4-26-2023

Exploring Host-Parasite Interactions in the Euhaplorchis Californiensis-Fundulus Parvipinnis System

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NSUWorks Citation

Bennett J. Perry. 2023. *Exploring Host-Parasite Interactions in the Euhaplorchis Californiensis-Fundulus Parvipinnis System.* Master's thesis. Nova Southeastern University. Retrieved from NSUWorks, . (135) https://nsuworks.nova.edu/hcas_etd_all/135.

Thesis of
Bennett J. Perry

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

April 2023

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NOVA SOUTHEASTERN UNIVERSITY

HALMOS COLLEGE OF ART AND SCIENCES

Exploring host-parasite interactions in the *Euhaplorchis californiensis-Fundulus parvipinnis* system

By

Bennett James Perry

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

Marine Science

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Abstract:

Parasites with complex life-cycles may alter their intermediate host's phenotype to increase the chance of successful transmission to their next host. This parasite-induced host modification often occurs in the form of behavioral changes, such as increased frequency of irregular locomotor behaviors (e.g., conspicuous behaviors), particularly in systems where the parasite is transmitted trophically through a predation event. However, most empirical evidence of host behavioral modification by parasites comes from a few model host-parasite systems and are frequently studied following a stressor (e.g., simulated predator attack). One host species studied is the California killifish *Fundulus parvipinnis*, which is infected by the brain-infecting trematode *Euhaplorchis californiensis.* Here, we assess the degree of behavioral modification of a population of *F. parvipinnis* by *E. californiensis* in the absence of a visual stressor, such as a predator or a human observer. In past studies, behavior modifications that have been observed include the tendency for fish to stay close to the surface and conspicuous swimming behaviors, such as darting, surfacing, and flashing. However, in this study, infection treatment alone did not significantly alter the proportion of fish in the top compartment. Similarly, infection treatment also did not significantly affect either the total conspicuous behaviors or any of the individual conspicuous behavior that were displayed by the fish. Taken together, these findings suggest that the nature of behavior modification by *E. californiensis* may be context-specific (e.g., presence of a visual stressor) and influenced by life history traits (e.g., parasite maturity, age of fish upon infection, coinfection).

Keywords: Parasites, Trematode, Behavior, Intermediate Host, California Killifish, Experimental Infection

Acknowledgements:

This project has been an incredible opportunity for me to further my comprehension of the field of research and expand my understanding of several sub-disciplines of marine science like behavior physiology and parasitology, and I have many people to thank for not only making this project possible but also help complete the project along the way. I would like to start off by thanking Dr. Lauren E. Nadler, my primary advisor throughout the majority of this project and the one who made this project possible in the first place. I would like to thank Dr. Nadler for many things including introducing me to the field of behavior physiology, furthering my ability to interpret scientific literature, all her incredible comments/feedback on my written work, and for having so much patience with me during the thesis process. I would also like to thank the other members of my committee, Dr. Kelly L.Weinersmith and Dr. Christopher A. Blanar. Both of these committee members have provided so much great feedback throughout the different stages of this project, helped further my skills as a scientific writer, and helped introduce me to the field of parasitology.

I would also like to take this opportunity to thank the undergraduate/graduate who assisted me with this project along the way. First, I would like to thank Victoria Heller for helping me learn about the conspicuous behaviors that I identified in this study as well as all of her invaluable assistance in video quality assessment and top-compartment analysis. Second, I would like to thank Laura Delgado Nicolas for her assistance in completing and double-checking the top compartment analysis portion of the study as well as helping me start analyzing the recorded videos for conspicuous behavior analysis. Finally, I would like to thank Celeste Jaworski for all her assistance in helping me complete and double-check the conspicuous behavior analysis portion of the study.

Finally, I would like to thank my family who has been there to support me throughout my graduate experience here at Nova Southeastern University, and all the amazing friends I met during my experience that encouraged me to keep going no matter what challenge I was facing.

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Introduction:

Parasites are highly diverse and represent a large proportion of biodiversity in marine ecosystems (Buck, 2019). Diversity of parasites within an ecosystem may even be a useful bio-indicator of ecosystem health (Mackenzie et al., 1995). Numerous studies have highlighted the key role of parasitism in structuring community interactions. For instance, parasites are capable of altering host mortality and reproductive rates, which can trigger a cascade effects at the population, community, and ecosystem level, such as altered trophic interactions throughout the food web (Pascal et al., 2020). However, not all parasites cause drastic shifts in host population size; in fact, parasites can induce more subtle changes in the host population through alteration of the host's phenotype (e.g., physiology, immunology, behavior).

The term "parasite increased trophic transmission" (abbreviated from here on as PITT) was first coined in 1999 (Lafferty, 1999). PITT describes how parasites may manipulate intermediate hosts (e.g., fiddler crabs, copepods) to increase predation by subsequent intermediate hosts and final hosts in that parasite's life cycle to promote trophic transmission (Lafferty, 1999). This infection-induced shift in phenotype (e.g., behavior, morphology, physiology) has since been described in several host-parasite systems. For example, amphipods infected by acanthocephalan parasites incurred a higher rate of successful attack by local shorebirds due to irregular locomotor behavior (Born-Torrijos et al., 2016; Dick et al., 2010). Another example is seen in the rust crayfish (*Faxonus rusticus*), which exhibits increased boldness in threatening situations following infection by *Microphallus* spp. parasites.
These behavioral changes increase transmission to its avian or mammalian final host (MacKay & Moore, 2021). The same phenomenon has also been discovered in a variety of vertebrate hosts. For example, long-tail southern cod (*Patagonotothen ramsayi*) and Atlantic chub mackerel (*Scomber colias*) infected with the acanthocephalan parasite *Corynosoma austral* have been shown to be more likely to be consumed by sea lion predators due to increased lethargy during predator attacks (Hernandez-Orts et al., 2019). Parasite-induced changes in predator avoidance have also been observed in African killifish (*Nothobranchius furzeri*). Individuals that are infected with *Apatemon* sp. trematode parasites (i.e., metacercariae) spend more time at the surface and execute less vigorous antipredator behavior following a simulated predator attack when compared to uninfected populations (Nezhybova et al., 2020). However, many host-parasite interactions lack clear documentation or experimental evidence that supports adaptive

host manipulation. Host-parasite interactions can be difficult to disentangle using exclusively naturally infected hosts. Wild hosts may have also been exposed to and infected by multiple parasite species that can interact synergistically or antagonistically to alter host phenotypes (Cezilly et al., 2014). These interactions make connecting a particular phenotypic modification to a specific parasite species difficult (Poulin & Maure, 2015). Experimental infections enable researchers to expose uninfected individuals to a specific species of parasite in controlled numbers to uncover drivers of phenotypic changes, which aids in resolving questions about the cause-and-effect of parasite-induced changes in host behavior, morphology, and physiology (Poulin, 2013). Experimental infections are critical in not only understanding how a parasite affects the phenotype of its host, but also the mechanisms of parasite manipulation if present (Helland-Riise et al., 2020).

