UNIVERSITY OF CALIFORNIA SAN DIEGO

Behavioral defense against parasites: California killifish move, dart, and scratch more during trematode cercaria exposure and attack.

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Marine Biology

by

Rebecca Noemi Hernandez

Committee in charge:

Professor Ryan F. Hechinger, Chair
Professor Phillip A. Hastings
Professor Martin Tresguerres

2019

The thesis of Rebecca Noemi Hernandez is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

_____

_____

_____

Chair

University of California San Diego

2019

DEDICATION

To all the friends, family and teachers that inspired, motivated and pointed me in the right direction over these past 19 years. It has been a very long journey that I could not have completed without them.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SUPPLEMENTAL FILES

Supplemental Tables and Figures, Hernandez_tables and figures.pdf

ABSTRACT OF THE THESIS

Behavioral defense against parasites: California killifish move, dart, and scratch more during trematode cercaria exposure and attack.

by

Rebecca Noemi Hernandez

Master of Science in Marine Biology

University of California San Diego, 2019

Professor Ryan F. Hechinger, Chair

With the ubiquity of parasites, many hosts have been selected to decrease parasite infection success by employing behavioral defenses, such as avoidance of infected habitats/conspecifics, grooming, grouping, altering swimming behavior, or even self-inducing behavioral fevers. California killifish, *Fundulus parvipinnis* – common to southern California and Baja California estuaries – are typically exposed to several trematode species that use them as 2nd intermediate hosts. At least one of these trematodes substantially impacts killifish fitness. We also know killifish likely perceive trematode infectious propagules (cercariae). However, we

do not know whether they employ behavioral defenses. We experimentally exposed killifish (originating from two San Diego wetlands) individually and in groups to two of their trematode species: *Euhaplorchis californiensis* – which infects the killifish brain and manipulates host behavior to increase predation rates – and Small Cyathocotylid, which infects connective and muscle tissues. To assess killifish behavioral response to exposure, we quantified several behavioral traits: average number/type of potential defensive behaviors (PDBs), activity, vertical position in the water column, and group size before and during exposure to parasites. Our results showed that killifish individually-exposed (both previously infected and naïve) to parasites increase their average number of PDBs, but not their activity. However, in groups, parasite-exposed killifish increase both their average number of PDBs and activity. Conversely, neither average vertical position (in either experiment) nor group size was influenced by parasite exposure. In sum, parasites can alter killifish behavior after infection, but also during anti-parasite behavioral defense, with implications for host-parasite coevolution, and host social and ecological interactions.

Introduction

Castration, loss of control over one's own body, phenotypic alteration to increase 'attractiveness' to predators, and death are only some consequences associated with parasitic infection. Given the severe outcomes associated with parasitic infection, it is not surprising that hosts have been selected to employ a wide range of anti-parasite defenses. Such defenses are usually considered in terms of immunological defense. However, animals also employ a diverse array of behavioral anti-parasite defenses such as animal grouping, grooming, or even fly repelling (Hart, 1992).

Anti-parasite behavioral defense, an organism's first line of defense, has been extensively examined in terrestrial ecosystems for over 50 years, yet research in aquatic systems only began receiving attention much more recently (Behringer et al. 2018). Despite this, a growing number of empirical studies in aquatic systems suggest that behavioral defense may play an important role in helping organisms to defend against parasite exposure and attack. In aquatic systems, anti-parasite behavior can take many forms. For instance, after using various types of cues, (Kiesecker et al. 1999), organisms have been shown to defend themselves against parasites by altering activity levels (Poulin et al. 1991; Genna et al. 2005; Thiemann and Wasersug 2000; James et al. 2008; Koprivnikar et al. 2006; Koprivnikar et al. 2012; Koprivnikar et al. 2014; Bui et al. 2017; Bui et al. 2018), shoal size (Mikheev et al. 2013; Krause and Ruxton 2002; Poulin and Fitzgerald 1989; Stumbo et al. 2012), avoiding infected conspecifics (Kiesecker et al. 1999; Barber et al. 1998), or areas harboring infectious parasite stages (Lowenberger and Manfred 1994), self-inducing behavioral fevers (Mohammed et al. 2016), displaying a greater frequency of swimming bursts (Bui et al. 2017) or leaps (Atkinson et al. 2018), or even traveling to fresh-water sources to engage in fresh water baths (Bui et al., 2018; Birkeland and Jakobsen 1996).

Regardless of the specific tactics, anti-parasite behavioral defenses should be favored if they confer a net fitness advantage by decreasing a host's risk of exposure and infection, or by combatting the negative effects of infection (Wisenden et al. 2009; Behringer et al. 2018).

Aquatic animals can detect parasite infectious stages using visual (Szuroczi and Richardson 2012; Koprivnikar and Penalva 2015; Klemme and Karvonen 2016; James et al. 2008), olfactory (Nadler et al. 2016; James et al. 2008), and chemical (Kiesecker and Skelly 2000; Rohr et al. 2008; Baker and Smith 1996; Koprivnikar and Penalva 2015) cues, alone or in combination (Kiesecker et al. 1999). Then, depending on the host-parasite system, animals can mount appropriate behavioral defense mechanisms, some of which are very similar to anti-predator defenses. For instance, prey join larger groups to decrease individual risk of predation via the 'dilution' or 'many-eyes' effect (Krause and Ruxton 2002). The larger the group, the lower the chance that any particular individual will be attacked by a predator (Krause and Ruxton 2002). The same situation occurs to potential hosts during exposure to parasites, as animals in larger groups decrease their number of individually-acquired parasite infectious propagules (Poulin and Fitzgerald,1989; Richards et al. 2010; Stumbo et al. 2010; Barber et al. 1998; Mikheev et al. 2013). However, this only applies to parasites *not* transmitted directly or via close contact. Directly transmitted parasites such as some monogeneans or some copepods actually benefit from large aggregations of aquatic animals (Richards et al. 2010). In this situation, smaller group size would be a much more effective anti-parasite behavioral defense.

