijos et al. 2016). However, the effect of brain infection on other personality traits and behavioural repeatability has not been examined before.

This study aimed to explore the impact of *C. ovicorpus* on the behaviour of the mottled triplefin (*Forsterygion capito*), one of the two known second intermediate host species of this parasite, by investigating two aspects: (1) whether the intensity of *C. ovicorpus* infection correlates with any commonly studied personality traits in the fish (exploration, boldness, activity, and aggressiveness) (Coats et al. 2010) as well as the fish's response to a predator stimulus, and (2) the effect of *C. ovicorpus* infection on the repeatability of fish behaviours. The latter was assessed by comparing behavioural scores between two trial days and examining the effect sizes of the statistical models. Although *C. ovicorpus* does not penetrate brain tissue like previously studied brain-encysting parasites (e.g., Fredensborg and Longoria 2012; Born-Torrijos et al. 2016), a significant correlation between infection intensity and behavioural traits was still expected as the parasite is in a location likely to affect the host's neurobiological processes and behavioural outputs. Specifically, we predicted higher scores in exploration, activity, and aggressiveness, as *F. capito* is a benthic species that typically spends much of its time sitting still on the seafloor of the marine littoral zone (Feary and Clements 2006; Roberts et al. 2015). At low tide, they typically hide under rocks, while at high tide they can wander into waters several meters deep, all within the range where diving shags forage. Increased movement would make them more easily detected and predated on by shags, along with an increase in boldness. Additionally, we expected greater infection intensities to result in lower repeatability in behaviour; whether adaptive or a mere pathological side effect, this outcome could also lead to increased risk of avian predation.

2 | Materials and Methods

2.1 | Sampling and Husbandry

All procedures described below followed Animal Use Protocol (AUP) number 23–84, approved by the University of Otago Animal Ethics Committee. In February 2024, 34 mottled triplefins (*Forsterygion capito*) were captured at Broad Bay (45°50′ 49.9′ S 170°37′ 23.5″ E), Portobello, Otago, New Zealand. All individuals used in the study were captured by hand at low tide, from small pools (a few mm deep) under rocks. All fish were equally accessible and easy to collect. Basically, every fish found was collected, ensuring no selection bias during capture. The fish were held in containers with oxygen provided by a battery-operated aerator during transport to the Department of Zoology, University of Otago. Fish were randomly distributed into one of two identical 250 L recirculating tanks fitted with a biofilter. The conditions in both tanks were controlled to match the natural habitat of the fish at the time of the experiment, including a temperature range of 14°C–16°C, shelter provided by 15 PVC pipe sections, salinity of 35 ppt, and a 12-h light/dark cycle. Tanks were covered by nets to prevent fish from jumping out. A 30% water change and *ad libitum* feeding with aquaculture pellets took place every two days in the morning, while ammonia levels, salinity, and fish body condition were monitored daily. Fish were acclimatized to the laboratory conditions for two weeks before the behavioural tests were carried out.

2.2 | Behaviour Test

Mottled triplefins ($n = 34$) were tested for all behaviours, unless stated otherwise. For each batch, four fish were gently moved from the holding tank into one of four identical individual observing tanks (23 W × 36 D × 26 cm H). The inside of the observing tanks was covered with white opaque material to visually separate the fish from one another and to reduce reflection, with one side being removable to expose the fish to a reflective side of the tank serving as a mirror later in the trial. The water level was filled to 20 cm (Figure 1). Half a terracotta pot (8.5 cm diameter) was provided in the tank as a shelter. One GoPro Hero 6 camera was attached above two tanks to record the behaviour (Figure 1). Fish were given time to acclimate overnight for 15 h in the observing tank before the behavioural test took place on the following morning. Bubblers were provided in each tank during the acclimation period to keep the water oxygenated.

The trial began with the observation of exploratory behaviour, measured as the time spent outside the refuge or “exploration” within the first 20 min of the trial's start. A fish was considered to exit the shelter when its full body was observed outside and to enter the shelter once the tip of its snout entered. Within the same 20 min, total time spent “observing” was recorded when the fish had at least its full head or a significant portion of its body outside the shelter while keeping the rest of its body sheltered. This behaviour ended once the full caudal fin was visible outside the shelter or when the full head could not be seen as the fish retreated into the shelter.