Ecologically-relevant experimental parasite infections can be generated in the lab, but the procedure comes with a suite of challenges as well. Wild fish accumulate parasites often in nature via repeated parasite exposures throughout ontogenetic development; these parasite intensities are often greater than those generated experimentally and may occur through many more parasite exposure events than is feasible in a lab setting (Helland-Riise et al., 2020). For example, a single experimental exposure of the California killifish *Fundulus parvipinnis* with the brain-infecting trematode *Euhaplorchis californiensis* yielded parasite loads that were orders of magnitude lower than those found in fish naturally infected in the wild (Renick et al., 2016; Shaw et al., 2009). Developing experimental infection protocols that achieve infection intensities like those found in natural environments is necessary to potentially active parasite manipulation and better understand how these parasites influence the physiology and behavior of its host (Helland-Riise et al., 2020). However, this procedure relies on knowledge of all hosts in a parasite's life cycle, the ability to maintain multiple host species in a lab setting, and a protocol to produce infective parasite stages for the focal host species. If these challenges can be overcome, studying host-parasite interactions via experimental infections poses several advantages over studying wild host-parasite associations.

One host-parasite system in particular that has been the focus of multiple studies is the association between *Fundulus parvipinnis* and *Euhaplorchis californiensis*, which was first described by Martin (1950). *Fundulus* spp. are found in estuarine ecosystems across North America, including California, Alabama, Texas, and Florida (Hernandez & Fredensborg, 2015b;

McNeff, 1978; Shaw & Øverli, 2012; Smith, 2001). The most common species found in estuaries in southern California and Baja California is the California killifish (*F. parvipinnis*) (Allen et al., 2006; Hechinger et al., 2007; Shaw et al., 2010). The host-parasite system between *F. parvipinnis* and *Euhaplorchis californiensis* is one of the most well-studied examples of PITT. The free-living cercariae stage of *E. californiensis* exits its first intermediate host, the California horn snail (*Cerithideopsis californica*), and goes in search of its second intermediate host, the California killifish. Once a suitable host is encountered, the cercariae attaches to the fish's skin, drops its tail, and penetrates the skin and tissues of the fish where it will travel via blood vessels and nerves to the brain where the parasite will encyst (developing into the metacercaria stage). Infected *F. parvipinnis* display significantly more behaviors that draw an observer's attention to a particular fish (referred to from here as conspicuous behaviors) compared to uninfected individuals, which increases their detection and capture by predatory piscivorous marsh birds, the final host for *E. californiensis* (Lafferty & Morris, 1996; Shaw et al., 2010). This modified behavior could be a result of parasite manipulation of the neurotransmitters, such as dopamine and serotonin, following infection (Shaw et al., 2009; Shaw et al., 2010). These behavioral changes suggest that the parasite may have an indirect effect on the diet of its definitive host, potentially aiding in transfer of energy from an aquatic to a terrestrial environment (Lafferty, 2008). Studies show that infection by *E. californiensis* was associated with increased frequency of conspicuous behaviors in *F. parvipinnis* (Lafferty & Morris, 1996). There has been research on behavior changes of this specific host-parasite interaction in both unstressed and stressed environments. For instance, Lafferty & Morris (1996), behavior of infected *F. parvipinnis* was analyzed by a human observer with no clear method of obscuring the observer from the *F. parvipinnis*. Fish were also frequently exposed to stress in the form of a hand net. In contrast, in Weinersmith et al. (in press), the experimentally infected *F. parvipinnis* were exposed to neither of these forms of stress during the observation period. The experimentally infected *F. parvipinnis* from Weinersmith et al. (in press) displayed a much lower changed in conspicuous behaviors compared to the stressed population of infected *F. parvipinnis* from Lafferty & Morris (1996). However, research on whether *E. californiensis* alters the locomotor behavior of *F. parvipinnis* in an unstressed environment is still limited compared to research on the locomotor behavior of the host in a stressed environment, such as following handling stress from an observer in an experimental setting. This project furthers our understanding of whether *E.*

californiensis alters the behavior of its intermediate host under routine conditions in the absence of a stressor using wild-caught and uninfected killifish that were experimentally infected repeatedly over several months. This study will also help us to understand if behavioral modifications of *F. parvipinnis* following *E. californiensis* infection is context specific, which will allow us to further comprehend the degree of behavioral modification in this host-parasite interaction. I hypothesize that *F. parvipinnis* shoals infected with *E. californiensis* will display an increase in the frequency of conspicuous behaviors (e.g., surfacing, darting, flashing, and scratching) and greater tendency to stay near the surface when compared to uninfected shoals, making them more easily detected and captured by their primary predators and the next host in the parasite's life cycle, piscivorous marsh birds.

Methods:

Fish collection and husbandry

In October 2017, we collected the California killifish (*F. parvipinnis*) using a two-pole seine from San Luis Rey River in Oceanside, California (33.20°N, 117.39°W, n = 114 fish) and Devereux Slough in Santa Barbara, California (34.41°N, 119.88°W, n = 292 fish), two populations known to lack *E. californiensis* (confirmed based on dissections of six fish per site, and visual confirmation that each site lacked the first intermediate host for *E. californiensis, C. californica*). All fish were > 5 cm standard length, but work by Fritz (1975) suggests that early spawned young (April-May) can be >5 cm standard length by October within a single breeding year. Thus, we likely collected a combination of early spawned young and the adult stage or approximately between 6-14 months of age at the time of capture. Following collection, all fish were transported to the laboratory at the Scripps Institution of Oceanography in San Diego, where they were housed in 38L glass tanks (51 x 27 x 32 cm, n = 17) in groups of 22 to 25 fish from October 2017 to April 2018 (n=5 tanks for fish collected in Oceanside; n=12 tanks for the fish collected in Santa Barbara). Each tank was fed twice daily ad libitum with a mixture of Skretting aquaculture feed, frozen then thawed blood worms, and macerated peas. Water temperature and light cycle mimicked natural ambient conditions in the southern California winter (18°C and 11:13h light:dark cycle, respectively).

Experimental Infection

We used the experimental infection procedure outlined in Helland-Riise et al. (2020) as a guide, with some minor modifications as outlined below. The first intermediate host for *E. californiensis*, the California horn snail (*C. californica*), was collected from the University of California Kendall-Frost Marsh Reserve in San Diego, California. The California horn snail can host up to 19 different species of trematode parasite, so snails were shed individually in compartment boxes and cercariae were identified using Hechinger (2019). Those snails that harbored *E. californiensis* were used for experimental infections of *F. parvipinnis* and housed between infection events in mudflat mesocosms operating with a simulated tidal cycle that matched the tide schedule in the local area.