Changes in activity have been observed during exposure to parasites. In tadpole-trematode systems, activity manifests itself as evasive maneuvering to avoid parasites present in the water column or remove them after making contact to prevent establishment (Berhinger et al. 2018; Bui et al. 2017; Bui et al. 2018; Koprivnikar et al. 2006; Koprivnikar et al. 2014;

Thiemann and Wassersug 2000). Furthermore, the efficacy of activity as an anti-parasite behavioral defense was demonstrated by Koprivnikar et al. (2006) when they showed that more active tadpoles acquired lower parasite burdens. Contrary to tadpole-trematode studies, less active salmon acquired lower parasite burdens during exposure to copepod infectious stages (Bui et al. 2017; Genna et al. 2005). Since copepods respond to water movement, shadows, and pressure (Genna et al. 2005), less movement throughout the water should confer an advantage to salmon seeking to avoid detection. Again, this shows that the specific anti-parasite behaviors employed by hosts will depend on the host-parasite system in question.

Exposure to parasite infectious stages can even cause animals to breach the surface of the water. For instance, Bui et al. (2017) quantified the frequency of jumping/rolling, bursts, and twitches for three salmonid species, Atlantic salmon (*Salmo salar*), Chinook salmon (*Oncorhynchus tshawytscha*) and sea trout (*Salmo trutta*) during exposure to copepods. They found that the frequency of jumping increased for both the salmon species (e.g., Atlantic and Chinook), suggesting that it may function as an anti-parasite defensive strategy. A subsequent study confirmed that this increase in jumping does, in fact, lead to lower sea-lice abundances (Atkinson et al. 2018), indicating the defensive behavior's efficacy. Interestingly, sea trout did not increase the frequency of jumping upon parasite exposure, suggesting that this might not be an anti-parasite behavioral defense employed by this fish species. The authors concluded that this difference was possibly due to the fish's diverging life histories. Whereas sea trout have more frequent access to freshwater where they might be able to engage in freshwater baths, Atlantic and Chinook salmon lack this resource. As a result, this has allowed Atlantic and Chinook salmon to evolve a different suite of anti-parasite behavioral defenses to combat parasite infestation.

Spatial avoidance is another common and effective anti-parasite defense particularly when parasites are easily detectable. For example, Poulin and Fitzgerald (1989) showed that sticklebacks, *Gasterosteus aculeatus,* in parasite-free tanks preferred to be closer to the tank bottom. However, in aquaria containing the ectoparasite, *Argulus canadensis*, sticklebacks shifted up in the water column to move away from the source of infection as *A. canadensis* preferred to be near the tank bottom.

Exposure to parasites is typical for California killifish, *Fundulus parvipinnis.* Common to Baja and Southern California estuaries (Fritz 1975) killifish frequently serve as 2nd intermediate hosts for several trematode (parasitic flatworm) species (Hechinger et al. 2007), including the well-known brain-infecting trematode, *Euhaplorchis californiensis* (EUHA) (Martin 1950). Larval trematodes such as EUHA emerge from their first intermediate California Horn snail host, *Cerithideopsis californica*, and actively seek out killifish where they develop into a resting stage awaiting ingestion by the final bird host. The interactions between killifish and EUHA have been used as a classic system for behavior modification and parasite increased trophic transmission. After infecting its killifish host, EUHA encysts on the brain's surface where it alters the fishes' serotonin and dopamine levels (Shaw et al. 2009) to modify its behavior, causing killifish to display 4x more conspicuous behaviors which make them 10-30x more susceptible to predation by their visual bird predators (Lafferty and Morris 1996). Hence, it seems clear that EUHA negatively impacts killifish by modifying its behavior after parasites have established on the brain. However, no research has examined whether killifish employ behavioral defense to avoid exposure or counter EUHA cercariae attack. The strong fitness impacts of EUHA on killifish led us to hypothesize that killifish will employ anti-parasite behaviors during cercariae exposure to reduce infection risk. In addition to examining behavioral responses to EUHA, we included

another common trematode, Small Cyathocotylid (SMCY). Despite having similar life cycles, EUHA and SMCY are quite different. SMCY functions as more of a generalist, as it infects various hosts as well as variety of host connective and muscle tissues (Martin 1972). Although we were unaware of whether SMCY alters killifish behavior, we know that this is a possibility and that it likely uses a different physiological mechanism to that of EUHA. Despite this, we expected that killifish would display anti-parasite behaviors during exposure to each parasite species.

To assess killifish behavioral responses during parasite exposure, we took advantage of a long-term, multi-facetted study which examined the effects of EUHA (and SMCY) on neurology, physiology and behavior throughout fish development. Treatments involved different exposure types: exposure to no parasites (controls), to different EUHA doses (low or high), and exposure to a different parasite species, SMCY, which served as a positive control. We then exposed killifish individually (removed from their home tanks to individually exposed them) or in groups (while in their home tanks) and quantified several behavioral traits likely to serve as anti-parasite defenses: (i) average number of specific "potential defensive behaviors" or PDBs (Table 2), (ii) activity, (iii) vertical position, and (iv) shoal size. The selected PDBs were specific behaviors that we have observed killifish employ that would likely serve to avoid questing cercariae or dislodge recently attached ones. These PDBs included darting, scratching, surfacing, flashing, and twitching. We predicted that if each of the behavioral traits we quantified functioned as behavioral anti-parasite defenses for killifish during exposure to parasites, then: (i) the total number of PDBs should increase, (ii) activity should increase, (iii) vertical position should decrease (fish should move closer to the tank bottom) and (iv) shoal size should increase. The directionality of these predictions comes from considering the basic physics of the

