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Thesis of
Delaney Farrell

Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science
Marine Science

Nova Southeastern University
Halmos College of Arts and Sciences

August 2023

Approved:
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NOVA SOUTHEASTERN UNIVERSITY

HALMOS COLLEGE OF ARTS AND SCIENCES

Parasites Versus Predation: The Role of Chronic and Acute Parasite Exposure in Infection Risk and Anti-Predator Behavior

By

Delaney Farrell

Submitted to the Faculty of
Halmos College of Arts and Sciences
in partial fulfillment of the requirements for
the degree of Master of Science with a specialty in:

Marine Science

Nova Southeastern University

August 2023

Abstract

Parasites with complex, multi-host lifecycles often engage in host behavior manipulation to increase transmission between successive hosts. In intermediate fish hosts, previous research has measured increased frequency of conspicuous behaviors, decreased swimming performance, and reduced antipredator behavior, which would collectively increase the fish's risk of predation. In ecosystems where this type of parasite increased trophic transmission (PITT) occurs, parasites can play a substantial role in food webs. In this study, I investigate how chronic versus acute exposure to the trematode *Euhaplorchis* sp. A. affects the antipredator behavior of the Gulf killifish *Fundulus grandis*. Using a fully crossed design, I examine how acute versus chronic exposure to infectious larval parasites (i.e., cercariae) alter the fish's primary antipredator behavior, the fast-start escape response, which is a high-speed anaerobically fueled burst swimming behavior used primarily to evade attacking predators. Chronic parasite exposure had no significant impact on the reaction timing (i.e., latency) or kinematic performance (i.e., average turning rate and distance covered) of the fast-start escape response. Acute exposure reduced average turning rates, regardless of previous infection history, indicating a decline in agility in the presence of infectious cercariae. All fish used in this study were experimentally infected in the laboratory, so I also examined the factors influencing infection risk, by investigating how body size and sex influence infection intensity. Infection intensity increased significantly with body length and was significantly greater in males than females. *Fundulus* spp. are found in marsh habitats worldwide; this study aids in understanding the ecological role of parasite exposure in defining the ecological niche of these widespread fishes.

Keywords: parasites, *Euhaplorchis*, trematodes, *Fundulus grandis*, killifish, fast-starts, escape responses, predation risk, behavioral manipulation, trophic transmission

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Introduction

Parasites are a diverse and successful group, accounting for nearly 40% of all species worldwide (Dobson et al., 2008; Hammerschmidt et al., 2009). Within marine communities, parasites are a major component of biomass and can often exceed predator biomass (Hammerschmidt et al., 2009). As a prevalent group, it is unsurprising that parasites can have a profound influence on community structure and ecosystem functions (Allan et al., 2020; Hatcher et al., 2012; Pascal et al., 2020). Parasite infection can increase host mortality and reduce reproductive fitness by drastically increasing physiological stress (Loot et al., 2001). While some parasitic organisms seek to kill the host (such as parasitoids), most do not and instead induce sublethal consumptive effects of varying intensity (Buck, 2019; Lafferty & Kuris, 2002). These sublethal effects can have far-reaching effects on host traits, altering morphology, development, physiology, behavior, and metabolism (Buck, 2019). The direct impacts of parasitic infection are often associated with the added pressures on host metabolism (Allan et al., 2020; Nadler et al., 2021a; Robar et al., 2011). Reduction in metabolic capacity due to parasite infection can indirectly impact host behavior, but there are numerous examples of parasites directly manipulating the behavioral phenotype of their host (Adamo, 2013; Allan et al., 2020; Poulin, 2010). One of the most famous cases of host manipulation is that of *Toxoplasma gondii*; hosts infected with *T. gondii* are attracted to the scent of feline urine (Berdoy et al., 2000). Infected hyenas are bolder and take more risks in the presence of lions, leading to a higher mortality rate (Gering et al., 2021). This lack of fear in infected hosts is advantageous for the parasite as felines are the final host for *T. gondii*.

Many parasites exhibit complex life cycles (CLCs), where multiple hosts are required to complete the parasite's lifecycle (Choisy et al., 2003). The addition of one or more intermediate hosts can increase the transmission rate, especially in cases where the intermediate host is preyed upon by the definitive host (Choisy et al., 2003). Spreading out their life cycle through multiple hosts can allow a parasite to specialize in different life stages (Benesh, 2016). For example, the first intermediate host may support larval development, the second intermediate host may be the site of maturation for the parasite, and the final host allows the parasite to reproduce (Benesh, 2016). Splitting a life cycle can be beneficial, especially when intermediate hosts are more common than the definitive host (Choisy et al., 2003). However, this benefit is dependent on reliable transmission between hosts (Auld & Tinsley, 2015; Choisy et al., 2003). If the parasite

fails to transmit to the correct host, it will fail to complete its life cycle and will not be able to reproduce; infecting the wrong host is often a dead end for the parasite (Parker et al., 2009). With transmission being crucial to their success, parasites with CLCs often participate in host manipulation to increase transmission from one host to another (Hughes, 2014).

Alterations in host behavior designed to increase transmission through the food chain are known as parasite increased trophic transmission, or PITT (Lafferty, 1999). Through PITT, parasites can significantly increase predation on intermediate hosts via simple alterations in host phenotype (e.g., behavior, morphology, physiology) (Poulin & Maure, 2015). This phenomenon was found in many host-parasite systems across genera but was not named until 1999. Since the term was coined, PITT has been observed in many more host-parasite systems. In fish, infection by trophically transmitted parasites has been linked to increases in conspicuous behavior, reductions or a complete loss in escape response performance, loss of shoal cohesion, and a reduction in swimming performance; all of these behavioral changes can drastically increase predation risk (Allan et al., 2020; Blake et al., 2006; Demandt et al., 2020; Lafferty & Morris, 1996; Nezhybová et al., 2020; Sacco et al., 2021). Essentially, infected prey makes for an easy meal. For many predators, the benefit of readily available food may outweigh the metabolic costs of parasite infection (Buck et al., 2018; Øverli & Johansen, 2019). In some cases, the density of the initial host is correlated to the abundance of the definitive hosts (Hechinger & Lafferty, 2005; Hechinger et al., 2007). Due to PITT encouraging transmission through food webs, parasites are involved in approximately 75% of food web links (Hammerschmidt et al., 2009; Hatcher et al., 2012). Predators can eat large amounts of infected prey in a short amount of time, allowing for quick parasite accumulation that can increase the parasite's fitness (Born-Torrijos et al., 2016; Brown et al., 2001).

Hosts can exhibit a range of changes in their anti-predator behavior following infection, including escape responses. In many fish, the fast-start escape response is the primary mechanism for predator evasion. Fast-start escape responses are high-speed anaerobically fueled responses to threats and are crucial behavior for avoiding predation (Domenici & Hale, 2019). Fast-start responses are triggered by specialized neurons known as Mauthner cells in response to threat stimuli (Eaton et al., 2001; Hecker et al., 2020). Escape responses involve an array of behavioral and kinematic components, including responsiveness, latency, reaction distance,

turning angle, swimming speed and acceleration, and directionality (Domenici, 2010; Domenici & Hale, 2019). The most prevalent form of prey escape response is the C-start, which consists of three stages (Figure 1). Stage 1 is a unilateral muscle contraction that bends the fish's body into a C shape, prepping it for movement and turning it away from the threat (Blake et al., 2006; Domenici & Blake, 1997; Domenici & Hale, 2019). In stage 2, the muscles contract on the other side of the body, propelling the fish away from the threat (Domenici, 2010). Stage 3 includes continuous swimming or gliding, bringing the fish further away from the threat (Domenici & Blake, 1997). While the fast-start response was previously thought to be an innate response lacking plasticity, we now know that this response can be altered by an array of factors, such as environmental cues or parasite infection (Allan et al., 2020; Blake et al., 2006; Domenici, 2010).

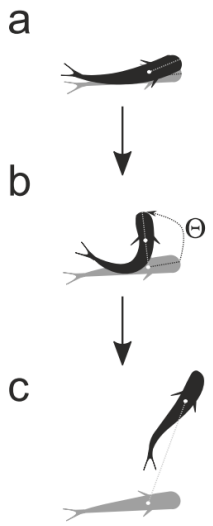

Figure 1. The three stages of a fast-start escape response. The image in (a) shows the initial reaction following a stimulus; in (b), the fish exhibits the C curve achieved at the maximum turning angle, turning the fish away from the threat and preparing the fish to accelerate rapidly—this is stage 1; the fish in (c) shows the second lateral bend (stage 2) that propels the fish away from the predator. Following (c), the fish would continue to swim away (stage 3). The grey fish indicates the starting position at the time of the predator stimulus and the black fish represents the position following the respective stage of the escape response. Figure is taken from (Nadler et al., 2021b)

Parasites are linked to reductions in escape response and swimming performance, often leading to an increase in predation mortality (Allan et al., 2020; Lafferty & Morris, 1996; Sacco

et al., 2021). Attacks by a lone gnathiid (a micropredator) significantly reduce both swimming and escape performances in ambon damselfish *Pomacentrus amboinensis* (Allan et al., 2020). Following gnathiid infection, the fish's cortisol levels spike (Allan et al., 2020). Infections by endoparasites also affect escape responses. Cestode (*Shistocephalus solidus*) infection is linked to a reduction in escape response and swimming capacity in three-spined sticklebacks *Gasterosteus aculeatus* (Blake et al., 2006). *Shistocephalus solidus* infection also impairs grouping behavior. Infected sticklebacks react slower to threats than uninfected fish, reducing shoal cohesion and increasing predation risk for all fish in the shoal (Demandt et al., 2021; Demandt et al., 2020). Infected sticklebacks tend to take higher risks in the presence of predators and fish at extreme infection levels may not react to a threat at all (Blake et al., 2006). While *S. solidus* infection has a significant impact on stickleback behavior, infection with a trematode, *Bunodera* sp.,has no effect (Blake et al., 2006). It is important to note that the effect of parasite infection on anti-predator behavior is highly variable amongst species and infection intensities, with many parasite species having no effect on escape response performance.