Following the first 20 min, an anti-predator test was conducted by striking a heron head model into the tank (39.5 cm tall, with a 25 cm head + beak and 2 cm thickness) (Figure 2). The strike penetrated water and reached half of the depth (10 cm) and was positioned above the fish without contact to simulate an aerial predator attack (Figure 2). The fish's “predator avoidance” was recorded as a binary response: whether the fish reacted (i.e., swam away immediately, or headed for the shelter) to the bird strike or remained undisturbed; the ‘bird strike’ was conducted on all 34 fish. However, only 19 fish ($n = 19$) that were outside the shelter during this portion of the trial and had their anti-predator responses recorded

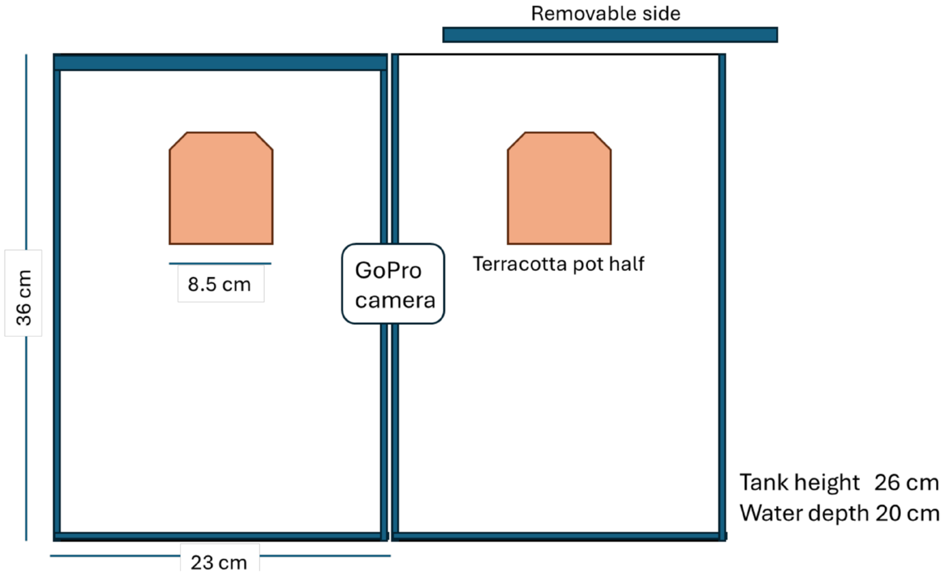

FIGURE 1 | Top view of the observation tank setup. The inside of the tanks was covered by opaque material to visually separate fish from one another, with one side being removable to expose the fish to a reflective side of the tank serving as a mirror later in the trial. Two tanks were placed side by side, with a GoPro Hero 6 camera positioned above to record fish behaviour. Two sets of observation tanks were arranged in this manner.

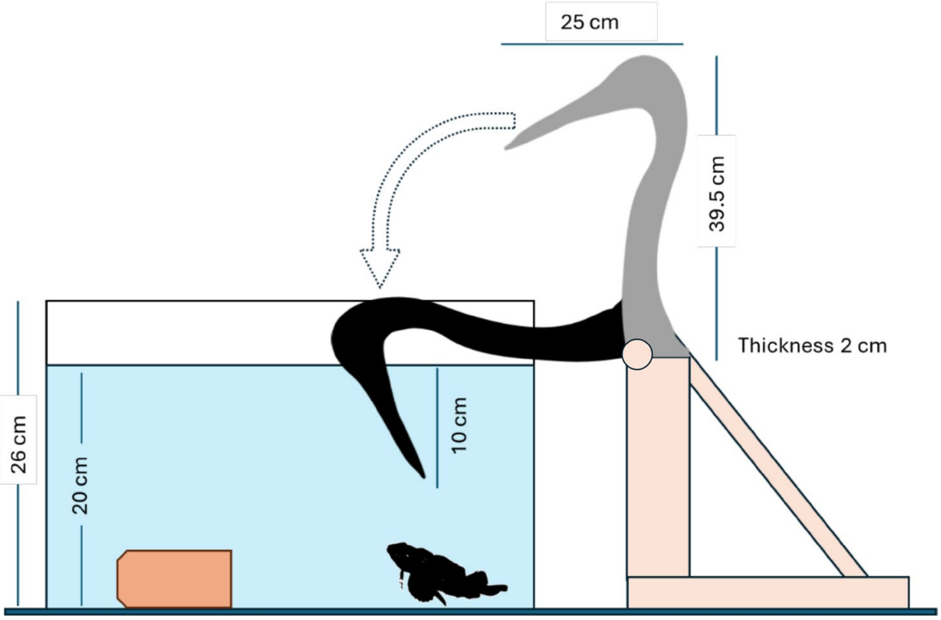

FIGURE 2 | Side view of the anti-predator test setup. The bird head can be lowered into the tank, reaching half of the water depth (10 cm), and pulled back by an attached string.

on both days were included in the analysis of this behaviour. "Boldness" was recorded as the time taken for the fish to leave the shelter after the bird strike, with a maximum cut-off at 30 min, following the procedures in Hammond-Tooke et al. (2012). Fish inside the shelter during the bird strike were also assessed for boldness, as they were likely to sense the disturbance. The analysis of this behaviour included only 17 fish ($n=17$) that remained inside the shelter after the bird strike on both days, either by retreating into it following the strike or by staying there beforehand.