interaction and by considering parallel systems in the literature. The number of PDBs should increase given that some might operate as pre-contact parasite avoidance behaviors (darting, flashing, and twitching), while others may serve as post-contact defensive behaviors (surfacing and scratching). Killifish activity levels should increase during exposure to parasites because this behavior has previously been shown (in a different system) to be an effective strategy in helping to decrease risk of exposure and infection by parasites (Koprivnikar et al. 2006). Our prediction for vertical position was founded on our understanding regarding cercarial behavior, particularly that of EUHA. As EUHA cercariae are positively phototactic (move towards light) and negatively geotactic (move against gravity) (Weinersmith et al. 2018), we predicted that they would become aggregated closer to the surface of the water, therefore causing killifish to shift downwards in the water column. Although we have yet to evaluate the behavior of SMCY in relation to light and gravity, we predicted that it would induce a similar response in killifish vertical position. While we predicted that killifish vertical position would decrease, we were aware that we might not see a response, particularly for the individual exposures due to the small tank size. Furthermore, we were also aware that we might not be able to detect a change in vertical position for fish exposed in groups given that we did not conduct a thorough evaluation of cercarial distribution in the water column. Nevertheless, we still assessed this behavioral trait in case there was a response. Lastly, we predicted that shoal size would increase during exposure to parasites as fish in larger groups are able to dilute their individual infection risk more efficiently via the 'dilution' or 'many eyes' effect (Krause and Ruxton 2002).

As mentioned above, the PDBs we quantified were modified from Lafferty and Morris (1996). In their study, they assessed the influence of EUHA *infection* on killifish behavior. Thus, they referred to these behaviors as 'conspicuous behaviors,' as they were associated with

behavioral modification mediated by EUHA infection. Although we tracked the same behaviors in our study, we reclassified them as 'potential defensive behaviors' as we examined them in the context of *defense* during *exposure* to parasites, *not* infection. Whereas *infection* refers to the successful establishment of parasites on or within a host, *exposure* represents hosts that are in the state of becoming infected as they are in close proximity to infectious parasite stages.

Materials and Methods

General overview

This study was part of a larger, multi-facetted project examining the influence of EUHA (and SMCY) on killifish neurology, physiology, and behavior throughout fish development. That project generated two batches of lab-reared, experimental fish, one starting in 2016 and the other in 2017. The first batch of lab-reared fish originated from parents coming from two wetlands, San Elijo (SE) and Kendall Frost (KF), while the second batch originated only from KF. We reared fish in small groups in replicate treatment "home tanks". Treatments involved different exposure types. Fish reared in 2016 belonged to one of four treatment groups: control, low EUHA dose, High EUHA dose (3x more parasites than low EUHA dose) and SMCY, a different parasite species. Those reared in 2017 had only control, high EUHA, and SMCY treatments. For the current study, we used the above fish to examine their behavioral response to acute cercarial exposure in two general ways: (1) individual exposures, where we removed fish from their home tanks to individually expose them, and (2) group exposures, where we exposed groups in their home tanks, with group sizes ranging from ten to twenty individuals/tank. For individual exposures, we used lab-reared fish from the 2017 batch, supplemented with new wild-caught fish from KF. For group exposures, we used lab-reared fish from the 2016 batch, using videos from

one scheduled experimental infection (see Table 1 for summary). Below, we provide specifics on all the above aspects of the experiment.

Table 1: Summary of origin, previous infection status of fish exposed individually or in groups. Origin = collection site, killifish group used = lab-reared or wild-caught, batch = the group of lab-reared fish, Home tank treatment group = Treatment group fish were assigned to prior to their use in this study and Previously infected (Y/N) = whether or not killifish were previously infected by parasites via controlled experimental infections or via natural infections (wild-caught).

Experiment	Killifish group used	Origin	Batch (lab - reared only)	Home tank treatment groups	Previously infected (Y/N)
Individual Exposures	Lab – Reared*	Kendall Frost	2017	Control	N
				High EUHA	Y
				SMCY	Y
Individual Exposures	Wild-caught**	Kendall Frost	N/A	N/A	Y
Group Exposures	Lab – Reared***	San Elijo	2016	Control	N
				Low EUHA	Y
				High EUHA	Y
				SMCY	Y

** These lab-reared killifish were ~ 9 months old when used in individual exposures.*
***Wild-caught killifish were housed in their holding tank for approximately 8 months prior to their use in individual exposures.*
**** These lab-reared killifish were ~ 5 months old when used in group exposures.*

1.) Experimental fish rearing, maintenance, and long-term infection exposures

a.) Gamete collection and egg rearing

We collected adult wild killifish using a 2-pole seine from naturally-infected populations between July – August 2016 (SE and KF) and April – September 2017 (KF). Fish were

temporarily separated into 2 buckets by sex. Gametes were collected by applying gentle pressure to their ventral surface from operculum to the vent until eggs/sperm were expelled following (Hubbs & Strawn, 1956). Eggs and sperm (from a minimum of four males) were placed in a small petri dish with enough seawater to cover the eggs and gently stirred to encourage contact and subsequent fertilization. Fertilized eggs were transferred to Scripps Institution of Oceanography where they were housed for 17 days in glass fingerbowls (diameter: 100 mm) containing approximately 75 eggs in filtered, aerated seawater containing methylene blue (3mg/L; to minimize fungal growth on the eggs during development). Starting on day 18, we maintained the eggs in only filtered, aerated seawater, while conducting water changes every other day until hatching, at approximately 21 days post-fertilization. Dead or unfertilized eggs were removed daily. Eggs were kept on a light:dark cycle similar to that of natural day length of San Diego, CA (from 11:13 light:dark cycle in winter to 13:11 light:dark cycle in summer). Mass mortality of eggs originating from KF (2016 Batch) resulted in us having no replication of KF controls. Therefore, we present only SE data (Table 1).