Manipulation is often conducted by parasites that reside in or on the central nervous system, where host behavior is altered by releasing substances that interfere with normal neural communication (Adamo, 2013; Lafferty & Shaw, 2013). Modifications in serotonergic activity by parasites are associated with behavioral manipulation (Helland-Riise et al., 2020; Shaw et al., 2009). Inhibition of dopamine signaling is also found in infected hosts (Shaw & Øverli, 2012). Despite interrupting normal neurotransmission, infected hosts can often maintain their body condition and reproductive activity (Shaw et al., 2009). Behavioral manipulation can vary between host populations and with parasite life stages. While mature parasites may alter fish behavior to increase trophic transmission, immature parasites can enhance the host's anti-predator behavior (Gopko et al., 2015; Hammerschmidt et al., 2009; Parker et al., 2009). By reducing vulnerability to predators while developing, parasites can improve their odds of reaching the correct life stage before entering the next host (Gopko et al., 2015; Parker et al., 2009). This can be problematic for researchers examining host-parasite systems involving behavioral manipulation, as the time after infection can affect the level and the type of manipulation. Variations in behavioral manipulation are also found between populations of the same host (Franceschi et al., 2010). Hosts from naïve populations have been found to be more sensitive to parasite manipulation than those from naturally infected populations, indicating that

fish populations with long term exposure may develop a resistance to manipulation (Franceschi et al., 2010). These variations can make it difficult to compare the behavior of naturally infected fish to naïve control fish, resulting in a need for experimental infections of naïve fish. Experimental infections can be extremely difficult to complete, as a naïve population must either be found or created through lab rearing and there must be a reliable source of the infective stage of the parasite (Franceschi et al., 2010; Helland-Riise et al., 2020). Ensuring successful infection of hosts can be incredibly difficult and time consuming; it may not be possible for all host-parasite systems.

The complex life cycle of the trematode *Euhaplorchis californiensis* has been extensively investigated, as well as its impact on its second intermediate host, the California killifish *Fundulus parvipinnis* (Lafferty & Morris, 1996). *Euhaplorchis californiensis* begins its life cycle in the California horn snail *Cerithideopsis californica* before leaving as a free-swimming larval phase (i.e., cercaria) that will only infect *F. parvipinnis* (Lafferty & Morris, 1996). When an *E. californiensis* cercaria encounters a fish, it enters the body by burrowing through the skin or through the gill epithelium. Once inside the fish, the cercaria travels to the head where it encysts as a metacercaria between the skull and the surface of the brain (Figure 2) and is believed to alter the behaviors of its host by inhibiting serotonin and dopamine signaling (Lafferty & Morris, 1996; Shaw et al., 2010; Shaw & Øverli, 2012). Infected fish show four times more conspicuous behaviors than uninfected fish (Lafferty & Morris, 1996). Infection related alterations in behavior result in a 10-30x increase in predation risk (Lafferty & Morris, 1996). The presence of *E. californiensis* cercariae in the environment has been linked to altered metabolism in *F. parvipinnis*, increasing both activity levels and the metabolic rate; these effects were greater in previously exposed individuals (Nadler et al., 2021a). These findings indicate that fish can identify cercariae in the environment and recognize them as a potential threat.

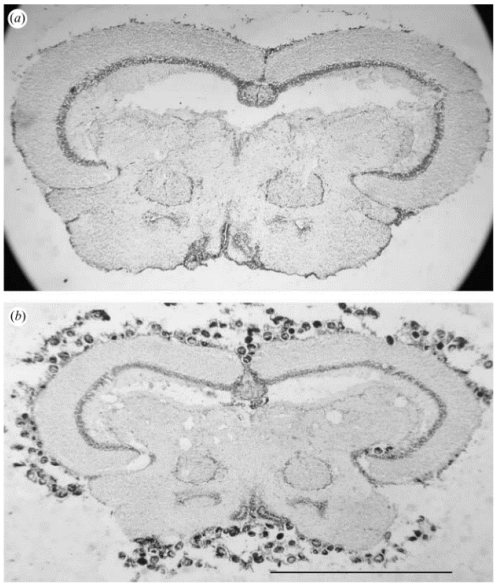

Figure 2. Histological sections of an uninfected *F. parvipinnis* brain and a *F. parvipinnis* brain infected with *E. californiensis*. The top panel (a) shows a histological section of the brain of an uninfected *F. parvipinnis*. The bottom panel (b) shows a histological section of the brain of a fish infected with *E. californiensis*. The black dots surrounding the brain in the bottom image are *E. californiensis* metacercariae. Figure is taken from (Shaw et al., 2009).

A related host-parasite system is found in the coastal waters of the Gulf of Mexico and off Florida's Atlantic coast. Here, the still-unnamed *Euhaplorchis* sp. A targets small-bodied mangrove fishes (Fredensborg & Longoria, 2012; Hernandez & Fredensborg, 2015). Unlike *E. californiensis,* this congeneric trematode parasite is a generalist at the second intermediate host stage, capable of infecting the Gulf killifish *Fundulus grandis*, the longnose killifish *F. similis*, the bayou killifish *F. pulvereus*, and the sailfin molly *Poecilia latipinna* (McNeff, 1978). Similar to *E. californiensis*, *Euhaplorchis* sp. A begins its life cycle in a snail, with studies linking this unnamed parasite to the ladder horn snail *Cerithidea scalariformis* on the Florida Atlantic coast and the plicate horn snail *C. pliculosa* in the western Gulf of Mexico (Fredensborg & Longoria, 2012; McNeff, 1978; Smith, 2001). While research on this system is limited, there is evidence that *Euhaplorchis* sp. A increases conspicuous behaviors in host fish, potentially increasing the rate of predation by marsh birds (Fredensborg & Longoria, 2012; Hernandez & Fredensborg, 2015). In this thesis, I aim to increase the understanding of this system by quantifying infection intensity following experimental infections and by examining the behavioral impacts of both long-term infection and acute exposure to *Euhaplorchis* sp. A cercariae.

First, I will examine the relationship among body size, sex, and infection intensity. In many host-parasite systems, including the *E. californiensis* system, larger individuals tend to harbor more parasites (Born-Torrijos et al., 2016; Helland-Riise et al., 2020; Shaw et al., 2010). Interestingly, this trend was not seen in the Florida system with naturally infected *F. similis* (Fredensborg & Longoria, 2012). Fredensborg and Longoria (2012a) examined wild-caught and naturally infected fish, so fish with higher infection intensities may have been more intensely preyed upon and thus not present in their surveys. I hypothesize that infection intensity increases with body size in experimentally infected *F. grandis*. Evidence from an array of different taxa indicates that males of a species often exhibit greater parasite prevalence and infection intensity (Stephenson et al., 2016; Zuk & McKean, 1996). Increased infection intensity in males is likely linked to the deviation of life-history strategies between males and females, as well as the presence of high testosterone levels in males (Grear et al., 2009). Testosterone is believed to act as an immunosuppressant, reducing parasite resistance in males (Folstad & Karter, 1992). In the California system, female *F. parvipinnis* show a higher parasite load than males, regardless of size (Shaw et al., 2010). This deviation may be tied to the slower growth of female *F. parvipinnis* or to heavily infected males being preferentially removed by predators (Shaw et al., 2010). I hypothesize that there is a significant difference in the infection intensity between male and female fish, with males having a more severe infection.

I will also examine the relationship between parasite exposure and fast-start escape responses, comparing uninfected versus infected fish (i.e., control fish versus fish experimentally exposed to infectious cercariae). *Euhaplorchis* sp. A infection increases surfacing and other risk-taking behavior in both *F. grandis* and *F. similis* (Fredensborg & Longoria, 2012; Hernandez & Fredensborg, 2015). I hypothesize that infected fish show a reduced capacity for fast-start escape responses. I will also compare the escape responses of both uninfected and infected fish in the presence of *Euhaplorchis* sp. A cercariae. The presence of *E. californiensis* cercariae increases the routine metabolic rate of *F. parvipinnis*; the effect is marginally higher in previously infected fish (Nadler et al., 2021a). I hypothesize the presence of cercariae reduces escape response readiness and the kinematics of the response, with the effect greater in previously exposed individuals.

Methods

All collection methods, husbandry protocols, experimental infections, behavioral trials, and euthanasia methods were approved by the Nova Southeastern University Institutional Animal Care and Use Committee (IACUC) under protocol #2021.10.LD4.