Fish were then allowed 15 min to rest between the anti-predator and subsequent activity tests. The shelter was then removed to test for activity. Fish were allowed a further 15 min to acclimate without shelter. During the next 10 min, the number of "surfacing" events was recorded. A surfacing event consisted of fish mostly clinging to one corner of the tank and swimming upwards toward the water surface, occasionally breaking the surface. As we were not able to track and measure the total swimming distance because the fish were mostly sedentary at the edge of the tank with minimal movement, we instead recorded the time spent performing "tank crossing," i.e., the time spent moving away from the edge areas of the tank (more than 2 cm from the edge). This was because the fish typically moved continuously when away from the edge until they reached the other side of the tank or returned to the same side where they initially were. The recording was conducted over the same 10-min period as a measure of swimming activity.

Lastly, the aggressiveness test was carried out. The opaque cover was removed from one side of the tank to expose the fish to the reflective glass, creating an image of a same-sized conspecific approaching the test fish, as in Hammond-Tooke et al. (2012). Fish were allowed 1 min to adjust to their reflection, and "aggressiveness" was then recorded over the next 3 min. However, clear strikes against the mirror image were not observed, as the fish swiftly swam back and forth from one side of the glass wall to the other, touching the glass (i.e., their reflected image) with their snout while swimming. Therefore, in this study "aggressiveness" was defined as the total time spent interacting in this manner with the reflective side of the tank.

Each fish underwent two trial sequences to quantify the repeatability of their behaviour. The first trial occurred after the overnight 15-h acclimation period, as mentioned above; whereas the second took place at the same time the following morning, which was approximately one day after the first trial sequence. Fish remained in the observing tank the entire time, with bubblers provided during the interval between trial sequences, and were therefore not returned to the social environment they experienced earlier. The short interval between trials should lead to relatively high repeatability of the behaviours tested. Tanks were cleaned, and water was changed after the second trial was completed for each batch before tanks were used for another batch of fish. Each batch was transferred to the observing tanks in the evening of the feeding days, about 8 h after being fed. No additional feeding was provided between trial days. All behaviours were recorded by a GoPro Hero 6 camera attached above the tanks; the footage was analyzed afterward using the BORIS application (Friard and Gamba 2016).

2.3 | Fish Dissection

After both trials were completed, the fish were euthanized using an overdose of Aqui-S (175 mg/L). Their length was then measured, and they were freshly dissected, starting with the brain case and the eyes, where *C. ovicorpus* had been previously found by Bennett et al. (2023). The digestive tract, body cavity, and gills were also dissected to search for other parasites, as triplefins (*Forsterygion* spp.) are important intermediate hosts of helminth parasites in Otago Harbour (Bennett et al. 2023), and other parasites could potentially contribute to behaviour changes. The sex of each fish was also determined from the gonads found in the body cavity.

2.4 | Statistical Analysis

All statistical tests were performed using R 4.4.0 (R Core Team 2023). For each of the seven behavioural traits, a generalized linear mixed model (GLMM) was generated using the packages *lme4* (Bates et al. 2015) and *lmerTest* (Kuznetsova et al. 2017). Continuous behaviour data—boldness, crossing, and aggression—were log-transformed ($\log_{10}(x+1)$) to meet the assumption of normality. Surfacing data already met the assumption; therefore, no transformation was required. In each model, the total number of *C. ovicorpus*, fish size, trial days, and fish sex were included as fixed effects, with fish ID as a random effect. All other parasites were also excluded from the models to reduce analysis complexity, as they were present at very low prevalence (mostly fewer than 5 infected fish per parasite species) and low intensity of infection. Additionally, correlation matrix and principal component analyses were run for each of the trial days to identify possible interactions between behaviours.

Exploration and observation were originally measured as continuous variables (i.e., time spent performing the behaviour). However, because there are a high number of zeros in the dataset (more than 10 fish did not perform the behaviour in one or both of the trials), a two-step approach was therefore taken to analyse these two behaviours. Firstly, the data were treated as binary (e.g., the fish either emerged from the shelter or did not) and tested using GLMM. Secondly, additional GLMMs were conducted using the continuous data for the fish that performed the behaviour on both trial days (i.e., all zero data were removed).

To test whether the intensity of *C. ovicorpus* affects the repeatability of fish behaviour, we calculated the difference in behavioural measures per individual between the two trials, which served as a measure of individual consistency. In this approach, smaller differences between trials indicate higher behavioural consistency within individuals. Data for all seven behaviours were then log-transformed ($\log_{10}(x+1)$) to meet the assumption of normality. Subsequently, a linear model (LM) was created for each behaviour, including parasite intensity and fish size as fixed factors, following the method used by Hammond-Tooke et al. (2012). For predator avoidance, which was measured as a binary variable, a generalised linear model (GLM) was used instead. Scatter plots were also generated for each behaviour to visualise the relationship between *C. ovicorpus* infection intensity and behavioural consistency in *F. capito*.