b.) Fish rearing

Hatched killifish were transferred to 37.8 L glass aquaria (51 x 27 x 32 cm) until tanks contained fish densities of approximately 20 - 21 individuals. We assigned fish from the 2016 batch to either a control (received only seawater), low EUHA dose, high EUHA dose, or SMCY treatment group. Fish from the 2017 batch were assigned to all of the above treatments, except the low EUHA treatment group. We reared fish in these tanks for a period of 13 months. Two horizontal lines drawn on the back of each tank created three distinct, equal-height sections to be used in vertical position assessment. Tanks were covered on three sides to prevent visual contact

between neighboring fish and moveable black curtains covered the front side of the tank (opened during feeding and controlled exposures) to prevent uncontrolled visual stimuli from the surrounding area. Tanks housing the 2016 batch of fish contained a thin layer of sediment. This sediment was absent from tanks housing the 2017 batch. During the first 12 weeks of life, fish were fed live-hatched *Artemia* sp. Then, they were transitioned to a more varied diet composed of blood worms, Skretting aquaculture feed, and mashed peas.

c.) Long-term infection procedure

Fish were exposed to treatments in their home tanks twice weekly throughout the 13-month rearing period. Control fish were sham-exposed (received only seawater), fish in low or high EUHA treatment groups were exposed to EUHA cercariae, while those in the SMCY treatment group were exposed to SMCY cercariae. High EUHA and SMCY doses were always capped at 300 cercariae/fish and Low EUHA doses contained ⅓ the amount of cercariae found in the high EUHA dose.

Cercariae for exposures originated from naturally infected California horn snails collected from KF and previously identified as harboring either EUHA or SMCY infections (identifications made following Hechinger (under revision)). We maintained these snails at SIO in mudflat mesocosms under an artificial tidal regime mimicking the local tidal cycle. Twenty-four hours prior to any specific experimental infection, we placed snails in a warm, humid environment for up to 24 hours. This permitted cercariae to accumulate prior to 'shedding' (i.e. experimental release of cercariae).

On experimental infection days, snails were placed in finger bowls (10 cm internal diameter) in groups of 11 – 12 with enough seawater to completely submerge the snails and held under fluorescent lights for 2 – 3 hours to stimulate cercaria release. Dissection scopes were used to count the approximate number of cercariae in each finger bowl. Initially, we used the 'count method' to prepare aliquots of cercariae by counting individual parasites (2 cercariae/fish) and used a glass pipette to place them into tank-specific, 20 mL scintillation vials (24 mm diameter and 61 mm height). Each scintillation vial was then topped off with seawater. Cercarial doses tripled every four weeks (throughout the entire rearing period) until reaching 300 cercariae/fish. After cercaria exposure dose exceeded eighteen cercariae per fish, we switched to aliquotting controlled volumes of cercaria-laden seawater using a 'volume method'. To do this, we pooled cercariae (by parasite species) shed from fingerbowls (housing ~ 11-12 snails each) into 600mL beakers and iteratively aliquoted 15 mL volumes of cercariae-laden water into tank-specific polypropylene 120 mL jars (56 mm diameter x 70 mm height) using a 30 mL volumetric pipette ("turkey baster"). Each jar was topped off with seawater. Aliquot numbers for the both the count and volume methods were determined based on the total number fish in each tank and the total number of parasites shed with high EUHA tanks receiving 3x more cercariae relative to the low EUHA treatment. SMCY exposure levels were prepared in a similar manner.

For each exposure, we lowered the tank-specific aliquot jars into the tanks using a piece of fishing line that was attached to the jar and hooked onto the outside of the tank for easy retrieval approximately 24 hours after exposure. To ensure sinking of the polypropylene jar, we used a hot glue gun to attach a small fishing weight to the jar exteriors. Jars for control tanks receive only filtered seawater.

As stated above, batch 2016 fish came from both KF and SE estuaries, which was contrary to our original plan. Initially, we planned to use fish only from KF. However, we were unable get enough adults for egg harvest. Therefore, we had to supplement with eggs from SE and planned to include data from both wetlands in our analyses. However, a mass mortality of eggs from KF, resulted in us having no replication for KF controls. Thus, we present only SE data.

2.) Individual exposures experiment

To perform individual exposures, we used lab-reared fish from the 2017 batch and wild caught fish (all from KF) with different infection states. Lab-reared uninfected (mean fish total length (TL) ± s.e; = 2.68 ± 0.06 cm, n = 20) were parasite naïve (not infected with parasites), while the lab-reared infected fish (mean TL ± s.e.; = 3.00 ± 0.10 cm, n = 20) had been experimentally infected twice a week for nine months (July 2017 – April 2018), with their last experimental infection taking place on April 3, 2018. Wild-caught killifish (mean TL ± s.e.; 6.36 ± 0.09 cm, n = 18) were all previously infected as they were collected from a naturally infected population in KF using a two-pole seine and maintained in a large holding tank (177.8 x 76.2 x 81.3 cm) for approximately 8 months (August 2017 to April 2018) under a blood worm diet. We performed a total of seven trials (days) using sets of eight individual fish. In each trial, we quantified individual fish behavior in response to sham- or parasite-exposure during three time periods (before, immediately after and 2 Hrs after continuous exposure). EUHA cercariae for parasite-exposure treatments was collected from mesocosm and wild-caught horn snails. Given that wild-caught horn snails are commonly found infected with various trematode species (Martin 1972), they were examined under the dissecting scope. We then quantified the total

number of potential defensive behaviors (PDBs) (Table 1), activity and vertical position of each individual fish in response to each exposure treatment during each time period.