Specimen Collection and Husbandry

Fundulus grandis (n = 182 fish) were collected between May and July 2022 from Spruce Creek Preserve in New Smyrna Beach, Florida over five collection trips. This population was selected based on preliminary studies indicating a lack of *Euhaplorchis* sp. A. Fish were collected at two locations within the preserve, Site 1 (29.078N, -80.953W) and Site 2 (29.083N, -80.964W) (Figure 3). Fish were collected using a 3-meter seine net (3mm gauge). Once fish were identified as *F. grandis*, fish were placed in a 10-gallon cooler with an aerator for transport back to the Nadler Marine Behavior and Physiology (NMBP) Lab at the Nova Southeastern University Oceanographic Campus. A maximum of 30 fish were added to each cooler. Upon arrival at the NMBP Lab, fish received a one-minute freshwater rinse to remove external parasites.

Figure 3. Map of collection sites within Spruce Creek Preserve. Two collection sites were used: Site 1 (29.078N, -80.953W) and Site 2 (29.083N, -80.964W). The majority of the fish used in this study were collected at Site 2.

Fish were housed in twelve 10-gallon tanks with no more than 25 fish per tank. Small sections of PVC pipes of assorted sizes were added to the bottom of the tanks for fish to use as shelters, which reduced territorial aggression between fish. The tanks were split evenly between two independent seawater systems, with six tanks on each system to prevent cercariae from entering the control groups during experimental infections. For each uninfected tank, there was a paired infected tank that was collected on the same date. The tank room was kept on a consistent day/night light schedule (13:10hr of light, from 07:30-18:40) designed to match the natural 24-hr summer light cycle. Fish were fed Omega One freeze-dried bloodworms twice a day, once in the morning (08:30-11:30) and once in the afternoon (13:30-17:00). Feeding occurred at least one hour after lights turned on and an hour before lights turned off, with at least two hours between feedings. Water quality parameters (pH, nitrate, nitrite, ammonia, and salinity) were tested daily using an API Saltwater Master Test Kit and a refractometer; salinity was kept between 25 and 28 ppt. All tanks received a quick daily cleaning prior to the morning feeding. During this cleaning, any deceased fish were removed and frozen at -20°C prior to dissection. Once a week, all tanks received a deep clean, along with the system's sump walls, filtration bags, and protein skimmer. Following a deep clean, a 25% water change was completed using fresh salt water (25 ppt; made with distilled water, Instant Ocean Aquarium Salt, and FritzZyme 9 Saltwater live nitrifying bacteria additive) to maintain a consistent water level and improve water quality.

Infected *Cerithidea scalariformis* were collected in June 2022 from the Indian River Lagoon in Fort Pierce, Florida. Only mature snails were collected and brought to the Smithsonian Marine Station in Fort Pierce, Florida. *Euhaplorchis* sp. A infected snails were identified through morphological examination of the cercariae shed using the same shedding protocol as for each infection event (detailed below). Snails with confirmed *Euhaplorchis* sp. A infections were shipped to the NMBP Lab. Snails were housed in small (1.5 L) plastic tanks with no more than five per tank. A damp paper towel was placed in the tank to prevent drying out, along with three algae wafers (Tetra PRO PlecoWafers). The paper towels and algae wafers were replaced daily.

Experimental Infections

Experimental infections took place from July to August 2022, following an infection protocol adapted from the protocol outlined in S. H. Helland-Riise et al. (2020). Prior to each

infection event, snails (n = 5) were placed in mesh bags and allowed to dry out overnight. Following drying, snails were placed in compartment boxes (one snail per box) and covered with warm seawater (28-30°C). The snails were left in the water for two hours under a heat lamp (28-30°C) before each compartment was checked for cercariae using a dissecting microscope. All water containing cercariae was placed into a beaker and the total volume of water was recorded. The number of cercariae in 2 ml of water was counted under the dissecting microscope then extrapolated to calculate the total number of cercariae shed. The water was divided into scintillation vials based on the number of fish in each tank so that each fish was exposed to between 200 and 250 cercariae per infection event (Table 1). Fish were left in their home tank for infections and water flow was turned off. Prior to the addition of cercariae (or seawater sham for uninfected treatment), tanks were drained halfway (to five gallons) before the addition of either sham seawater or cercariae. Paired tanks received the same treatment schedule during infections, with the only variation being in the presence or lack of cercariae. The low water level was maintained for four hours to allow time for an infection to occur and maximize the encounters between fish hosts and infectious cercariae. Fish were not fed before infection to prevent a reduction in water quality when water flow was turned off. Infection events were at least three days apart to prevent excessive stress on the fish. Each tank received seven infection events, with each fish exposed to 1400-1600 cercariae total (Table 1). One month following infection, the fish were tagged with visible implant elastomer (VIE) tags. These tags function as fluorescent subcutaneous tattoos, making fish uniquely identifiable; there is no evidence that VIE tags have any adverse effects on fish survival or growth (Hoey & Mccormick, 2006).

Table 1. Summary of experimental infections.

Tank (n = fish)	***Estimated number of cercariae per fish***	***Number of infection events***	***Date of last infection event***
1 (n =16)	1568	7	8/14/22
2 (n = 14)	1499	7	8/14/22
3 (n = 14)	1518	7	8/12/22
4 (n = 14)	1603	7	8/15/22
5 (n = 10)	1567	7	8/12/22

Behavioral Trials

Fish were split into four treatment groups, crossing cercariae exposure (sham, cercariae exposure) and long-term infection status (uninfected, infected): sham + infected (treatment 1; n = 14), cercariae + infected (treatment 2; n = 15), sham + uninfected (treatment 3; n = 12), and cercariae + uninfected (treatment 4; n = 13). Each fish was only used in one escape response trial, allowing for independence of all treatment groups. The fish was placed in a behavioral arena (Figure 4) with five gallons of salt water and allowed to acclimate for 45 minutes. Following placement of the fish in the arena, a white tarp was hung up around the tank's PVC frame to prevent any external visual stimuli from startling the fish. Fish that failed to acclimate and exhibited abnormal swimming behavior (i.e., darting, flashing, freezing) after 30 minutes were removed from the arena and returned to their home tank for at least 24-hours before attempting another trial. Only six fish (11%) required a retrial. Fish were observed via a convex mirror placed over the tank to prevent disruption of the acclimation period. After acclimation was completed, 50 mL of either sham seawater or seawater containing cercariae was added to the arena. Seawater was added from outside the tarped off arena via a syringe attached to a small line of tubing to reduce the risk of startling the fish (i.e., no stimulation from the researcher when adding this stimuli). Approximately 1000 cercariae per trial (200 cercariae per gallon) were added for treatments 2 and 4. The cercariae density was chosen based off fish behavior during preliminary exposures. Fish exposed to greater cercariae densities tended to freeze and not respond to external stimuli; at 200 cercariae/gallon the fish reacted to the addition of cercariae but remained active. The exposure was filmed for ten minutes at 30fps to measure activity levels. Following the exposure window, a small weight was remotely dropped into the arena via an electromagnet to stimulate an aerial predator attack. The stimulus was released behind a PVC half-pipe shield to prevent the fish from seeing the stimulus before it struck the water. The stimulus was only released when the fish is within 1-2 body lengths of (4-8cm) the stimulus. The release of the stimulus and the fish's response was recorded at 240fps to allow for examination of all components of the escape response. Following completion of the escape response, the fish's movements were recorded at 30fps for ten minutes to track recovery following the predator stimulus. After completion of the recovery window, the fish was removed from the arena. The tank was fully drained and wiped down with a clean cloth following each trial. Following the escape response trial, the fish was immediately euthanized via an overdose of MS222 anesthetic

(250mg/L, buffered with sodium bicarbonate) and frozen at -20°C. Fish were left submerged in MS222 for 30 minutes after the last gill movement was observed. All fish were dissected to confirm their infection status and intensity—all fish within the "infected" treatment groups had metacercariae within the brain and/or head region, at varying intensities.