3 | Results

3.1 | Parasite Infections

All 34 fish tested were infected by *Cardiocephaloides ovicorpus*, with the majority of metacercariae (larval trematode stages) encysted and found surrounding the brain; however, none had penetrated the brain tissue (Figure 3). Additionally, seven fish had up to three *C. ovicorpus* parasites present in either of their eyes. The total number of *C. ovicorpus* parasites in the brain and eyes ranged from 6 to 233 per fish, with an average of 36 parasites per fish, making it by far the most abundant parasite. Other parasites found included one species of monogenean, five trematodes, two nematodes, and six cestodes, most of which were located within the gastrointestinal tract. However, all of these parasites had low prevalence, infecting no more than 9 fish per species (26% of fish). Fish size averaged 73.8 mm and ranged from 62 to 94 mm. Among the 34 fish, 12 were female (35%) and 22 were male (65%).

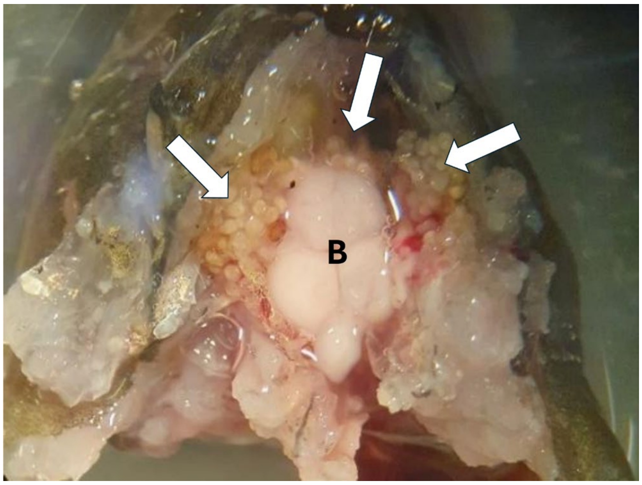

FIGURE 3 | Brain case of mottled triplefin (*Forsterygion capito*) infected by *Cardiocephaloides ovicorpus*. The parasite metacercariae (arrows) were encysted and surrounding the brain (B) without penetrating the brain tissue.

3.2 | Correlation Between C. Ovicorpus Infection and Fish Behaviour

The different behavioural measures covaried to some extent, especially surfacing and aggressiveness (Figure S1). This was the case in both trials, as indicated by the correlation coefficients and the PCA (Figure S1). However, as the correlation coefficients indicated weak to moderate associations (all coefficients ≤ 0.45), we treated each behavioural measure separately.

For exploration, the average number of *C. ovicorpus* per fish that did not fully emerge from the shelter during the 20 min was not significantly different compared to fish that fully emerged from the shelter at least once (Figure S2a; Tables 1 and S1). Similarly, fish that exhibited observing behaviour during the observation period had no significant differences in the number of *C. ovicorpus* per fish from those that did not perform observing behaviour (Figure S2b; Tables 1 and S1).

Regarding predator avoidance, most of the tested fish, 11 on the first trial day and 12 on the second trial day out of 19 fish, did not react to the simulated bird strike (Figure S2c). However, the GLMM indicated that the average intensity of *C. ovicorpus* did not differ significantly between fish that did not react to the bird strike and those that did react (Table 1). For boldness, the time taken for the fish to emerge from the shelter after the simulated

TABLE 1 | Results of generalised linear mixed models (GLMM) testing for correlations between behaviours of mottled triplefin (*Forsterygion capito*) measured as binary responses and *Cardiocephaloides ovicorpus* infection intensity.

Behaviour	Predictor	Estimate	SE	*z*	*p*
Exploration *n* = 34	Intercept	0.343	0.921	0.373	0.709
	Fish size	0.280	0.523	0.535	0.593
	Trial day	−0.274	0.526	−0.521	0.602
	Fish sex	0.519	0.623	0.833	0.405
	No. of *C. ovicorpus*	−0.788	0.538	−1.465	0.143
Observing *n* = 34	Intercept	1.420	1.091	1.301	0.193
	Fish size	0.892	0.668	1.335	0.182
	Trial day	−0.657	0.594	−1.106	0.270
	Fish sex	0.561	0.747	0.751	0.453
	No. of *C. ovicorpus*	−1.284	0.722	−1.778	0.076
Predator Avoidance *n* = 19	Intercept	−1.050	3.991	−0.263	0.793
	Fish size	1.107	3.262	0.339	0.734
	Trial day	−0.701	1.244	−0.563	0.573
	Fish sex	−1.110	3.181	−0.349	0.727
	No. of *C. ovicorpus*	−3.065	3.718	−0.824	0.410

Note: Fish size, trial day, and fish sex were also included in all models as fixed factors and fish ID as a random factor.

bird strike was also not significantly correlated with the number of *C. ovicorpus* per fish (Figure S3a; Table 2).