Fish were placed individually in tanks (18.0 x 11.2 x 13.2 cm), filled ¾ of the way with room temperature seawater (~22°C). Cardboard covering three sides prevented visual contact between neighboring tanks and two horizontal lines drawn on each tank denoted the top and halfway marks of the water level (to be used for assessment of vertical position). Each tank was fitted with two airline hoses. The first provided air through a bubbler stone and the second (injection hose) went out of the tank to a syringe that we used to provide the acute exposure treatments. The airflow hose was used to mask the bubbles associated with the injection of the exposure treatments. Preliminary tests using food coloring confirmed that the current generated by the air hose quickly dispersed the dye.

Trials were conducted each day from 1100 to 2200 hours from April 6 – 12, 2018 using both lab-reared and wild-caught fish. During each trial, a set of eight fish was removed from their home tanks. Wild-caught fish always came from the same home tank, while lab-reared fish came from various home tanks to allow us to maintain comparable fish densities across tanks. Lab-reared uninfected (LRUI) fish came from control tanks. Thus, they were parasite-naïve. Lab-reared infected (LRI) fish were obtained from high EUHA tanks, with their last long-term exposure event taking place on April, 3, 2018 (lab-reared fish were ~ 9 months old). Fish were randomly assigned to one of two acute exposure treatments: (1) sham-exposed (water control) or (2) parasite-exposed. Sham-exposed fish received only 10 ml of seawater while fish in the parasite-exposed treatment group received 500 EUHA cercariae in a total of 10 ml of seawater. We used this quantity of cercariae to help ensure a behavioral response. The source of cercariae for these exposures was EUHA-infected horn snails from our mesocosms (n = 30) supplemented

with freshly acquired wild-caught snails (n = 72). Each day we used a new batch of wild-caught snails given that snails did not shed large numbers of cercariae two days in a row. Both mesocosm and wild snails were shed following methods outlined above. However, since we were unaware of wild-caught horn snail infection status, we shed them individually in parts box compartments to screen out EUHA-infected snails. We then pooled enough EUHA cercariae released from wild snails with those of the mesocosm snails into two glass finger bowls (10 cm internal diameter) to conduct the day's parasite-exposures. Aliquots for acute exposures were prepared by placing 500 cercariae in groups of ten with a glass pipette in a Stender dish and then filling the dish to 10 ml total with room temperature seawater. Control shams received only 10 ml of filtered room-temperature sea water.

During each trial, a set of eight individual fish was allowed to acclimate for 30 mins in individual tanks positioned at two different heights. Among trials, we ensured interspersion of each acute exposure treatment and long-term infection status. Four fish were placed on tanks positioned on the first level, while the remaining four were placed on the second level. Thus, creating four vertical pairs of tanks. We placed a tripod-mounted camera directly in front of a pair of fish tanks to record two individual fish. Cameras began recording fish behavior during three time periods: pre-exposure (before exposure), post-exposure (occurring on average 1 min 40 secs *immediately* after initial exposure), and 2 hrs post-exposure (2 Hrs after *continuous* exposure), with each recording lasting 20 mins. Each fish was observed for a total of five minutes during each time period. Behavioral assessments from pre-exposure and 2 hours post-exposure videos were performed during the first 5 mins of each video. Those from post-exposure videos were processed during the 5 minutes immediately following exposure treatment delivery (after no more bubbles/liquid were seen flowing through the injection hose).

We quantified activity, vertical position and total number of potential defensive behaviors (PDBs) (Table 2) for each fish group during each time period. Behavioral assessments were made blind to the acute exposure treatment, but we were aware of the fishes' prior infection status. Experimental tanks were rinsed in between trials and allowed to dry overnight.

3.) Group exposures

We examined the behavioral response of killifish in groups (in home tanks) to cercariae exposure using lab-reared fish from the 2016 batch (~ 5 months old) during one of their scheduled infection events (March 28, 2017). We recorded fish behavior before and during exposure to parasites. The following treatments were represented: Control (n = 5), Low EUHA (n = 4), High EUHA (n = 6), and SMCY (n = 4). Control tanks were sham-exposed (received only seawater), Low EUHA received 32 – 47 cercariae/fish, High EUHA received 110 – 134 cercariae/fish and SMCY received 13 – 25 cercariae/fish. Although we sought to have the SMCY exposure dosage equal to the High EUHA dosage, SMCY were not shedding cercariae at high enough levels to permit this at this point in the season. We also quantified behavior during three disturbance types surrounding initial exposure for a supplementary analysis to ensure that parasite *exposure* and *not* infection was driving any potential behavioral changes.

We quantified killifish baseline behavior in the videos at three pre-exposure key time-points: 20, 15, and 10 minutes before exposure procedure initiation (before lifting the curtains covering tank fronts). We tracked individual focal fish, which was facilitated by assigning all fish in a tank a unique number in an image still taken at the beginning of each key time-point. We used a random number generator to randomly select 10 focal fish in each tank to quantify average shoal size, vertical position, and number of PDBs, and to select 5 focal fish for

quantifying activity level. We used videos to track focal fish for 1 min to quantify activity and number of PDBs, and used image stills to assess vertical position and group size.

We similarly quantified killifish behavior during parasite exposure, using key time-points 10, 15 and 20 minutes *after* the exposure vial went into tanks, to permit time for cercariae to be distributed throughout the tank. All group exposure videos were processed blind to the treatment.

Throughout the 13-month rearing period, some of our lab-reared fish developed a couple of health issues. Some developed buoyancy (positive or negative), while others developed fecal casts (long, stringy, white feces). Consequently, we did not quantify behavior for these 'unhealthy' fish. However, we did use the number of unhealthy fish per tank to calculate the proportion of healthy fish and included it in our analyses as a potential predictor variable.