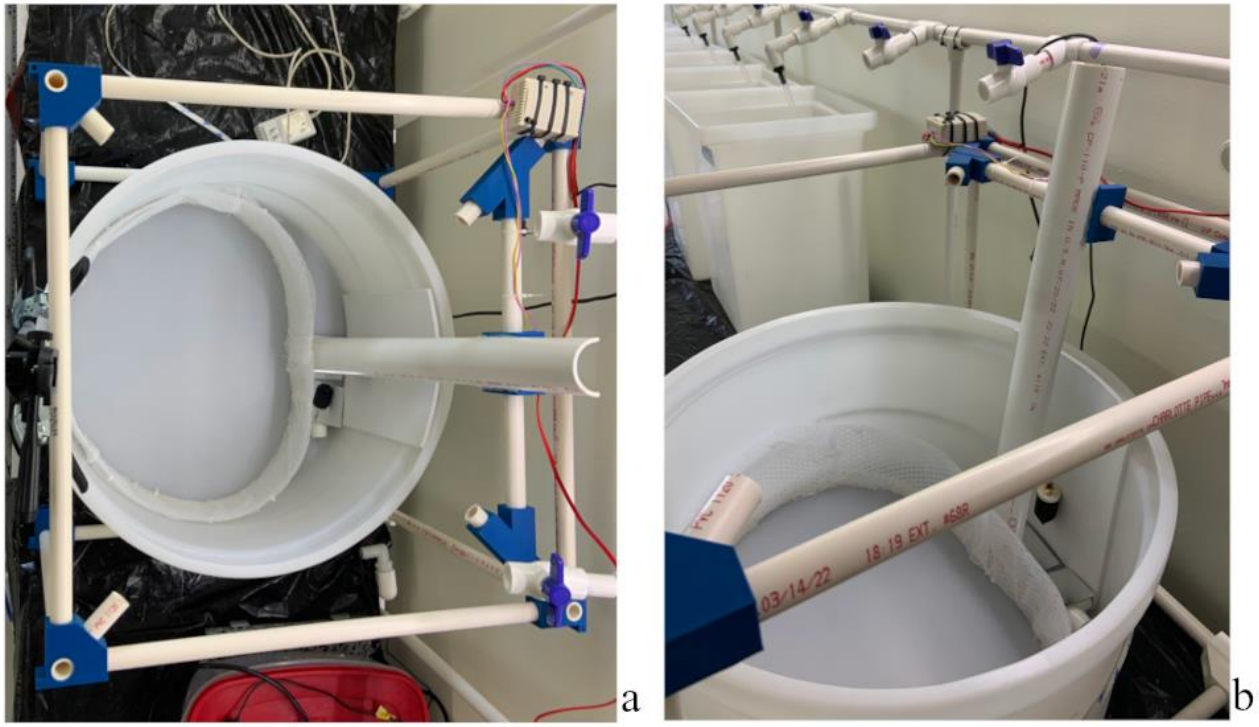

Figure 4. Behavioral arena. (a) Overhead view of the behavioral arena. The mesh circle is where the fish was placed to prevent it from swimming underneath the stimulus. The PVC frame surrounding the tank supported the camera tripod and the electromagnet that released the stimulus. (b) Side view of the area, highlighting the stimulus. The stimulus was dropped behind a PVC half pipe to prevent the fish from responding prior to the stimulus reaching the water surface. Mirrors were placed on the bottom of the tank to ensure the stimulus was still visible on camera.

Dissections

Fish from the completed fast-start experiment described above were dissected, as well as another set of experimentally infected fish used for a different study. In total, 117 *F. grandis* were dissected. For each fish, the body mass (to the nearest 0.001 g), standard length (to the nearest mm), total length (to the nearest mm), and sex (male, female) was recorded (Figure 5).

The brain and head—the preferred areas for *Euhaplorchis* sp. A to encyst—were examined. During initial dissections of fish that underwent acute cercariae exposure, extra care was taken to examine the dermis and head area for any new infections. No fresh infections were found, potentially due to the short exposure window and low exposure density or potentially from lack of magnification power preventing identification of new infections. The number of *Euhaplorchis* sp. A metacercariae found in the head or on the brain was recorded to quantify parasite prevalence and infection intensity following experimental infection. Prevalence is a descriptive tool that is defined as the number of individuals with at least one parasite within a given sample (Bush et al., 1997). Infection intensity is defined as the number of parasites found within a single host (Bush et al., 1997). The developmental stage of the metacercariae found was determined based on morphological features (e. g. eye spots, cyst wall thickness, folding in cyst wall). The external features (skin, gills, eyes, fins) and the organs within the body cavity (heart, swim bladder, intestines, gonads, kidneys, liver) were examined for any irregularities or additional parasite infections. Sex was confirmed via examination of the gonads to ensure accuracy.

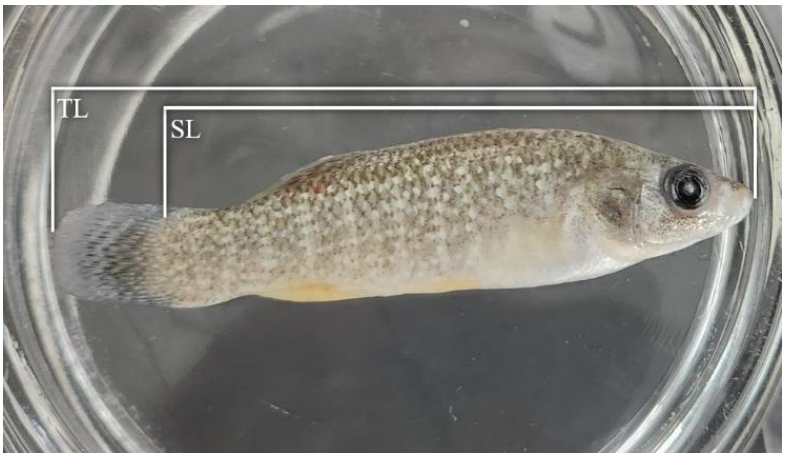

Figure 5. Lateral view of a *F. grandis* specimen. Total length (TL) and standard length (SL) measurement guidelines are marked above the fish. SL was used as the main body size measurement due to the fragile nature of the caudal fin.

Video Analysis

Fast-start response video analysis was conducted using DJV media player and ImageJ (Johnston et al., 2020; Schneider et al., 2012). Escape responses were assessed by measuring

latency, average turning rate, and distance covered (see Table 2 for definitions). Latency was measured by comparing the stimulus frame (i.e., the first frame in which the stimulus breaks the water's surface) and the response frame (i.e., the first frame showing movement by the fish following the stimulus). The frame difference was then converted to milliseconds (1 frame = 4.2 ms). Any video where the fish did not react to the stimulus within one second (240 frames) was considered a non-reaction and did not receive further analysis. Of the initial 54 recorded trials, six fish showed no reaction to the stimulus. Three fish had a latency time over 25.2 ms. These fish, along with the non-reactors, were excluded from statistical models as they do not qualify as "fast" Mauthner cell mediated responses, which are the main focus of this analysis (Hecker et al., 2020).

Table 2. Definitions of fast-start escape response components.

Latency	Time until fish responds to the initial predator stimulus
Average turning rate	The maximum angle achieved by the fish divided by the time it takes for the fish to reach the maximum angle
Distance covered	Distance traveled by the fish during the response

For analysis of average turning rate and distance covered, three screenshots were taken for each video. The first screenshot (t = 0 ms) is one frame before the fish shows initial movement in response to the stimulus. The second screenshot (stage 1) shows the maximum angle achieved by the fish's head, just prior to movement of the tail, during stage 1 of the fast-start response. The final screenshot (t = 42 ms) was taken ten frames after the initial frame (t = 0 ms)—most fish completed the fast-start response within this ten-frame window. Frame numbers were recorded for each screenshot. Distance covered by the fast-start response was measured by comparing the starting coordinates of the fish (t = 0 ms) and the final coordinates ten frames after the initial response (t = 42 ms). For average turning rate, the initial angle of the fish (t = 0 ms) was compared to the angle following completion of stage 1. This angle was divided by the time required to reach the maximum angle, which was found by subtracting the initial frame number (t = 0 ms) from the stage 1 completion frame number and converting from frames to milliseconds. The stimulus distance (distance from the fish's center of mass to the PVC stimulus

shield) was also measured on the initial frame (t = 0 ms) as a potential factor affecting fast-start responsiveness.

Statistical Analysis

All statistical analyses were completed using RStudio, running R Statistical Software v.4.3.0 (R Core Team, 2023). The effects of infection status (infected/uninfected), acute exposure (cercariae/sham), and stimulus distance on each fast-start response parameter (latency, average turning rate, and distance covered) were examined through generalized linear mixed-effects models (GLMM) using the package glmmTMB (Brooks et al., 2017). The fish's home tank was included in the models as a random factor. The assumptions for each model were testing using the package DHARMa (Hartig, 2022). Post-hoc analysis of the models was completed via an ANOVA using the packages car and emmeans (Fox & Weisberg, 2019; Lenth, 2023).

The impact of body size (represented by standard length) and sex on infection intensity was examined using a generalized linear model (GLM). A negative binomial GLM (supported by the package MASS) was used due to overdispersion in the data (Venables & Ripley, 2002). Overdispersion was found by examining the residuals. Significance of the predictors was determined using an ANOVA. A one-tailed t-test was used to determine which sex had greater *Euhaplorchis* sp. A count. Most fish included in the analysis were euthanized, however fish that were found deceased were also included in the analysis if the body was fully intact and the fish died at least one month following the final infection event. Standard length was used to represent body size instead of total length or weight, both of which were measured for each fish. Total length was not always measurable due to the fragile nature of the caudal fins and weight may not be accurate due to the freezing and thawing process.

Results

Experimental Infections

Fifty-nine fish were exposed to cercariae (referred to as infected) as a part of the experimental infection protocol. Only two of the infected fish failed to show visible *Euhaplorchis* sp. A infection—a 96.61% infection prevalence. No control (referred to as uninfected) fish exhibited *Euhaplorchis* sp. A infection, indicating there was no contamination between systems. A mean abundance of 165 ± 150 metacercariae were found in each fish. Metacercariae counts in infected fish ranged from 0 to 711. There was a significant difference in

infection intensity between tanks (Kruskal-Wallis $\chi^2 = 23.708$; df = 4; $p < 0.001$). Tank 3 had the lowest average infection intensity (49 ± 50) and tank 4 had the greatest (307 ± 191) (Table 3). Both failed infections (exposed fish with no *Euhaplorchis* sp. A metacercariae observed) originated from Tank 3. Tank 4 had the greatest variation in infection intensity, with metacercariae counts ranging from 41 to 711. Tank 5 had the lowest variation, with counts ranging from 51 – 187.