All fish holding tanks were systematically assigned to one of two treatments (alternating between the two treatments in the holding rack): 1) control treatment (sham exposed using only seawater; n=10 tanks), or 2) infected treatment (repeatedly exposed to infectious *E. californiensis* cercariae; n=8 tanks). Cercariae for experimental infections were acquired from the snails previously identified to harbor *E. californiensis,* which were held in a dry but humid environment for at least 24 hours prior to each infection event (which promoted maximal shedding rates). Snails were shed in groups of 7-10 in glass finger bowls (10 cm ID) for a period of 2-4 hours (70-198 snails shed total for each infection event), after which the number of cercariae shed was estimated under a dissecting microscope. The cercaria dose per infection event increased incrementally, starting at 50, 100, and 200 cercariae per fish for the first through third infection event, respectively. From the fourth infection event onwards, fish were exposed at a rate of 300 cercariae per fish. Fish were batch exposed in their home tank, and the cercariae for the entire group was aliquoted into an assigned Qorpak jar (one per tank; 5.6 cm diameter x 7.0 cm height and volume = 120 mL). Based on the number of cercariae that shed, as many tanks as possible were exposed to parasites in each infection event (ranging from 1 to 7 tanks from the infected treatment in a single infection event). Each jar was weighted to ensure negative buoyancy and lowered to the bottom of its respective tank using fishing line. A similar protocol was used for control tanks as well as any tanks from the infected treatment that did not receive a parasite exposure during an infection event, except their Qorpak jars were filled with UV-sterilized and parasite-free seawater. Jars were removed 17 to 24 hours after each infection event. Each tank in the infected treatment experienced 14 to 15 infection events during the course of this study (October 2017 to April 2018) for a total average exposure per fish of 3650 to 3950 cercariae. Starting dates for infection events for tanks in infected treatment ranged from 10/17/2017 to 11/3/2017. Dates for the final infection event for these tanks ranged from 4/6/2018 to 4/17 /2018. Number of days between subsequent experimental infections ranged from 3 to 42 days. Experimental infections were confirmed to be successful through the dissection of 12 random fish from the infected treatment, which indicated that the average infection intensity was 265 +/- 44 (mean+/- SE). The range of number of metacercaria in these 12 fish was 65 to 540.

Video Recording and Behavioral Data Collection

Video recordings were taken at 0800 prior to feeding and exposure to cercariae on five dates from 2017-2018 that were separated by approximately one month (12/8/2017, 1/12/2018, 2/9/2018, 3/9/2018, 4/13/2018), during which time the shoals were repeatedly exposed to *E. californiensis* cercariae as described above. Each tank had an Amcrest ProHD 1080P (2.1MP) Wi-Fi Security Camera mounted in front of it to execute these recordings. During these recording sessions, no people were in the room in order to avoid disturbing the fish, and the tanks were covered so the fishs' behavior would not be affected by what was happening in the lab. The camera software was also started remotely so the fish would not be disturbed before the recording session. Recordings were viewed using VLC media player for behavioral analyses. All videos were analyzed blind to treatment to prevent observation bias. Analyses included the proportion of fish in the top third (8 cm) of the tank ("top compartment analysis") and frequency of conspicuous behaviors ("conspicuous behavior analysis").

Top Compartment Analysis

To determine the number of fish in the top 8 cm of each tank, screenshots of the tank were taken at 6 different time points separated by 1 min (i.e., at times 0:00,1:00, 2:00, 3:00, 4:00, 5:00 relative to the start of the video). The top compartment (from the tank surface to 8 cm depth) was noted on each tank using white tape around the tank's exterior (sides and back). Two observers counted the number of fish in the tank and the number of fish in the top compartment at each designated time point. Number of fish in tank (i.e., shoal size) for control and infected tanks across the 5 observation periods is listed in Table 1. The proportion of the shoal in the top compartment in each screenshot was then determined by dividing the number of fish in the top compartment by the total number of fish in the shoal (i.e., number of fish in each tank).

Table 1. Shoal size in tanks used for both top compartment and conspicuous behavior analysis at all five observation dates.

Tank	Treatment	Date	Shoal Size
2	Control	12/8/17	18
4	Control	12/8/17	16
8	Control	12/8/17	19
11	Control	12/8/17	17
15	Control	12/8/17	15
17	Control	12/8/17	17
32	Control	12/8/17	20
36	Control	12/8/17	19
2	Control	1/12/18	19
8	Control	1/12/18	19
11	Control	1/12/18	17
15	Control	1/12/18	15
17	Control	1/12/18	17
34	Control	1/12/18	19
36	Control	1/12/18	20
2	Control	2/9/18	19
8	Control	2/9/18	18
11	Control	2/9/18	17
15	Control	2/9/18	14
17	Control	2/9/18	17
32	Control	2/9/18	20
34	Control	2/9/18	21
36	Control	2/9/18	20
2	Control	3/9/18	19
8	Control	3/9/18	19
11	Control	3/9/18	17
15	Control	3/9/18	14
17	Control	3/9/18	17
32	Control	3/9/18	19
34	Control	3/9/18	20
36	Control	3/9/18	19

8	Control	4/13/18	20
11	Control	4/13/18	17
15	Control	4/13/18	14
32	Control	4/13/18	19
1	Infected	12/8/17	21
3	Infected	12/8/17	24
7	Infected	12/8/17	23
9	Infected	12/8/17	22
16	Infected	12/8/17	17
33	Infected	12/8/17	19
1	Infected	1/12/18	21
3	Infected	1/12/18	24
7	Infected	1/12/18	24
12	Infected	1/12/18	17
16	Infected	1/12/18	16
3	Infected	2/9/18	23
7	Infected	2/9/18	23
12	Infected	2/9/18	17
16	Infected	2/9/18	16
35	Infected	2/9/18	21
3	Infected	3/9/18	24
7	Infected	3/9/18	23
12	Infected	3/9/18	17
16	Infected	3/9/18	15
35	Infected	3/9/18	19
7	Infected	4/13/18	23
9	Infected	4/13/18	22
16	Infected	4/13/18	13
33	Infected	4/13/18	18

Conspicuous Behavior Analysis

The number of conspicuous behaviors was recorded for 10 increments of 10 seconds each (i.e., for a total observation time of 100 s per tank). Classification of conspicuous behaviors was completed using the definitions listed in Table 2. The type and starting time of each conspicuous behavior that took place in a specific quadrant was recorded. Videos were analyzed by two observers to confirm the total number and type of conspicuous behaviors present in the recording.

Table 2. Definitions for each conspicuous behavior recorded (Weinersmith et al., in press). Unlike in Weinersmith et al., (in press), contorting behaviors were not included in analysis due to how it was only seen once across all tanks.

Dart	The fish suddenly and rapidly moves forward a distance of at least 1 body length but abruptly slows down. The movement is no more than a distance equal to 3/4 the height of the tank. Multiple darts occurred when the fish darted multiple times with less than a second in between darts. The number of regular darts and the number of darts seen during any multiple dart event were all counted together as the number of total darts for the tank. Rapid movement up and down the side of the tank would indicate pacing along one side of the tank, and is an artifact of the laboratory environment. This pacing behavior does not count as a dart.
Surface	Any part of the fish's body makes contact with the water surface. Only count a new surface event if the fish moves down from the surface of the water by at least half a body length (head to tail), and then moves up again.
Flash + Scratch	A flash is when the fish moves forward quickly and turns laterally so that one side of the body faces upward. This behavior would cause the light at the surface to reflect off the

	silver coloration on the fish’s ventral side. A scratch is when the fish rubs its body against a hard surface in the tank. Hard surfaces include the PVC pipe, airlines, the filter, and the glass in the tank itself.

Statistical Analysis

All models were generated in RStudio (version 2022.07.01) (R-Studio Team, 2022) running R (version 4.2.2) (R Core Team 2022) using the packages glmmTMB (Brooks et al., 2017), car (Fox & Weisberg, 2019), and emmeans (Lenth, 2022). To examine the effect of infection on behavior, including total conspicuous behaviors, individual conspicuous behaviors (dart , flash + scratch, and surface), and fish in the top compartment, we used generalized linear mixed effects (GLME) models and Akaike Information Criterion (AIC) to determine the family (poisson or negative binomial as all data were counts), if a zero-inflation correction was needed, and the best-fit model in terms of inclusion of fixed effects and their interactions as well as the random effect.