Our main focus was to compare the behavioral response of previously infected killifish before and during exposure to parasites. The comparison with the control (previously uninfected and unexposed) fish permitted asking whether any observed response to exposure simply represented a generic killifish response to the disturbances caused by implementing the exposure event (raising and lowering the curtains, inserting the exposure vials). However, because long-term infection can influence killifish behavior (see introduction), including response to stress (Shaw et al. 2009), it is possible that an observed response would not be to cercaria exposure *per se*, but an established parasite-induced modified response to disturbance. Therefore, we also quantified killifish behavior during the 'disturbance' period, which was in between our pre-exposure and exposure time periods. This 'disturbance' period involved three, sequential disturbance types: (i) CU = Curtain Up, (ii) VI = Vial In, and (iii) CD = Curtain Down. CU was the video time when the curtain was rolled up. VI was the time when the infection vial (i.e. infection jar) touched the water surface. Lastly, CD was the time when the curtain was rolled

down. CU and CD serve as visual disturbances, while VI served as a mechanical disturbance. However, exposure to parasites initially began during VI and continued through CD, leaving the initial disturbance, CU, as the only time point completely lacking a confound of cercaria exposure. Hence, if any observed response during exposure was due to cercaria exposure and not an already established parasite-induced response to disturbance, we predicted to see behavioral responses that increased over time from CU, to VI (earliest possible exposure), to CD (increasing time for contact), to our "Exposure" period, 20 minutes later.

3.) Quantified Behavioral traits

(a) Potential defensive behaviors (PDBs)

PDBs (Table 2) were categorized as darting, scratching, flashing and twitching modified from (Lafferty and Morris 1996). However, we used the terms darting and twitching instead of jerking and shimmying, respectively. In quantifying darts, we noticed that when individually-exposed fish darted in their small tanks, they would hit a tank wall resulting in a series of rapidly repeated darts (occurring within 1 s of one another), thus artificially inflating the number of darts. We therefore recorded only the total number of 'independent darts,' operationally defined as those occurring at least 1 sec following a previous dart. Whereas (Lafferty and Morris 1996) defined flashing as a lateral turn that exposed a fishes' silvery belly, while simultaneously chafing the tank bottom, we split up this definition into two distinct terms. In our study, flashing was used to describe only the lateral turn that resulted in a 'sparkle of light', while scratching referred to the chafing aspect of the behavior (Table 2).

Table 2: Description of potential defensive behaviors (PDBs) quantified over the course of 5 minutes (individual exposures) and 1 minute (group exposures).

Behavior	Description
Darting (Individual Exposures)	Independent, fast, forward movement of at least 0.5 body lengths/sec followed by a clear sudden stop. Each change in direction is considered a dart. Occurred at least 1 sec following a previous dart.
Darting (Group Exposures)	Fast, forward movement least 1 body length and then rapid deceleration. Each change in direction is considered a dart.
Scratching	Fish rubs its body against a hard surface in the tank. The movement is often rapid and associated with the fish turning on its side as it scratches its body against a hard surface. Hard surfaces include the airline, air stone and the tank sides. Often referred to as 'chafing' in the literature.
Surfacing	Fish body makes contact with the water surface (every contact with the surface counts as one surface).
Flashing	Fish moves forward quickly and turns laterally, exposing its silvery ventrum, causing it to reflect the light and display a 'sparkle' while in the water column.
Twitching	Fish jerks its head rapidly side to side, while keeping its tail relatively straight. The fish does NOT move forward rapidly or dramatically during this time.

The same PDBs were quantified in the group-exposures experiment with a minor adjustment to the darting definition, given the larger tank sizes (Table 2). We also tracked an additional PDB, surfacing, which we defined following (Lafferty and Morris 1996). We did not track this behavior for the individually-exposed fish because we expected that the small size of

those tanks (and low water depth) would preclude meaningful variation in surfacing rates, particularly for the larger wild-caught fish).

(b) Activity

In the individual exposures experiment**,** activity was defined as the proportion of time a fish is *actively* swimming forward (e.g., continuous forward motion), operationally defined as moving forward at least 0.5 body length per second. Fish exhibiting passive upward, backward or downward motions (i.e. non head-directed movement) were *not* considered active. The body length of each fish was measured and activity was calculated based on the amount of time a focal fish was actively moving or not for 1 sec at each of the 15 second time intervals over the course of 5 mins.

In the group exposures experiment, activity was defined as the amount of time a fish was actively moving forward at least one body length per second. To quantify killifish activity, we recorded the amount of time each randomly selected focal fish was inactive (i.e. not moving forward at least one body length per second) and subtracted this amount from the total observation time of one minute at each replicate.

We modified the definition for activity for fish exposed individually or in groups based on two criteria: (i) tank and (ii) fish size. Whereas the smaller lab-reared fish could swim forward several body lengths at a time, larger, wild-caught fish could at most, swim only between 0.5-1 body length. As a result, we decided to standardize the distance aspect of the activity definition to a minimum of 0.5 body lengths. Conversely, fish exposed in groups were all lab-reared and housed in much larger tanks. Thus, we decided to use one body length/sec (instead of 0.5 body lengths/sec) as a measure of activity.

(c) Vertical Position

To measure vertical position during the individual exposures, we recorded the position of the fish's head in the water column in each of the two tank sections. Over the course of 5 mins, we recorded either a 0 or a 1 if the fish was located on the bottom or top half of the tank, respectively, at 15 sec time intervals. We used these values to calculate the mean vertical position of each fish. Given the large size of the wild fish and the small tank size, we did not expect to see a change in vertical position for this fish group.