Table 3. Infection intensities by tank.

Tank of origin	***Mean metacercariae count ± standard deviation***	***Metacercariae count range***	***Estimated number of cercariae per fish*** ***(from Table 1)***
1	134 ± 141	2 – 475, 473	1568
2	235 ± 180	54 – 695, 641	1499
3	49 ± 50	0 – 167, 167	1518
4	307 ± 191	41 – 711, 670	1603
5	132 ± 42	51 – 187, 136	1567

Sex, Body Size, and Infection Intensity

Standard length (SL) had a significant effect on total *Euhaplorchis* sp. A count ($\chi^2 = 5.34$; df = 1; $p = 0.021$), as did sex ($\chi^2 = 5.04$; df =1; $p = 0.025$) (Figure 6). Males had a significantly higher infection intensity than females ($t = -2.52$; $p = 0.008$). Based upon Akaike information criterion (AIC) scores, a model excluding the interaction between SL and sex was selected as the model of best fit. Females had an average standard length of 5.48 ± 0.93 cm, with a range of 3.91-7.85 cm. Males had an average standard length of 5.2 ± 0.72 cm, with a range of 3.76-7.01 cm. There was no significant difference in the standard lengths between the sexes ($t = 1.58$; $p = 0.120$).

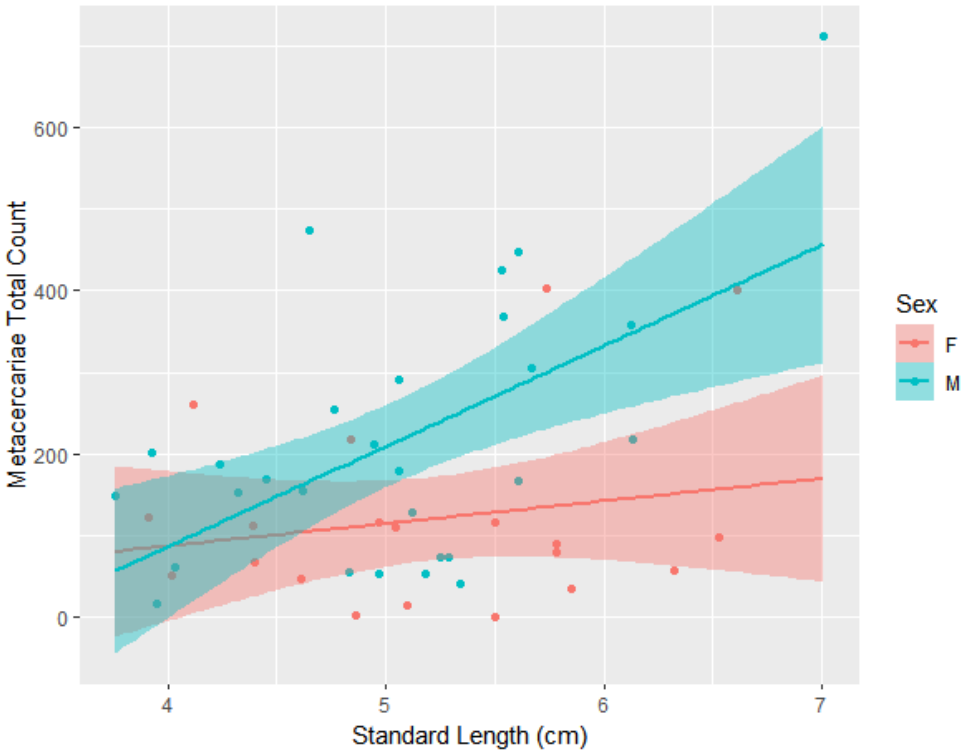

Figure 6. *Euhaplorchis* sp. A metacercariae counts versus standard length for male and female *F. grandis*. Females are represented by the magenta, males by the blue. There is a significant increase in metacercariae count as standard length increases. Males have a significantly higher metacercariae count than females.

Latency

There was no significant difference in latency between fish with different infection statuses (infected/uninfected) (χ^2 = 1.3155; p = 0.251) or between exposure treatment (cercariae/sham) (χ^2 = 0.9746; p = 0.987). There was no significant interaction between the treatments (χ^2 = 0.0003; p = 0.987). Stimulus distance did not have an effect on latency (χ^2 = 0.0184; p = 0.8920).

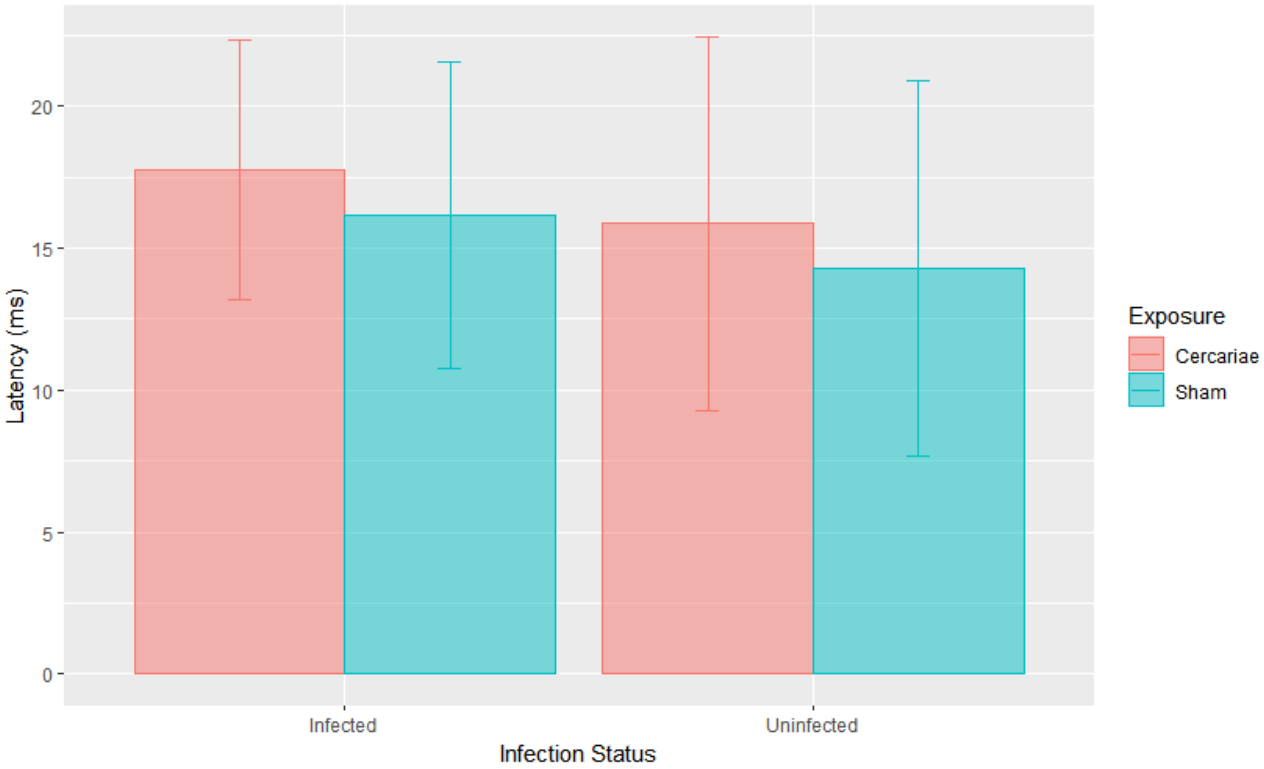

Figure 7. Bar plot comparing the latency between the four treatment groups. No difference was detected for infection or exposure treatment ($p > 0.05$).