All models exhibited overdispersion so used a negative binomial distribution (nbinom1), except for the darts model, which used a Poisson distribution, and the proportion of fish in the top-compartment model, which used a binomial distribution. We tested if each model conformed to its assumptions using the DHARMa package (Hartig, 2022). Each model included infection status (Infected or Uninfected), video date, shoal size,and 2- and 3-way interactions) as fixed effects, with tank nested within locality (Oceanside, Santa Barbara) as a random effect. Shoal size was used as a continuous predictor to allow the model to determine the best fitting relationship between the response variable and shoal size. The shoal size covariate on counts accounts for the differences in shoal size among tanks when considering how the shoal size may affect the overall number of conspicuous behaviors.

Results:

Models of best fit for each different response variable were determined using AIC scores as summarized in Table 3. Of the five models that were analyzed, four of the models of best fit included treatment. However, treatment was only statistically significant when involved in an interaction with one or more other fixed effects. In each case, the most complex model included the observation date ("Date"), parasite treatment ("Treat"), the size of the shoal in the observed tank ("SS"), and all associated interactions. The most complex model also included a random effect for tank nested within locality of collection ("RE"). A "✓ " indicates that the term was included in the model. Models are presented in ordered rank based on Akaike Information Criterion ("ΔAIC "). Presented models with ΔAIC less than 2 are bolded while models with ΔAIC greater than 25 were not included (Burnham & Anderson, 2002).

Table 3: Model rankings for linear mixed-effect models for top compartment and conspicuous behavior analysis

Date*Treat*SS	**Date*Treat**	**Date*SS**	**Treat*SS**	**Date**	**Treat**	**SS**	**RE**	**ΔAIC**
Proportion of Fish in Top Compartment								
				✓	✓	✓	✓	**0**
				✓	✓		✓	**0.6**
				✓			✓	**1.4**
				✓		✓	✓	**1.6**
					✓	✓	✓	5.1
				✓				5.8
	✓		✓	✓	✓	✓	✓	7.9
		✓	✓	✓	✓	✓	✓	9.2
	✓	✓		✓	✓	✓	✓	13.9
	✓	✓	✓	✓	✓	✓	✓	15.6
Sum (total conspicuous behaviors)								
				✓		✓		**0**
				✓		✓	✓	4
				✓	✓	✓	✓	5.6
			✓	✓	✓	✓	✓	6.9
	✓		✓	✓	✓	✓	✓	11.3
				✓	✓		✓	15.7
	✓	✓	✓	✓	✓	✓	✓	17.9
✓	✓	✓	✓	✓	✓	✓	✓	19.6

Darts								
	✓			✓	✓	✓	✓	**0**
	✓			✓	✓		✓	**1.5**
	✓		✓	✓	✓	✓	✓	**1.6**
				✓	✓	✓	✓	2.3
	✓	✓	✓	✓	✓	✓	✓	3.4
			✓	✓	✓	✓	✓	3.5
Surfacing								
				✓	✓	✓		**0**
				✓		✓		**0.9**
				✓	✓	✓	✓	4
				✓		✓	✓	4.9
			✓	✓	✓	✓	✓	5.1
	✓			✓	✓	✓	✓	10
	✓		✓	✓	✓	✓	✓	10.7
				✓	✓		✓	12.1
	✓	✓	✓	✓	✓	✓	✓	15.4
					✓	✓	✓	17.6
Flash and Scratch								
				✓	✓	✓		**0**
				✓				**0.1**
				✓		✓		**1.2**
				✓		✓	✓	2.2
				✓	✓	✓	✓	3.7
				✓	✓		✓	5.3
			✓	✓	✓	✓	✓	5.7
	✓		✓	✓	✓	✓	✓	12.6
	✓	✓	✓	✓	✓	✓	✓	12.6
✓	✓	✓	✓	✓	✓	✓	✓	13.7

Proportion of Fish in Top Compartment

While treatment was included in the model of best fit based on ΔAIC (Table 3), treatment was not found to significantly affect the proportion of fish displayed by the fish ($\chi 2_1=0.37$, $p=0.54$). The date that the fish were recorded significantly affected the proportion of fish seen in the top compartment of the tank ($\chi 2_4=2.73$, $p=0.02$, Figure 1). While shoal size was included in the model of best fit and there appears to be a weak positive relationship between shoal size and the proportion of fish in the top compartment, shoal size was not a significant effector in this model ($\chi2_1$=2.7, p=0.10, Figure 2).

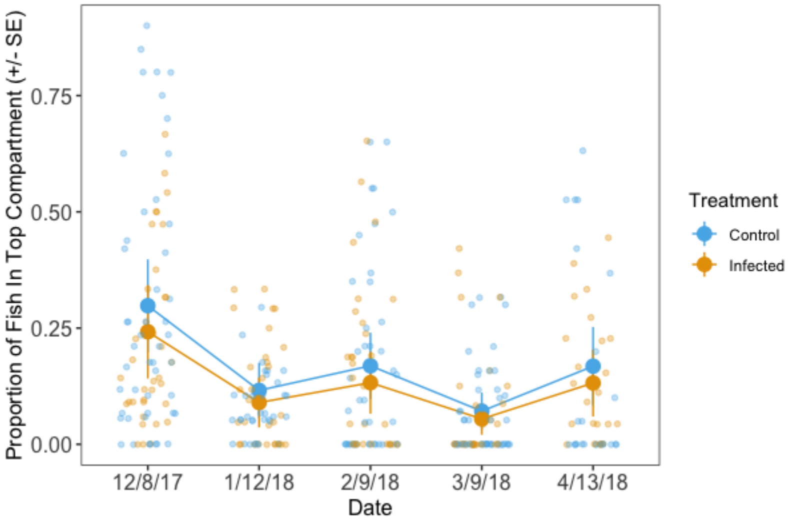

Figure 1- Proportion of fish in top compartment in monthly observation dates from December 2017 to April 2018 in uninfected (control) versus infected treatment tanks. Large dots represent the estimated marginal mean (± SE) from generalized linear mixed-effects model analysis (controlling for shoal size). Small dots represent individual proportion values of fish in the top compartment.

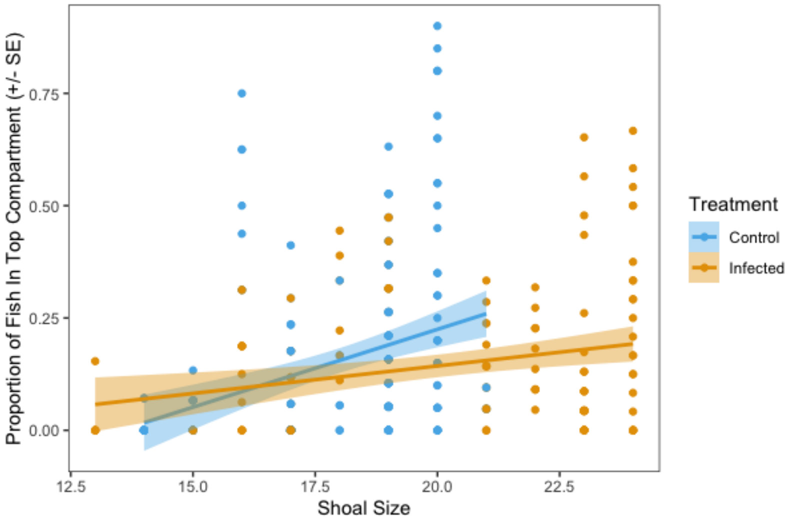

Figure 2-Proportion of fish in the top compartment as a function of shoal size in the tank in uninfected versus infected treatment tanks. Each data point represents one proportion value, with lines indicating the linear regression between shoal size and number of fish in the top compartment by infection treatment (uninfected, infected). Shading represents the 95% CI around the linear regression.