In terms of fish activity, there was a positive correlation between the number of *C. ovicorpus* per fish and surfacing behaviour (Figure 4), and the GLMM suggested that this trend was statistically significant (Table 2). However, the crossing behaviour was not significantly correlated with *C. ovicorpus* (Figure S3b; Table 2) or the fish aggressiveness (Figure S3c; Table 2).

Overall, the correlation between fish size and the number of *C. ovicorpus* was positive and strong (Pearson's correlation coefficient = 0.81). However, fish size was not significantly correlated with any of the seven behavioural traits measured in this study (Tables 1 and 2). Additionally, there was no significant effect of the trial day or fish sex on any of the behaviours (Tables 1 and 2).

3.3 | Behaviour Repeatability

Although the GLMM results suggested no significant differences between trial days for any of the seven behaviours (Tables 1 and 2), some behaviours were non-repeatable because individuals behaved differently on each trial day without affecting the average behaviour score (Figure S4).

The linear model (LM) testing for consistency suggested that *C. ovicorpus* infection intensity was significantly correlated with the repeatability of surfacing behaviour (Figure 5; Table 3). The positive correlation shown in both the LM and the scatter plot indicated that higher infection intensity decreases the consistency of surfacing behaviour, as differences in behavioural expression between trials increased with greater infection intensity. However, the infection intensity was not significantly correlated with any of the other six behaviours (Figure S5; Table 3).

4 | Discussion

The variation in behavioural traits among individuals from a single population may have multiple explanations. These include infection by trophically transmitted parasites, as parasite-induced changes in behaviour are likely to enhance the predation rate on the host and thus promote parasite transmission to their next host (Fredensborg and Longoria 2012; Hammond-Tooke et al. 2012; Dubois and Binning 2022). In this study, the prevalence of the brain-encysting trematode, *Cardiocephaloides ovicorpus*, was 100% among the tested mottled triplefin, *Forsterygion capito*, with infection ranging from 6 to 233 parasites per fish. This wide range of infection levels allowed us to test for correlations between infection and behaviour expression. Our analyses revealed that the intensity of infection by *C. ovicorpus* was positively correlated with the occurrence of surfacing behaviour in *F. capito*, as well as the consistency of this surfacing behaviour over time, i.e., across two trial days. However, the intensity of *C. ovicorpus* had no statistically significant correlation with the other six studied behaviour traits, nor was it associated with significant changes in the repeatability of those behaviours across two trial days.

Considering these findings, we propose that '*C. ovicorpus*' manipulation of the fish host may target primarily the host's surfacing behaviour over other behaviour traits. Increases in surfacing could expose triplefins to a higher risk of being seen

TABLE 2 | Results of generalised linear mixed models (GLMM) testing for correlations between behaviours of mottled triplefin (*Forsterygion capito*) measured as continuous variables and *Cardiocephaloides ovicorpus* infection intensity.

Behaviour	Predictor	Estimate	SE	*t*	*p*
Boldness df = 108 *n* = 17	Intercept	2.725	0.177	15.403	< 0.001
	Fish size	0.145	0.137	1.063	0.302
	Trial day	0.168	0.088	1.916	0.068
	Fish sex	−0.265	0.179	−1.480	0.156
	No. of *C. ovicorpus*	−0.056	0.138	−0.403	0.691
Surfacing df = 169.5 *n* = 34	Intercept	12.490	3.052	4.092	< 0.001
	Fish size	−1.391	2.210	−0.630	0.533
	Trial day	0.912	1.491	0.611	0.545
	Fish sex	1.700	2.639	0.643	0.525
	No. of *C. ovicorpus*	**4.873**	**2.160**	**2.258**	**0.030**
Tank crossing df = 195 *n* = 34	Intercept	1.297	0.209	6.201	< 0.001
	Fish size	0.027	0.123	−0.216	0.830
	Trial day	−0.044	0.116	−0.379	0.707
	Fish sex	0.196	0.147	1.333	0.192
	No. of *C. ovicorpus*	−0.170	0.118	−1.436	0.082
Aggressiveness df = 202 *n* = 34	Intercept	1.374	0.229	5.997	< 0.001
	Fish size	0.037	0.156	0.236	0.815
	Trial day	0.094	0.117	0.799	0.430
	Fish sex	0.218	0.187	1.170	0.250
	No. of *C. ovicorpus*	0.096	0.149	0.646	0.766

Note: Fish size, trial day, and fish sex were also included in all models as fixed factors and fish ID as a random factor. Significant effects are shown in bold.