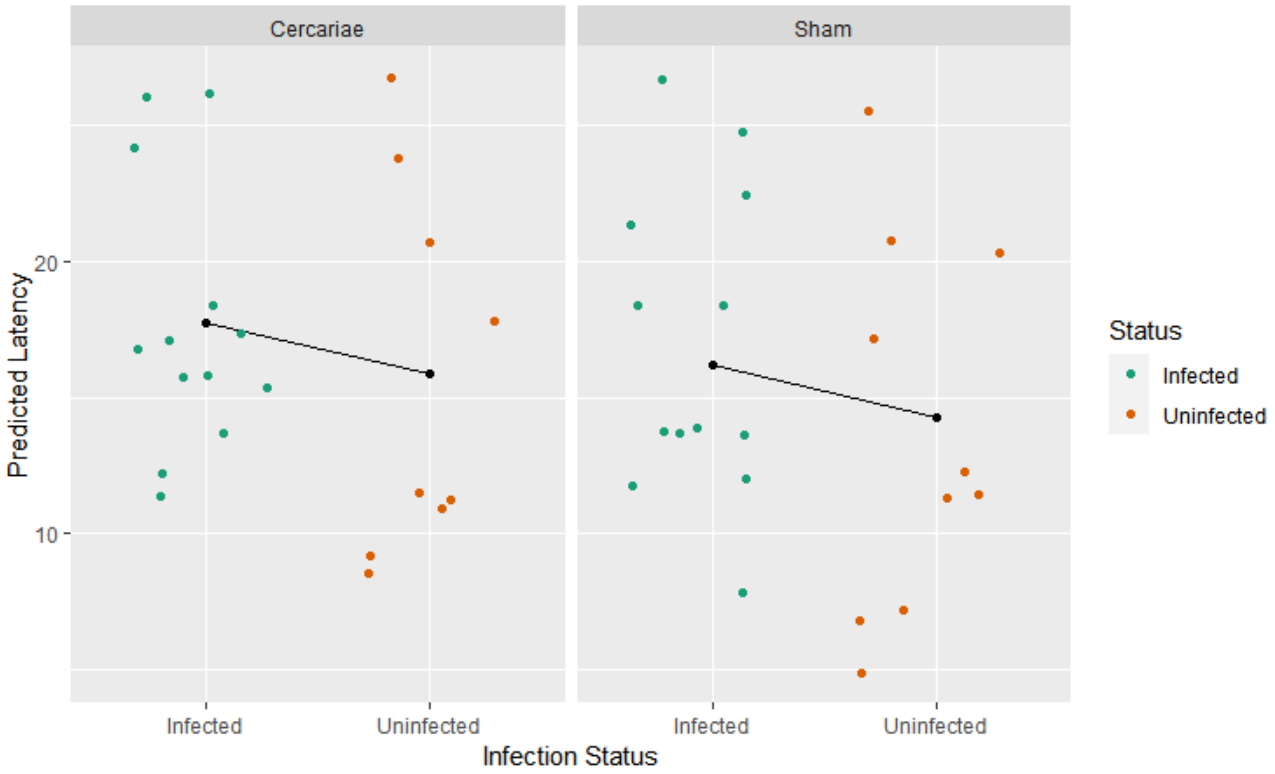

Figure 8. Predicted latency (ms) values for infected and uninfected fish in both exposure treatments. Predictions are based upon GLMM analysis.

Average Turning Rate

There was no significant difference in average turning rate (ATR) between infected and uninfected fish (χ^2= 0.0266; p = 0.870). However, there was a significant difference between the exposures, with sham exposed fish showing a greater average turning rate than the cercariae exposed fish (χ^2= 5.0041; p = 0.025) (Figure 10b). There was no significant interaction between the infection and exposure (χ^2= 1.6689; p = 0.196) (Figure 9 & 10a). Stimulus distance did not have an effect (χ^2= 0.5141; p = 0.47338).

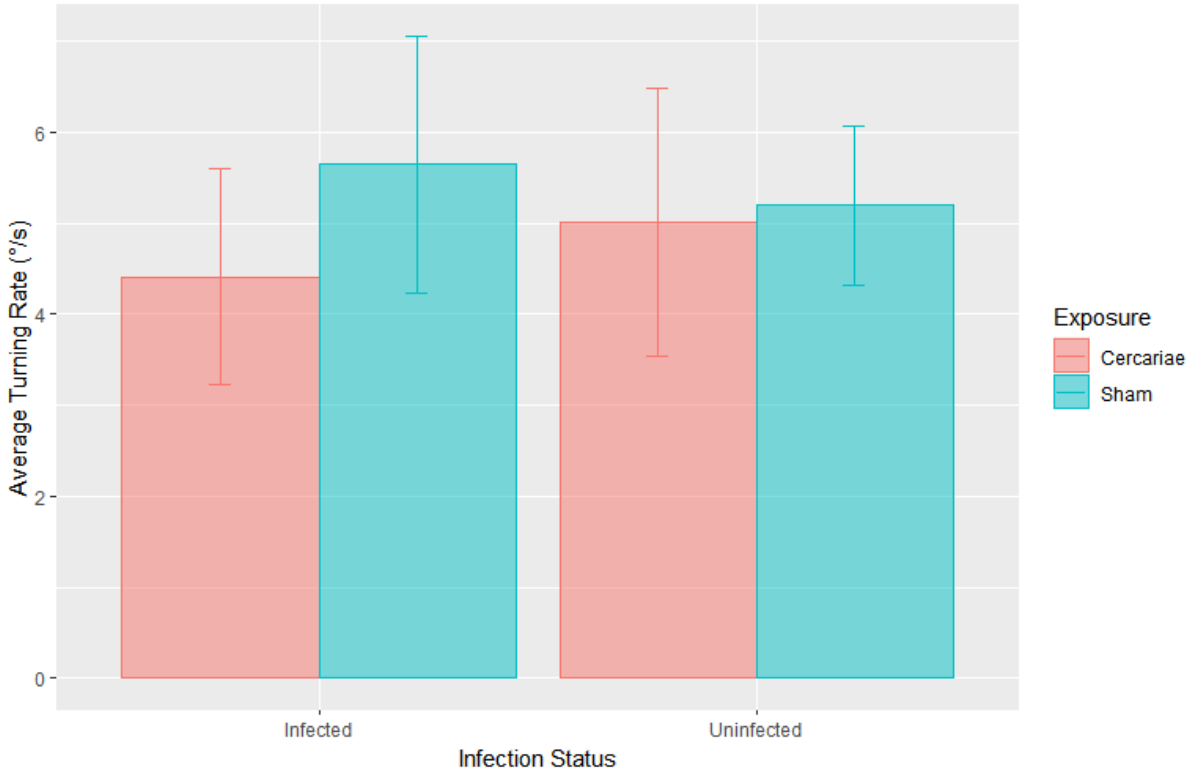

Figure 9. Bar plot comparing the average turning rate between the four treatment groups. Sham exposed fish had a significantly higher turning rate (χ^2= 5.0041; p = 0.025). Infected fish exposed to cercariae had the lowest turning rate values.

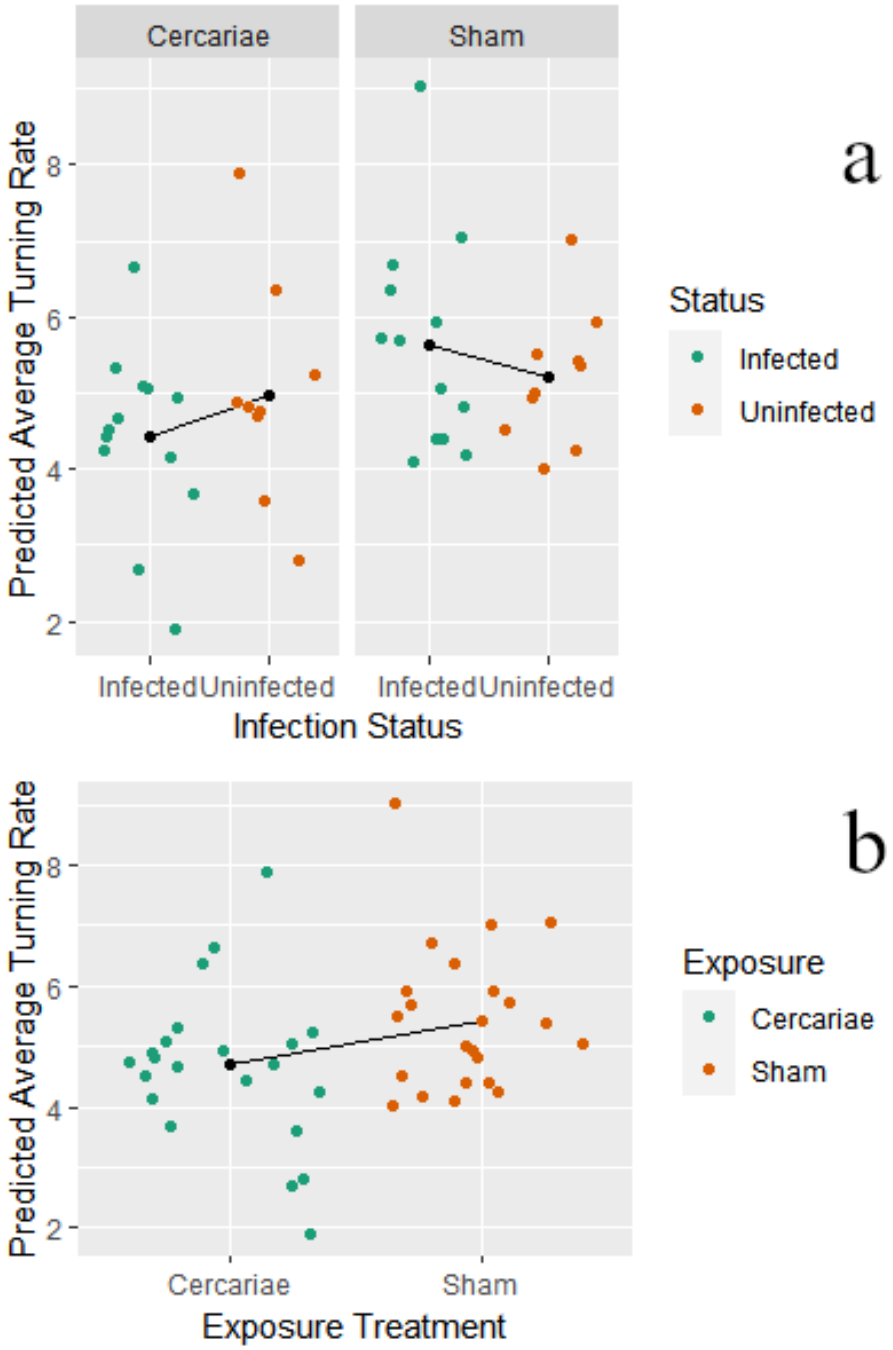

Figure 10. Predicted average turning rate (°/s) values for infected and uninfected fish in both exposure treatments. Predictions are based upon GLMM analysis. Fish in the sham treatment exhibit significantly higher ATR values (χ^2= 5.0041; p = 0.025) (b). All other comparisons are not significant (a).