Total Conspicuous Behaviors

Treatment was found to not significantly affect the total number of conspicuous of behaviors as there was only one model with AIC<2, treatment was not included in this model (Table 3). The date that the fish were recorded significantly impacted the number of total conspicuous behaviors displayed by the fish in the tank ($\chi2_4$ =102.37, p<0.0001, Figure 3). The number of conspicuous behaviors observed appears to have a direct relationship with shoal size ($\chi2_1$=16.83, p<0.0001, Figure 4).

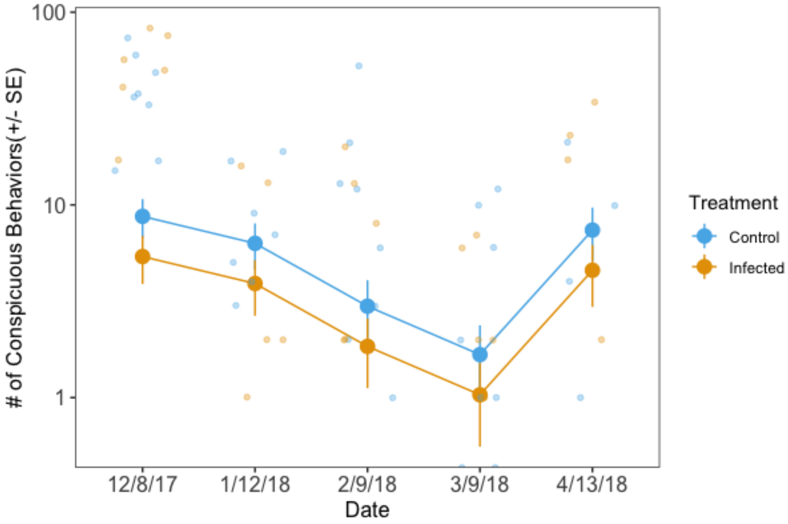

Figure 3- Number of total conspicuous behaviors displayed by fish during monthly observation dates from December 2017 to April 2018 in uninfected (control) versus infected treatment tanks. Large dots represent the estimated marginal mean (± SE) from generalized linear mixed-effects model analysis (controlling for shoal size). Small dots represent individual counts of total conspicuous behaviors.

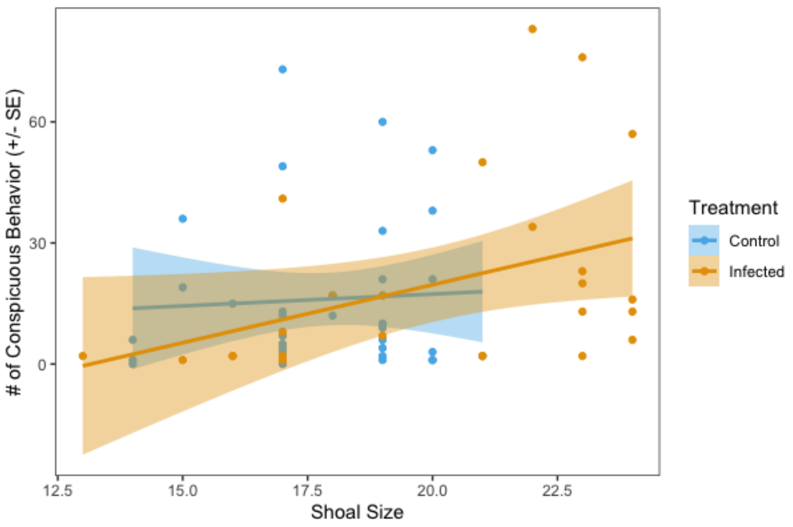

Figure 4- Total number of conspicuous behaviors as a function of shoal size in the tank in uninfected vs infected tank treatments. Each data point represents one count of total number of conspicuous behaviors, with lines indicating the linear regression between shoal size and number of conspicuous behaviors by infection treatment (uninfected, infected). Shading represents the 95% CI around the linear regression.

Darts

There were 3 models within AIC<2, and all of the models included a two-way interaction between date and infection treatment, which in the top model significantly affected the total number of darts seen in the tank ($\chi2_4 = 10.00$, p=0.04, Figure 5 & 6). In infected tanks, there was significantly more darts displayed by fish in December 2017 compared to all observations dates except April 2018 ($p_{8Dec17\text{-}12Jan18}<0.0001$, $p_{8Dec17\text{-}9Feb18}=0.014$, $p_{8Dec17\text{-}9Mar18}<0.0001$). This same trend was not seen in the uninfected tanks. The date of recording also seem to significantly affect the total number of darts observed ($\chi2_4 = 71.09$, $p<0.0001$, Figure 5) though there was not a consistent trend between December 2017 and April 2018. There also appears to be a direct relationship between number of darts and shoal size ($\chi2_1=4.19$ p=0.04, Figure 6).

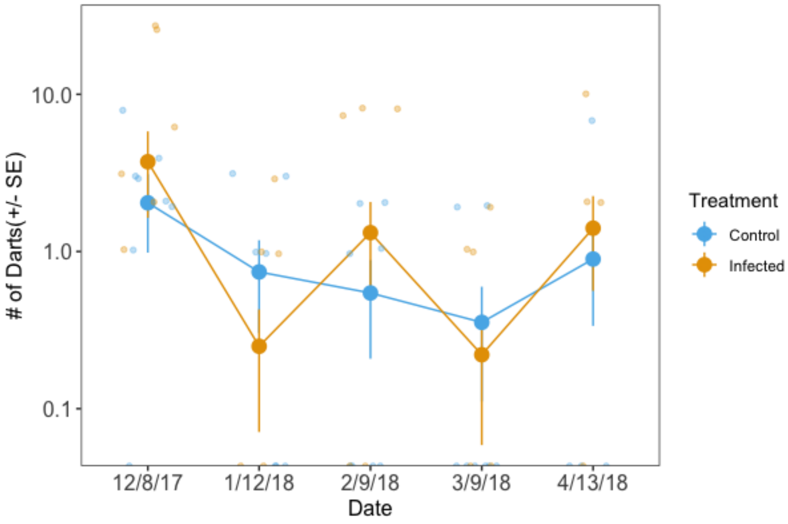

Figure 5- Number of darts displayed by fish during monthly observation dates from December 2017 to April 2018 in uninfected (control) versus infected treatment tanks. Large dots represent the estimated marginal mean (± SE) from generalized linear mixed-effects model analysis (controlling for shoal size and two-way interaction between date and infection treatment). Small dots represent individual counts of darts