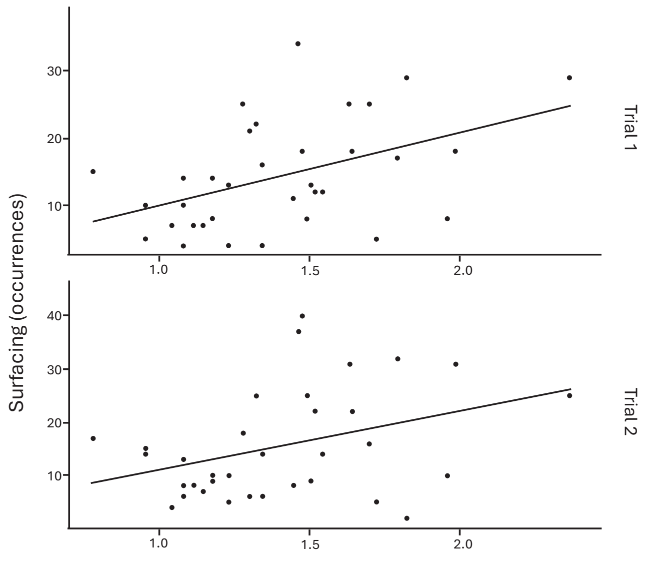

FIGURE 4 | Relationships between the infection intensity of *Cardiocephaloides ovicorpus* and surfacing behaviour of mottled triplefin (*Forsterygion capito*), shown separately for each trial day (*n* = 34).

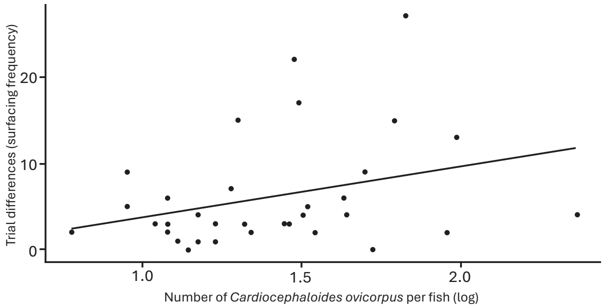

FIGURE 5 | Relationships between the infection intensity of *Cardiocephaloides ovicorpus* and consistency of surfacing behaviour of mottled triplefin (*Forsterygion capito*) (*n* = 34). The consistency was measured by differences in behavioural expression between the two trial days.

by avian predators. Our results also aligned with the other studies on brain-encysting trematodes such as *Euhaplorchis* spp. (Fredensborg and Longoria 2012) and *Cardiocephaloides longicollis* (Born-Torrijos et al. 2016), in which the parasites were found to alter the swimming patterns and surfacing behaviour of their fish hosts. Although *C. ovicorpus* does

TABLE 3 | Results of repeatability analysis on behaviours of mottled triplefin (*Forsterygion capito*).

Behaviour	Predictor	Estimate	SE	*t*	*p*
Exploration *n* = 34	Intercept	1.643	0.176	9.346	< 0.001
	Fish size	−0.151	0.310	−0.486	0.630
	No. of *C. ovicorpus*	−0.449	0.310	−1.449	0.157
Observing *n* = 34	Intercept	1.683	0.177	9.497	< 0.001
	Fish size	−0.199	0.313	−0.637	0.529
	No. of *C. ovicorpus*	−0.101	0.313	−0.324	0.748
Boldness *n* = 17	Intercept	2.012	0.256	7.869	< 0.001
	Fish size	0.085	0.431	0.196	0.847
	No. of *C. ovicorpus*	−0.327	0.431	−0.760	0.460
Surfacing *n* = 34	Intercept	0.704	0.058	12.168	< 0.001
	Fish size	−0.153	0.102	−1.500	0.144
	No. of *C. ovicorpus*	**0.223**	**0.102**	**2.182**	**0.037**
Tank crossing *n* = 34	Intercept	1.314	0.085	15.546	< 0.001
	Fish size	< 0.001	0.149	0.006	0.996
	No. of *C. ovicorpus*	−0.049	0.149	−0.327	0.746
Aggressiveness *n* = 34	Intercept	1.367	0.072	18.863	< 0.001
	Fish size	−0.044	0.128	−0.343	0.734
	No. of *C. ovicorpus*	0.052	0.128	−0.410	0.685

Behaviour	Predictor	Estimate	SE	*z*	*p*
Predator avoidance *n* = 19	Intercept	−2.449	1.115	−2.196	0.028
	Fish size	3.033	1.951	1.555	0.120
	No. of *C. ovicorpus*	−3.161	1.943	−1.627	0.104

Note: The infection intensity by *Cardiocephaloides ovicorpus* was included as a fixed factor. Significant effects are shown in bold.

not penetrate brain tissue like the other two parasites, the positive correlation between its infection intensity and surfacing frequency suggests it can similarly manipulate host surfacing behaviour. Additionally, Born-Torrijos et al. (2023) and Helland-Riise, Nadler, et al. (2020) suggested that brain-encysting parasites have a pattern of distribution within the brain (i.e., not encysting randomly by entry point) that targets specific areas of the brain. Therefore, it is possible that brain-encysting parasites, including *C. ovicorpus*, settle in or on the specific area of the brain controlling surfacing behaviour of their fish hosts. This could also explain why only surfacing was significantly correlated with infection but not the other behavioural traits of the fish.