In the group exposures, tanks were subdivided into three sections instead of two. Each section was assigned a number denoting the position in the water column (1 = bottom, 2 = middle, 3 = top). We then calculated weighted average vertical position using these values and the proportion of healthy fish in each tank section at each replicate. Unhealthy fish were excluded from these calculations.

(d) Shoal Size

Shoal size was quantified only in the group exposures. Here, a shoal was defined as the number of fish within two body lengths of a focal fish (Delacourt and Poncin 2012; Krause and Godin 1994). To quantify shoal size, we measured the body lengths of each of our 10 randomly selected focal fish (at each replicate), and used them to count the number of fish within two body lengths. Unhealthy fish (i.e. those with buoyancy or fecal cast issues) never served as focal fish. Despite this, they were included as part of a shoal *only* if they were within two body lengths of the focal fish.

Statistical Analysis

1.) Individual Exposures

We used generalized linear mixed models (GLMMs) (Bolker et al. 2009) and linear mixed models (LMMs) to examine how the number of PDBs, activity and vertical position in the individual exposures were influenced by time period (i.e. pre-exposure, post-exposure and 2 Hours post-exposure), prior infection status (i.e. LRUI, LRI and WI), treatment (sham- vs. parasite-exposed) and fish total length (cm). Tank ID, block and fish ID were treated as random effects, and all others as fixed effects. Initial data exploration revealed collinearity between fish total length (cm) and prior infection status (total length of WI fish was different from that of lab-reared). Therefore, fish total length was dropped from subsequent analyses (Zurr et al. 2010). We considered the main effects of treatment, time period and prior infection status as well as all their two- and three-way interactions.

We analyzed the average number of PDBs using a GLMM with a Poisson error distribution and a zero-inflation parameter that varied with prior infection status (Brooks et al. 2017), as zeroes composed 27% of data. To assess zero-inflation, we fit three models: one that excluded the zero-inflation parameter, one that modeled zero-inflation as equal for all observations and another where zero-inflation varied by prior infection status. This was followed by model comparison using Akaike's Information Criterion corrected for small sample size, AICc (Burnham and Anderson 2002; Wagenmakers and Farrell 2004). In addition, we used a multinomial logistic regression in JMP®, Version 13.0, to examine how specific types of PDBs varied among time periods. To analyze activity and vertical position data of individually-exposed fish, we applied a logit transformation (Warton and Hui 2011) prior to conducting an LMM. This was followed by model selection and averaging.

2.) Group Exposures

Here**,** we used GLMMs to examine the effects of treatment (i.e. control, low EUHA, High EUHA and SMCY), time period (pre-exposure, disturbance and exposure), temperature and proportion of healthy fish on the average number of PDBs, activity and vertical position, as well as group size. Treatment, time period, proportion of healthy fish and temperature served as fixed effects while tank ID and block served as random effects. Given the narrow temperature range (~1℃) among tanks, we did not include it in our analyses. We considered the main effects of treatment, time period and proportion of healthy fish as well as the interaction between treatment and time period.

We performed a similar analysis to analyze the average number of PDBs in the group exposures (see above). However, zero-inflation was not present in this dataset. Activity, vertical position and shoal size of group-exposed fish, were also analyzed using a GLMM with a Poisson error distribution. Analysis of vertical position and shoal size was followed by model selection and averaging. Prior to statistical analysis, group exposures data was converted to integers (as required by Poisson regression) by multiplying the data by a factor of ten and rounding to the nearest whole number. Data was back-transformed and rescaled, where appropriate, prior to plotting.

The influence of disturbance on fish exposed in groups was analyzed as described above. However, here we considered the main effects of treatment, disturbance type (CU, VI and CD) and proportion of healthy fish as well as the interaction between treatment and disturbance type.

Model selection, averaging, and adequacy

We examined the relative performance of the global model and all nested sub-models using AICc (Burnham and Anderson 2002; Wagenmakers and Farrell 2004), for data from both experiments. Model adequacy for models without zero-inflation were assessed by examining Pearson residuals (Dunn and Smyth 1996; McCullagh and Nelder 1989) for normality and homogeneity of variance (Cox and Snell 1968; Quinn and Keough 2002). Zero-inflation models do not provide Pearson residuals. Therefore, we used simulation-based quantile residual plots to assess model adequacy (Dunn and Smyth 1996). If there was no clear favored model (one with an AICc at least 2 smaller than the next best model), we performed model averaging using all models with $\Delta AICc < 2$ (Burnham and Anderson 2002; Symonds and Moussalli 2010). We dropped random effects if they did not explain significant variation in the data (Bolker et al. 2009)

We conducted all the above statistical analyses with R statistical software (version 3.5.1, R Development Core Team 2009), unless otherwise stated. We used the package 'MuMIn' (Bartón 2019) to conduct model selection and model averaging. GLMMs were performed using the package 'glmmTMB' (Brooks et al. 2017), while the packages 'lme4' (Bates et al. 2015) and 'lmerTest' (Kuznetsova et al. 2017) were used for LMMs. Lastly, we used the 'DHARMa' package (Hartig 2019) to plot and examine quantile residuals.

Results

(a) Potential Defensive Behaviors (PDBs)

Concerning individually-exposed fish, AICc scores (Table 3) strongly favored the model with the interaction between treatment and time period and with prior infection as a main effect

(ΔAICc = 0.0). Consistent with our predictions, this indicated that the effect of treatment varied across time period (Table 3), with killifish individually exposed to parasites displaying approximately a two-fold increase in PDBs/5 mins compared to sham-exposed fish (Fig. 1). Interestingly, the response to exposure was consistent across prior-infection statuses, with naïve, previously infected, and wild-caught previously infected fish all exhibiting a peak in PDBs upon exposure, followed by a decrease two hours after continuous exposure (Fig. 1).