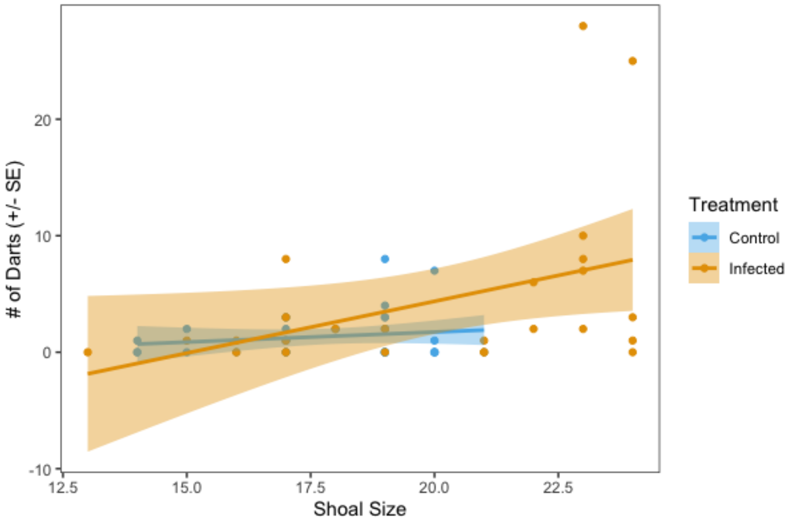

Figure 6- Total number of darts as a function of shoal size in the tank in uninfected vs infected tank treatments. Each data point represents one count of number of darts, with lines indicating the linear regression between shoal size and number of darts by infection treatment (uninfected, infected). Shading represents the 95% CI around the linear regression

Scratches and Flashes

While treatment was included in the model of best fit based on ΔAIC (Table 3), treatment was not found to significantly affect the number of scratches and flashes displayed by the fish ($\chi 2_1=0.75$, $p=0.39$). The date that the fish were recorded significantly affected the number of flashes and scratches seen in the tank ($\chi 2_4=84.94$, $p<0.0001$, Figure 7). The number of flashes and scratches observed seems to have a direct relationship with shoal size ($\chi 2_1=3.91$, $p=0.05$, Figure 8).

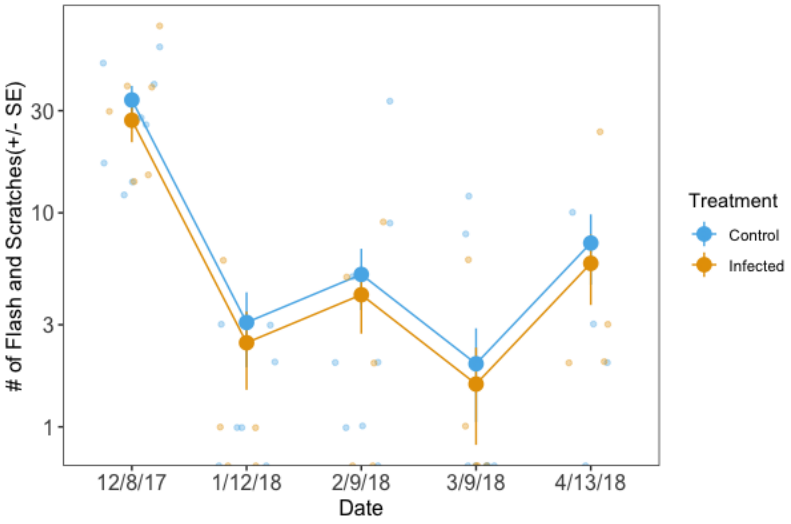

Figure 7- Number of flashes and scratches displayed by fish during monthly observation dates from December 2017 to April 2018 in uninfected (control) versus infected treatment tanks. Large dots represent the estimated marginal mean (± SE) from generalized linear mixed-effects model analysis (controlling for shoal size). Small dots represent individual counts of flashes and scratches

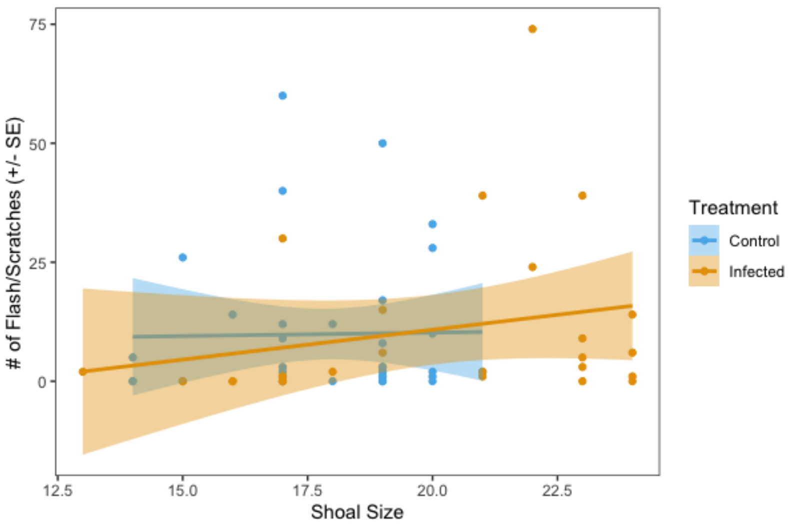

Figure 8- Number of flashes and scratches as a function of shoal size in the tank in uninfected vs infected tank treatments. Each data point represents one count of number of flashes and scratches, with lines indicating the linear regression between shoal size and number of flashes and scratches by infection treatment (uninfected, infected). Shading represents the 95% CI around the linear regression

Surfaces

While treatment was included in the model of best fit based on ΔAIC (Table 3), treatment was not found to significantly affect the number of surfaces displayed by the fish ($\chi 2_1=2.76$, p=0.10). The date that the fish were recorded significantly affected the number of surfaces seen in the tank ($\chi 2_4=18.76$, p=0.0009, Figure 9). The number of surfaces observed seems to have a direct relationship with shoal size ($\chi 2_1=9.42$, p=0.002, Figure 10).

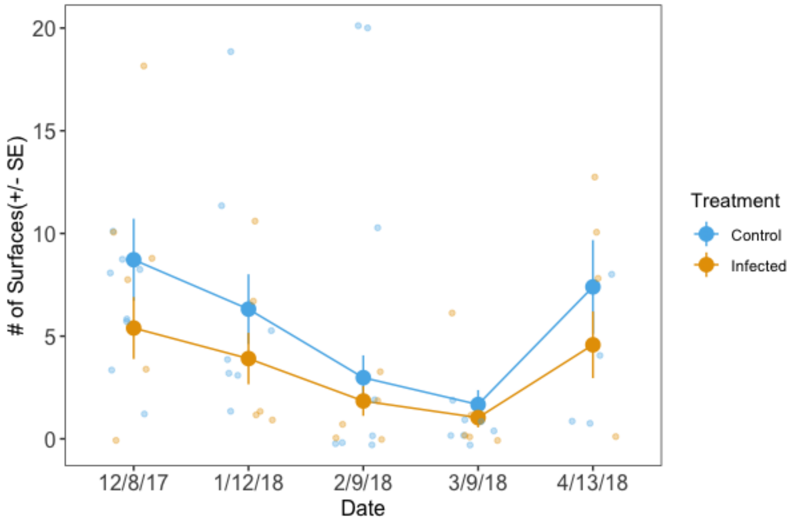

Figure 9- Number of surfaces displayed by fish during monthly observation dates from December 2017 to April 2018 in uninfected (control) versus infected treatment tanks. Large dots represent the estimated marginal mean (± SE) from generalized linear mixed-effects model analysis (controlling for shoal size). Small dots represent individual counts of surfaces.