Distance Covered

Infection status had no significant effect on the distance covered by fish post-stimulus (χ^2= 0.0428; p = 0.8360), nor did exposure treatment (χ^2= 0.0006; p = 0.9809) (Figure 12). There was no interaction observed between status and exposure (χ^2= 1.1600; p = 0.2815) (Figure 11). Stimulus distance did not have a significant effect (χ^2= 0.5592; p = 0.4546).

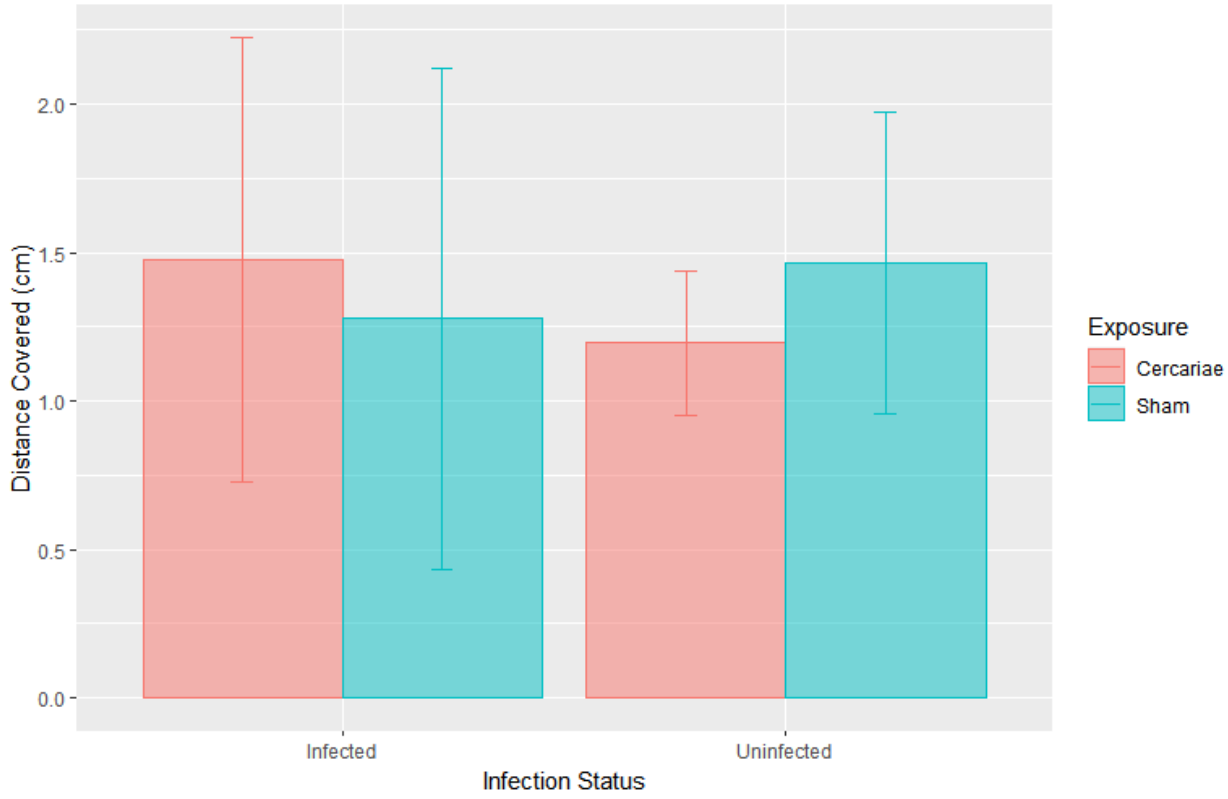

Figure 11. Bar plot comparing the distance covered between the four treatment groups. There was no significant difference between the treatments (p > 0.05).

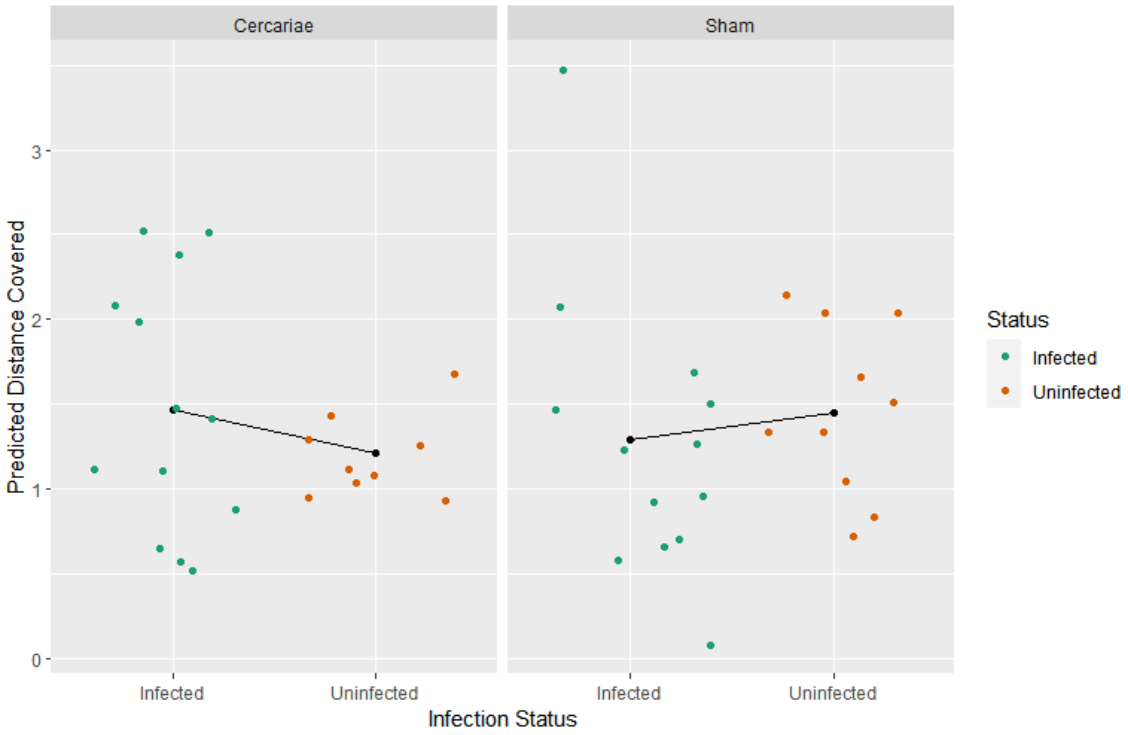

Figure 12. Predicted distance covered (cm) values for infected and uninfected fish in both exposure treatments. Predictions are based upon GLMM analysis.

Discussion

The ability to successfully perform anti-predator behaviors is crucial to the individual success of a fish. In this study, we found that *Euhaplorchis* sp. A chronic infection did not impact fast-start responses in *F. grandis* despite previous evidence showing that infection increases conspicuous behaviors (Fredensborg & Longoria, 2012). Acute exposure to *Euhaplorchis* sp. A cercariae significantly reduced average turning rate in both infected and uninfected fish, indicating that the presence of infectious cercariae may trigger a fear response in *F. grandis* that results in a reduction in agility. The fish used in this study were collected from a naïve population and experimentally infected with *Euhaplorchis* sp. A. Infection intensities varied significantly with both size and sex. Larger fish had greater metacercariae counts, likely due to their size increasing the potential for interaction with cercariae. Males had significantly higher metacercariae counts than females. This is a common trend across phyla and may be linked to differences in life history and to testosterones role as an immunosuppressant (Grear et

al., 2009). Together, these results show how infection impacts subsects of fish populations differently and how anti-predator behavior may vary between these groups.

Factors Impacting Euhaplorchis sp. A Infection Intensity in Experimentally Infected Fundulus grandis

The experimental infection protocol utilized in this study was successful, with 96.61% of the exposed fish developing *Euhaplorchis* sp. A infections. The two fish with failed infections were used in the behavioral trials as there is evidence that repeated parasite encounters can still alter the behavior of resistant hosts (Vindas et al., 2023). We detected a great deal of variation in infection intensity within and among tanks. This result indicates that infection prevalence and intensity are dictated by factors other than the number of parasites fish are exposed to. Evidence suggests that infection success is influenced by environmental factors and genetic variation in both the parasite and the host (Vale & Little, 2009). Infection susceptibility can vary spatially between genetically connected host populations, suggesting that ecological and behavioral factors play a significant role in susceptibility (Gibson et al., 2016). Fish received the same treatment across all tanks to reduce the impact of environment on infection success. The high variation in infection intensity between tanks is likely driven by differences in behavior.