Trophically transmitted parasites may have the ability to adaptively manipulate host behaviour to enhance their transmission between hosts; however, pathological side effects are another possible cause of behavioural changes (Herbison et al. 2018). Although *C. ovicorpus* is likely to be able to manipulate host behaviour by positioning itself within the braincase of triplefins (Helland-Riise, Nadler, et al. 2020; Helland-Riise, Vindas, et al. 2020; Lafferty and Shaw 2013), Nadler et al. (2020) have found that infections caused by brain-encysting parasites also affect metabolic processes. This results in acute elevation in metabolism and increased fish activity as they cope with the infection (Nadler et al. 2020). The immune response (Gradito et al. 2024; Herbison et al. 2018) and the stress response, marked by increased cortisol levels (Allan et al. 2020), may also raise oxygen demands following parasite infection. As a result, increased surfacing could be a response to meet this higher oxygen demand. Moreover, hunger may have elevated the activity level of fish, prompting them to forage and explore a larger area of the arena (Hansen et al. 2015; Ruehle and Poulin 2021), especially during the second trial, as the fish had not been fed for over 24 h. However, we observed negative correlations, though not significant, between *C. ovicorpus* intensity and exploration as well as crossing activity, which possibly reflected energy conservation to cope with the infection rather than the increased foraging in response to hunger (Kirsten et al. 2018). Furthermore, in most cases, we observed that the fish did not break the surface when performing surfacing behaviour, suggesting minimal evidence of elevated oxygen demand. Additionally, the fish were acclimatised in the laboratory for two weeks prior to behavioural testing; therefore, any acute effects on metabolism should have already subsided.

Variation in fish personalities could also contribute to the differences in the level of expression of each behavioural trait among individuals (Bell and Sih 2007; Dingemanse et al. 2010; Thomson et al. 2020). It is possible that the extent of surfacing behaviour may also differ due to variation in fish personalities and coincidentally correlated with the intensity of parasite infection. However, it is unlikely that fish surfacing more frequently acquire more parasites, because the unidentified first intermediate host of *C. ovicorpus* is likely a bottom-dwelling gastropod, consistent with the typical life cycle of parasitic trematodes (Leung et al. 2009). Also, triplefins primarily feed on benthic invertebrates (Feary et al. 2009), and only occasionally swim higher in the water column, potentially to feed on small swimming crustaceans (personal observation in wild fish). Therefore, surfacing behaviour is unlikely to be the cause of higher parasite acquisition, as the infection likely occurs along the seabed.

Although it is plausible that increased levels of surfacing behaviour in *F. capito* are the result of adaptive manipulation by the brain-infecting *C. ovicorpus*, we have yet to rule out other possible explanations, as the causal direction between *C. ovicorpus* infection intensity and fish behavioural variation has not been determined in this study. Establishing causality would require experimentally infecting fish to compare pre- and post-infection behaviours (Gradito et al. 2024; Petkova et al. 2018; Ruehle and Poulin 2021). In our case, experimental infection was not feasible, as the cercarial (infective) stage required for infection can only be obtained from the first intermediate host—a gastropod that remains unidentified for *C. ovicorpus* in Otago Harbour, despite our extensive surveys of gastropod species likely to be intermediate hosts within the area where the fish were collected. Future studies should aim to resolve the complete life cycle of *C. ovicorpus* by identifying the unknown first intermediate host, enabling experimental infection of fish to ascertain the causality of infection versus host behaviour.

Trophically transmitted parasites may not only alter the expression level of behavioural traits in their host but also reduce the consistency of behaviour across different contexts and over time (Gradito et al. 2024; Kortet et al. 2010; Poulin 2013). It has been hypothesized that the decrease in repeatability caused by parasites may not change the average level of behavioural expression at the population level but rather increase the likelihood of mismatched responses to stimuli (such as predator cues) by individual hosts, thereby raising the risk of predation for the host (Coats et al. 2010; Poulin 2013). Our results support this possibility, as the average level of behaviour expression did not significantly differ across the two trial days for any of the seven behaviours studied. However, the linear model revealed a decrease in the repeatability of surfacing behaviour, as it showed a significant positive correlation between *C. ovicorpus* infection intensity and the difference in surfacing frequency between the two trials. This may further suggest that the alteration of surfacing behaviour is a primary target of *C. ovicorpus* manipulation of the host to enhance its transmission to birds by not only increasing surfacing visits but also reducing the repeatability of this trait. In addition, no significant effect of infection on repeatability was found for any of the other six behavioural traits. Other studies examining the effect of parasite infection on the repeatability of behaviour have also found reduced consistency in the host's activity following infection. For instance, Gradito et al. (2024) reported a decrease in the repeatability of exploration and activity in pumpkinseed sunfish (*Lepomis gibbosus*) after infection with trematodes and cestodes, which use the fish as their second intermediate host. Similarly, Coats et al. (2010) observed reduced repeatability in the phototaxis of the amphipod *Paracalliope fluviatilis* after infection with the trematode *Microphallus* sp. The reduction in behavioural repeatability reported in these studies supports our findings. Although there have been relatively few studies in this area, emerging evidence suggests that parasite infections may lead to less consistent activity patterns—in our case, surfacing—among intermediate hosts.