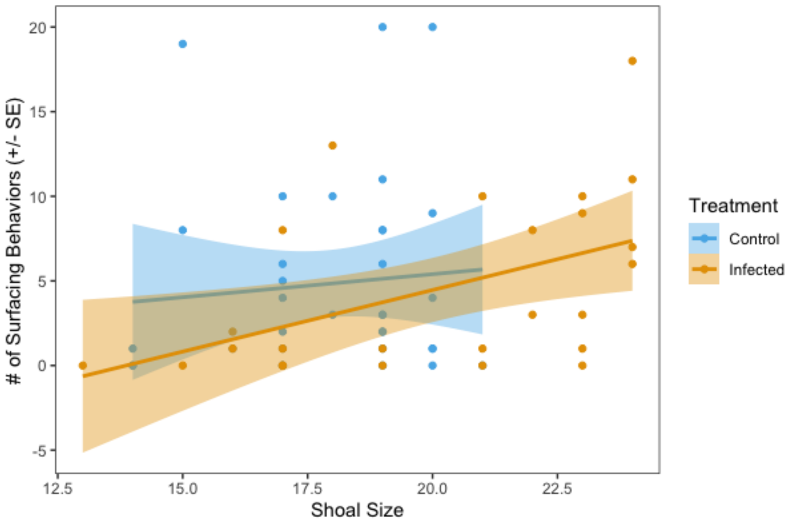

Figure 10- Number of surfaces as a function of shoal size in the tank in uninfected vs infected tank treatments. Each data point represents one count of number of surfacing behaviors, with lines indicating the linear regression between shoal size and number of surfaces by infection treatment (uninfected, infected). Shading represents the 95% CI around the linear regression.

Discussion:

Our study does not support the proposed hypothesis that California killifish that are infected with *E. californiensis* display more conspicuous behaviors and have a greater tendency to aggregate near the water's surface in comparison to uninfected fish. The infection treatment by itself did not significantly affect any of the behaviors observed during this study. These observations are in contrast to previously published work; for example, Lafferty & Morris (1996) found that several conspicuous behaviors observed, including flashing, scratching, jerking, and the total sum of conspicuous behaviors, were more frequent in parasitized fish compared to unparasitized fish. As we did not find comparable differences in behavior in *F. parvipinnis* following infection by *E. californiensis*, it suggests that many parameters likely influence the magnitude of behavioral modification, such as the host's environment and life history.

Differences in the level of stress that infected fish in this study were exposed to prior to testing could explain the lack of a difference between uninfected and infected fish. In Lafferty & Morris (1996), both uninfected and infected *F. parvipinnis* were exposed to stressors. These stressors include an observer who recorded conspicuous behaviors for 30 minutes in which the fish had an unimpeded view of the observer and a hand net that was used to capture fish from the tanks that would be dissected to measure intensity of metacercariae. Further, in the accompanying field study, the authors found that parasitized fish were 10-31 times more susceptible to predation compared to the unparasitized fish. This increase in conspicuous behaviors among infected fish that were exposed to a similar stressor as those in Lafferty & Morris (1996) is seen in interactions between other *Euhaplorchis* spp. parasites and their *Fundulus* spp. hosts. The mean number of conspicuous behaviors displayed by Gulf killifish (*Fundulus grandis)* infected with *Euhaplorchis* sp. A was significantly higher than uninfected *F. grandis*. Similar to Lafferty & Morris (1996), behavior analysis was done with an observer. This observer was 3 meters away from the tank and observed each individual fish for 15 minute periods (Hernandez & Fredensborg, 2015a). Similarly, the frequency of surfacing behavior increased directly with *Euhaplorchis* sp. A infection intensity in the longnose killifish (*Fundulus similis)* (Fredensborg & Longoria, 2012). Like in Hernandez & Fredensborg, (2015a), the researcher directly observed the *Fundulus* spp. host one at a time, with a 3-meter distance between the tank and the observer. As the authors specify no strategies to prevent the fish from seeing the observer, the focal fish was likely stressed by the presence of the observer, which may have stimulated an infection-induced increase in conspicuous behaviors in response to this stress. In Weinersmith et al., (in press), researchers used a comparable protocol to the one used in our study, in which cameras were triggered remotely and thus the fish could not see the observer. While infected fish had a 1.5 fold increase in the number of conspicuous behaviors compared to uninfected fish, the effect-size of infection treatment was much lower than those previously described in the literature. The lack of significant effect of treatment on conspicuous behavior and number of fish in top compartment in our study, and the similarity in results to Weinersmith et al. (in press), could suggest that stress plays a key role in behavioral modification *F. parvipinnis* by *E. californiensis*.

The degree of host behavior modification can also be influenced by infection intensity. It is possible that a threshold intensity of *E. californieinsis* was not achieved in the majority of experimentally infected fish and as a result, frequency of conspicuous behaviors in infected *F. parvipinnis* did not significantly differ from that of the control population. In previous studies with this host-parasite system, experimentally infected fish harbored hundreds to thousands of *E. californiensis* before behavior observations. In Lafferty & Morris (1996), naturally infected fish, who displayed a larger number of conspicuous behaviors compared to the uninfected fish, exhibited infection intensities that exceed 1000 *E. californiensis* metacercariae per host. In contrast, other studies where experimentally infected *F. parvipinnis* had a much lower infection intensity compared to Lafferty & Morris (1996) saw little to no change in the frequency of conspicuous behaviors compared to uninfected. For example, experimentally infected *F. parvipinnis* with an average *E. californiensis* infection intensity of 49 metacercariae per fish did not experience a significant change in anti-predator behaviors compared to the control (Renick et al., 2016). This observation suggests that a threshold infection intensity must be reached for *E. californiensis* to significantly modify *F. parvipinnis* behavior. The average infection intensity in this study was 265 +/- 44 (mean+/- SE), which is much lower than the infection intensity in Lafferty & Morris (1996) and the natural infection intensities reported in Shaw et al. (2010). These observations suggest that the lack of change in swimming behavior in infected fish could be a result of experimentally infected fish not reaching the infection threshold necessary to generate behavior modification.