As hypothesized, infection intensity increased with standard length. This echoes the California system, where larger fish exhibit greater parasite loads (Born-Torrijos et al., 2016; Helland-Riise et al., 2020; Shaw et al., 2010). In the wild, larger animals often have more intense infections. As fish grow with age, parasites accumulate over time, resulting in older and larger fish developing more severe infections (Shaw & Dobson, 1995). However, this pattern was not observed in wild-caught specimens of the closely related *F. similis* infected with *Euhaplorchis* sp. A (Fredensborg & Longoria, 2012). If parasites alter fish behavior to increase trophic transmission, large fish with high parasite loads may be absent from a population due to preferential removal by predators (Fredensborg & Longoria, 2012; Rousset et al., 1996; Shaw et al., 2010). Larger fish with previous exposures to a parasite may also be more resistant to future infections through mechanisms of acquired immunity (Fredensborg & Longoria, 2012). The pattern observed in this study indicates that larger fish will develop more intense *Euhaplorchis* sp. A infections, even over a short exposure period. Cercariae infect fish by burying through their skin or gill epithelium, so it is possible that larger fish with a greater skin surface area are simply easier targets for cercariae. The fish used in this study were collected from a naïve population of

F. grandis; wild-caught fish from a population with a known *Euhaplorchis* sp. A presence should be examined to compare the differences between naturally and experimentally infected fish.

No evidence of sexually dimorphic size variation in *F. grandis* was found in this study; there was no significant size difference between males and females. Despite the lack of size difference, there was a difference in infection intensity between sexes. Males exhibited a significantly higher infection intensity than females. This result diverges from previous literature, as Hernandez and Fredensborg (2015) found no association between sex and infection status in *F. grandis*. Higher parasite loads in males is a common trend across many taxa, including fish (Stephenson et al., 2016; Zuk & McKean, 1996). This variation in parasite resistance may be derived from a difference in life history strategies, as well as variation in hormonal composition between the sexes (Folstad & Karter, 1992; Rolff, 2002). Reproduction is more energetically expensive for females, driving them to invest in in parasite resistance to ensure longevity and lasting reproductive success (Stephenson et al., 2016; Zuk, 2009). Males maximize reproductive fitness by mating with as many females as possible, reducing the importance of longevity (Stephenson et al., 2016; Zuk, 2009). There is also evidence that testosterone reduces immunocompetence, increasing infection risk in males (Folstad & Karter, 1992; Grear et al., 2009). In addition to reducing resistance, high testosterone levels are associated with risky behaviors that could increase the risk of parasite exposure and subsequent infection (Grear et al., 2009). Males must weigh the risks of increased infection intensities with the benefits of sexual characteristics associated with testosterone.

The Hamilton-Zuk hypothesis suggests that females prefer males with well-developed secondary sexual characteristics as they function as an honest indicator for strong parasite resistance (Hamilton & Zuk, 1982). Increased parasite resistance or tolerance will improve reproductive odds for males in species where sexual dimorphism and female choice are present. Male *F. grandis* undergo coloration changes during spawning periods, indicating that there is some role of sexual selection present (Greeley & Macgregor, 1983). No secondary sexual characteristics (e.g., male brightness) were measured in this study. Examining potential variations in infection intensity between males based on coloring could provide insight on the Hamilton-Zuk hypothesis in killifish. As with the association between body size and infection

intensity, a study on fish from a naturally infected population may provide further insight on variations in parasite resistance and tolerance between male and female *F. grandis*.

Impact of Chronic Euhaplorchis sp. A Infection and Acute Exposure on Fundulus grandis Fast-Start Response Behavior

Chronic *Euhaplorchis* sp. A infection had no significant effect on any of the fast-start response components. These results fail to support the hypothesis that *Euhaplorchis* sp. A infection reduces the escape capacity of *F. grandis*. However, *Euhaplorchis* sp. A may still be driving PITT. Increased conspicuous behaviors, such as flashing, jerking, surfacing, and scratching have all been observed in both experimentally and naturally infected *F. grandis* and *F. similis* (Fredensborg & Longoria, 2012; Hernandez & Fredensborg, 2015). In conspicuous behaviors, the Florida system mirrors the California system (Lafferty & Morris, 1996). The increase in conspicuous behavior driven by *E. californiensis* has been linked to greater predation rates, ranging from 10-30x (Lafferty & Morris, 1996; Shaw et al., 2010). The lack of evidence for reduced fast-start response performance in either system indicates that both *Euhaplorchis* species may be relying on increased conspicuous behaviors to increase their trophic transmission.

Acute exposure to infectious *E. californiensis* cercariae is linked to an increased routine metabolic rate in *F. parvipinnis* (Nadler et al., 2021a). We hypothesized that the presence of *Euhaplorchis* sp. A cercariae would reduce fast-start response performance in *F. grandis* based on the assumption that increased metabolic stress will reduce capacity for anti-predator behavior. Acute exposure had a significant effect on average turning rate. Fish exposed to live cercariae exhibited a lower average turning rate compared to sham-exposed fish, indicating that exposure to cercariae reduces agility. The reduction in turning rate was observed in both infected and uninfected fish, suggesting that the presence of cercariae may trigger a fear response in *F. grandis* regardless of previous exposure history (Daversa et al., 2021). Acute exposure had no significant effect in infected or uninfected fish for latency or for distance covered. As this study required uninfected *F. grandis* to function as a control group, the fish were collected from a population historically naïve to *Euhaplorchis* sp. A. Alterations in behavior seen in experimentally infected fish may be different from those seen in a natural system with fish that are continuously exposed to infectious cercariae. Behavioral manipulation may have detrimental effects on hosts and can increase mortality, which can drive antagonistic co-evolution between

the host and the parasite (Franceschi et al., 2010; Thompson, 1998). There is evidence that host populations where a parasite is common exhibit greater resistance to behavioral manipulation compared to naïve populations (Franceschi et al., 2010). In the amphipod *Gammarus pulex* the effects of behavioral manipulation by the acanthocephalan *Pomphorhynchus laevis* are significantly reduced in previously exposed populations compared to naïve populations (Franceschi et al., 2010). *Euhaplorchis* sp. A is known to infect *F. grandis* in the wild; comparing behavioral changes in naturally versus experimentally infected fish may provide more insight into how manipulation changes with exposure (Hernandez & Fredensborg, 2015).

Infection intensity is often lower in experimentally infected fish compared to naturally infected fish; as conspicuous behavior is known to increase with parasite density, it is possible that the reduced severity of experimental infections may dull the effect of infection (Fredensborg & Longoria, 2012). Wild *F. parvipinnis* naturally infected with *E. californiensis* can exhibit infection intensities in the 1000s, while experimentally infected *F. parvipinnis* often have greatly reduced infection intensities (Lafferty & Morris, 1996; Renick et al., 2016). *Fundulus similis* naturally infected with *Euhaplorchis* sp. A show infection intensities ranging from 55 to 549 metacercariae, suggesting that natural infection is lower for *Euhaplorchis* sp. A (Fredensborg & Longoria, 2012). Infection intensities in this study were low and highly variable, with metacercariae counts ranging from 0 to 711 and a mean abundance of 165 ± 150. The *F. grandis* in this study were exposed to ~1500 cercariae over seven independent infection events (Table 1). Wild populations would experience constant exposure, likely increasing infection success. The mean abundance is similar to that seen in wild *F. similis*, but many fish exhibited lower infection intensities than seen in wild populations (Fredensborg & Longoria, 2012; Hernandez & Fredensborg, 2015). Reduced infection intensity and the broad range of metacercariae counts may account for the lack of significant effects.

Potential for Future Studies

Future studies should compare infection intensity and behavioral changes in naturally infected and experimentally infected *F. grandis*. In addition to altering host behavior, parasites may also impact host personalities and potentially drive selection of particular personality traits (Barber & Dingemanse, 2010). Examining the differences between a fish's personality traits before and after infection with *Euhaplorchis* sp. A may provide insight into another parasite driven behavior change. For example, in this study we noticed that more fish were lost to

intraspecific aggression in the uninfected fish, which could indicate that *Euhaplorchis* sp. A reduces aggressive behavior. Personality tests for aggression, such as a mirror test, pre- and post-parasite exposure could be used to test this idea.

Conclusion

This study highlights the importance of morphological characteristics in infection risk, as well as the high variability in parasite resistance, even within a naïve population. *Euhaplorchis* sp. A is known to alter behavior in its intermediate hosts, but chronic infection does not appear to have a significant effect on fast-start escape responses in *F. grandis*. Latency was not significantly higher in infected fish than in uninfected fish. Any increased predation risk associated with *Euhaplorchis* sp. A is most likely driven by increased risky behaviors. Interestingly, acute exposure to *Euhaplorchis* sp. A cercariae did have a significant impact on fast-start behavior. Average turning rate was significantly lower in both infected and uninfected fish exposed to cercariae. This reduction in agility indicates that cercariae may trigger a fear response in *F. grandis* that in return increases predation risks. This is one of the few studies on the yet-unnamed *Euhaplorchis* sp. A and the first to examine its impact on fast-start escape responses. Our results broaden our understanding of this understudied system and the role it plays in fish behavior.

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