However, our results do not fully support that reduction in repeatability increases the likelihood of a mismatch between stimulus and response as suggested by Poulin (2013); we have yet to establish a linkage between reduction in consistency of surfacing and the risk of avian predation. It has been suggested that behavioural consistency may be context-dependent (Gradito et al. 2024; Kudo et al. 2021). For example, Hammond-Tooke et al. (2012) found decreased consistency in activity, aggression, and boldness in trematode-infected common bullies (*Gobiomorphus cotidianus*), but only when exposed to predator cues. Also, the overlap of confidence intervals between the models may suggest that the effect of reduction in repeatability might be subtle in normal conditions. Future studies could investigate whether the pattern we observed here holds or even strengthens when hosts are exposed to predation cues, as this would help elucidate the relationship between reduced behavioural repeatability due to parasite infection and the increased risk of predation.

In conclusion, our study provides correlative evidence that the brain-encysting parasite *C. ovicorpus* increases the frequency but decreases the repeatability of surfacing behaviour in the mottled triplefin (*F. capito*), potentially raising the fish's predation risk and enhancing the parasite's transmission to birds. None of the other six tested behaviours showed significant correlations with infection or changes in repeatability. This suggests that *C. ovicorpus* may selectively manipulate the surfacing or swimming behaviour to promote transmission in similar fashion to other previously studied brain-encysting parasites. However, due to the lack of experimental infection, we could not ascertain causal relationships or confirm that reduced repeatability directly increases predation. Future studies could aim to uncover the complete life cycle of *C. ovicorpus* and/or test behavioural repeatability under simulated predator exposure to better understand the linkage and causal directions between parasite infection, behaviour, repeatability, and predation risk.

Author Contributions

Sila Viriyautsahakul: conceptualization, methodology, formal analysis, visualization, writing – original draft, writing – review and editing, data curation, investigation. **Robert Poulin:** supervision, project administration, writing – review and editing, resources, methodology. **Jerusha Bennett:** writing – review and editing, visualization, supervision, resources. **Sheri L. Johnson:** writing – review and editing, formal analysis, resources, supervision, methodology.

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Ethics Statement

All procedures performed in our studies on fish were in accordance with the ethical standards of the University of Otago, where the studies were conducted.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The dataset and R codes used for this study are available from https://doi.org/10.6084/m9.figshare.29605601.v1.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Correlation matrix and principal component analyses of the behaviours of mottled triplefin (*Forsterygion capito*) infected by *Cardiocephaloides ovicorpus*. **FIGURE S2:** Relationships between the infection intensity of *Cardiocephaloides ovicorpus* and each of the behaviours of mottled triplefin (*Forsterygion capito*) measured as a binary response ($n = 34$ fish for exploration and observing, $n = 19$ for anti-predator response). **FIGURE S3:** Relationships between the infection intensity of *Cardiocephaloides ovicorpus* and each of the behaviours of mottled triplefin (*Forsterygion capito*) measured as continuous variables ($n = 18$ fish for boldness, $n = 34$ for tank crossing and aggressiveness). **FIGURE S4:** Comparison of individual scores for behaviours of mottled triplefin (*Forsterygion capito*) between two trial days for (a) tank crossing and (b) surfacing. **FIGURE S5:** Relationships between the infection intensity of *Cardiocephaloides ovicorpus* and each of the behaviour consistency of mottled triplefin (*Forsterygion capito*) ($n = 34$ fish, except $n = 19$ for anti-predator response and $n = 18$ for boldness). The consistency was measured by differences of behavioural expression between two trial days. **TABLE S1:** Results of additional generalised linear mixed models (GLMM) testing for correlations between exploration and observing behaviours of mottled triplefin (*Forsterygion capito*) when measured as continuous variables and *Cardiocephaloides ovicorpus* infection intensity. Fish size, trial day, and fish sex were also included in all models as fixed factors and fish ID as a random factor. Fish that did not perform the behaviour (i.e., behaviour measured as zero) were excluded from the model analysis.