Host behavioral modification could also be influenced by the parasite's developmental stage during the observation period. Previous research has suggested that immature parasites found in intermediate host are more likely to suppress conspicuous behaviors that could attract a predator when the parasite is not yet infectious to its final host (Dianne et al., 2011; Gopko et al., 2015; Parker et al., 2009). For instance, sheltering behavior was significantly more frequent in freshwater amphipods (*Gammerus pulex)* experimentally infected with immature *Pomphorhyncus laevis* cystacanths compared to uninfected individuals (Parker et al., 2009). This suppression of conspicuous behaviors among infected organisms is also seen in fish intermediate host species. In Gopko et al., (2015), rainbow trout (*Oncorhynchus mykiss*) with immature *Diplostomum pseudospathaceum* eye flukes displayed significantly less activity compared to uninfected individuals, which suggests that non-infective parasites can change the behavior of the fish host so that the chance of predation is lower until they become infective. Another non-infective parasite strategy is to avoid changing the swimming activity of its host when compared to uninfected members of the same shoal. Lake Malawi sardines (*Engraulicypris sardella*) infected with a mature *Ligula intestinalis* tapeworm displayed greater swimming activity and tended to position themselves higher in the tank compared uninfected individuals. In contrast, *E. sardella* infected with pre-infective larvae of *L. intestinalis* did not significantly differ from uninfected fish in terms of swimming activity and preferred to stay closer to the benthos compared to the fish with a mature *L. intestinalis* (Gabagambi et al., 2019). As *F. parvipinnis* in the infected treatment in this study were regularly exposed to infectious cercariae throughout the observation period (October 2017 to April 2018), it is possible that the number of immature metacercariae outweighed the number of mature metacercariae at several points during the observation, potentially resulting in the negligible effects on conspicuous behaviors and surfacing compared to the uninfected individuals.

The degree of behavior modification by *E. californiensis* can also be influenced by the timing of infection relative to the host's life stage. In this study, our fish were experimentally infected with *E. californiensis* as adults. This timing is in contrast to experimental infection protocols from other studies in *F. parvipinnis*. For instance, in both Helland-Riise et al., (2020) and Weinersmith et al., (in press), fish hosts were infected from soon after hatching for eight months. At this early life stage, organs like the brain are still developing in *F. parvipinnis* and this development may be impacted by chronic stress such as repeated infections with *E. californiensis* cercariae (Nardocci et al., 2014). Studies have illustrated that susceptibility to infection may decline with age, potentially impacting the nature and magnitude of impacts of infection on its host. For example, in rainbow trout (*Oncorhynchus mykiss*) that are less than one year old, the myxozoan parasite *Tetracapsuloides bryosalmonae* not only proliferated more quickly but also the disease severity increased when compared to older fish (Bailey et al., 2021). This trend is not only seen when comparing juveniles to adults, but also subadults to adults. For instance, subadult Nile tilapia (*Oreochromis niloticus*) were more susceptible to infection by gill dactylogyridean ectoparasites compared to mature adults (Wunderlich et al., 2022). These observations suggest that the success of infection and the parasite's scope for physiological manipulation is most likely affected by shifts as the host's immunity changes with age (Wunderlich et al., 2022). It is possible that like other complex life cycle parasites, *E. californiensis* has a greater chance of successfully infecting and manipulating the behavior of *F. parvipinnis* as a juvenile or subadult rather than an adult. This suggests that the timing of experimental infection in relation to age of *F. parvipinnis* may be important in ensuring realistic assessments of behavioral modification by *E. californiensis* in context-specific situations.

Co-evolution between the host and parasite may be an important component of parasite-induced behavior modification. Whether the population of fish collected for experimental infection co-occurs with the focal parasite of interest can impact both the type and the magnitude of the behavior modification experienced by the intermediate host. Collecting fish samples from locations that naturally lack the focal parasite of interest has been a common strategy of experimental infection protocols for many host-parasite studies. *F. parvipinnis* collected for experimental infections in previous studies were collected from a small isolated lagoon because the collection site historically lacked *Euhaplorchis* spp. and its first intermediate host (Hernandez & Fredensborg, 2015a; Shaw et al., 2009). The results of experimental infection in both these studies suggest that infection of *Euhaplorchis* spp. significantly influences the swimming behavior (Hernandez & Fredensborg, 2015a) and monoaminergic activity (Shaw et al., 2009). However, these noticeable physiological effects of *Euhaplorchis* spp. infection on its *Fundulus* spp. intermediate host could be a result of the population being naïve and more susceptible to manipulation by the parasite. It has been seen in other host-parasite interactions that host populations that co-evolve with the parasite of focus could be less susceptible to the manipulation by the parasite compared to naïve populations that have never been exposed to that parasite. For instance, the amphipod *G. pulex* sampled from a population that were naturally infected with acanthocephalan *P. laevis* were less susceptible to manipulation by the parasite compared to naïve populations (Franceschi et al., 2010). A small sample of our collected *F. parvipinnis* from each site were dissected to confirm the absence of *E. californiensis.* As the first intermediate host was also not observed at the collection sites, this evidence suggests that our target population was naive to the parasite, and while other systems suggest that naivety to the parasite increases manipulation, in our case, it perhaps decreases any observed behavior modification.

Co-infecting parasites may also result in antagonism that decreases observed behavioral changes with each parasite individually. When co-infecting parasite species have different final hosts, interspecific conflicts can arise. This conflict can result in several different outcomes, such as destroying the competing parasite or outcompeting the other parasite to manipulate the intermediate host in ways that maximize that parasite's fitness. Another parasite could also neutralize or sabotage the manipulation of its competitor without killing it (Hafer & Milinski, 2015). For instance, roundworm *Camallanus lacustis* and tapeworm *Schistocephalus soidus* antagonistically interact with each other when they both infect the copepod *Macrocyclops albidus*. Both parasites can reduce the manipulation of the competitor, though the degree of antagonism can depend on which of two parasites is the stronger manipulator. To illustrate this interaction, when *C. lacustis* is the more mature or infective parasite, the manipulation by *S. solidus* is eliminated; in contrast, when *S. solidus* is the more mature parasite, the manipulation by *C. lacustis* is only slightly reduced (Hafer & Milinski, 2016). These observations show that the outcome of these interspecific conflicts depend on multiple factors including maturity and strength of the manipulator. In our study, it is possible that these wild-caught fish were co-infected by another parasite that competed with *F. parvipinnis,* and thus reduced the magnitude of behavior modification.

While experimental infections are capable of creating infection intensities that replicate phenotypic changes in naturally infected intermediate hosts, experimental infection protocols have limitations that hinder how representative they are of natural infection processes. Evidence about how different lengths of time between exposures influences behavior modification is lacking. Further studies that analyze how the amount of time of subsequent exposure between the parasite of interest affect host-parasite interactions could help us improve the design of experimental protocols so that an experimentally infected population more accurately reflects how the host and focal parasite of interest interact in a naturally infected population

This study used a repeated experimental infection protocol to provide more information regarding the host-parasite interaction between *E. californiensis* and *F. parvipinnis*. Specifically, the results of this study contribute to understanding the impact of *E. californiensis* on the swimming behavior of its second intermediate host, *F. parvipinnis* , in a routine environment. Our results highlight that the nature of behavior modification of *F. parvipinnis* by *E. californiensis* is highly context specific. The documented absence of significant changes in conspicuous behaviors among infected *F. parvipinnis* generates new research questions to better understand the cause of this difference to previous studies. For instance, future studies can investigate the degree that stressors induce conspicuous behaviors by *E. californiensis*. Future investigations can also explore the role of parasite maturity of *E. californiensis* by increasing time between most recent experimental infections and observation periods. These types of studies would increase our understanding on what influences the magnitude of behavior modification by *E. californiensis* in its intermediate hosts. Overall, this study helps further our understanding of how factors ranging from life history traits or other interactions in an environment can influence how a trophically transmitted parasite interacts with its intermediate host.

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