Table 3: Model selection table for the 10 best GLMMs describing the variation in number of PDBs/5min displayed by individually-exposed fish in response to EUHA exposure. The models listed have the lowest AICc values from the 14 estimated models. The included variables are as follows: Treat = Treatment, TimePd = Time Period, and Prior Inf Stat = Prior infection status. Highlighted model in bold italics is the favored model.

Model	**df**	**AICc**	**△AICc**	***weight***
Treat X TimePd + Prior Inf Stat	***13***	***1190.698***	***0.00***	***0.95***
Treat X TimePd X Prior Inf Stat	23	1196.412	5.71	0.05
TimePd X Prior Inf Stat	14	1209.528	18.83	0.00
TimePd X Prior Inf Stat + Treat	15	1210.086	19.39	0.00
Prior Inf Stat + TimePd	10	1214.318	23.62	0.00
Treat + TimePd + Prior Inf Stat	11	1214.985	24.29	0.00
Treat X Prior Inf Stat + TimePd	13	1215.528	24.83	0.00
Treat X TimePd	11	1219.460	28.76	0.00
TimePd	8	1242.771	52.07	0.00
Treat + TimePd	9	1243.512	52.81	0.00

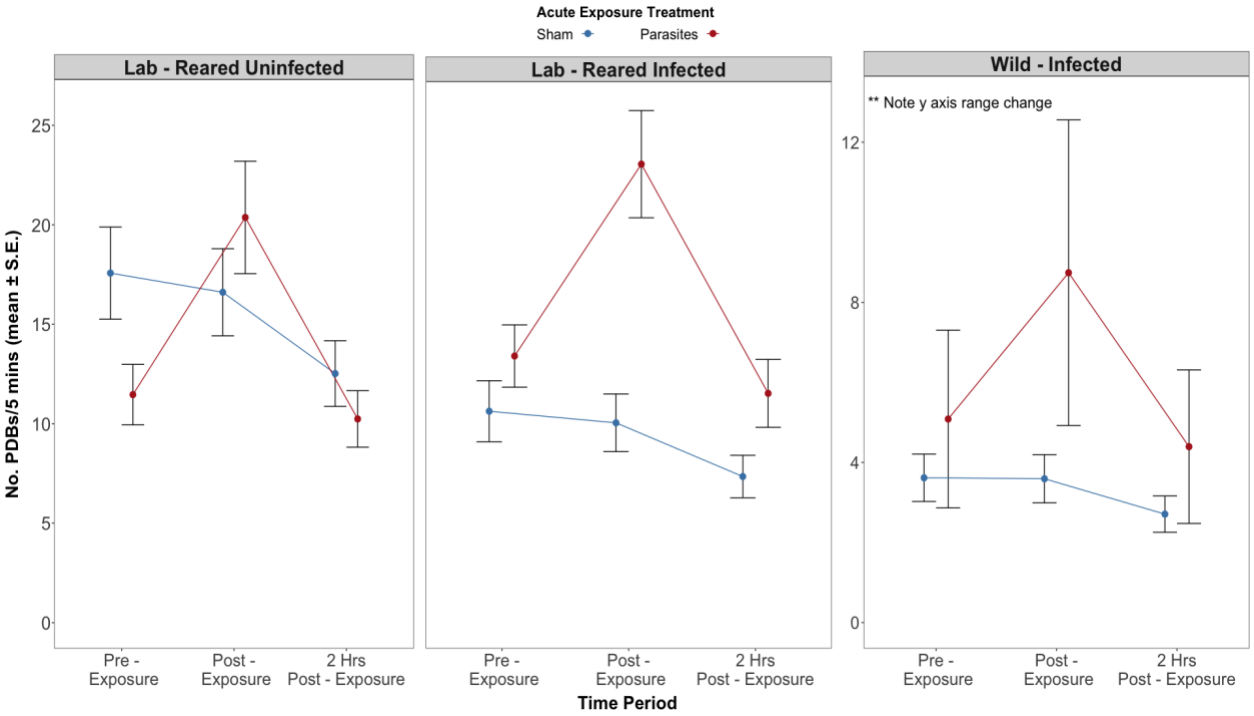

Figure 1: *Total number of potential defensive behaviors (PDBs)/5 mins displayed by individually-exposed lab-reared killifish in three different fish groups before exposure, immediately after and 2 Hrs after continuous exposure. The red peak during post-exposure indicates that all three fish groups responded similarly to parasite exposure. Figure was created using parameter estimates from favored model which included the 2-way interaction between treatment and time period and with prior infection status as a main effect.*

AICc scores from group-exposed fish provide parallel results to those from the individual exposures. The top model (ΔAICc = 0.0) also includes the interaction between treatment and time period (Table 4), reflecting differences in how sham- vs cercaria-exposed fish responded to exposure. Killifish exposed to parasites (regardless of dose or species) displayed more PDBs than sham-exposed fish (Fig. 2). Killifish exposed to the low and high EUHA doses displayed between 2.5 – 3x more PDBS/min. Additionally, the higher pre-exposure baseline of high EUHA resulted in an increase in PDBs being approximately the same as the low EUHA dose despite having had a larger absolute increase in PDBs. Further, killifish exposed to the other parasite species, SMCY, increased their average number of PDBs/min two-fold.

Table 4: Model selection table for the the 9 best GLMMs describing the variation in mean number of potential defensive behaviors (PDBs) of fish originating from San Elijo (SE). The models listed have the lowest AICc values from the 9 estimated models. The included variables are as follows: Prop = proportion of healthy fish, Treat = Treatment and TimePd = Time Period. The highlighted model in bold italics is the favored model.

Model	**df**	**AICc**	**△AICc**	***weights***
Treat X TimePd	***13***	***348.314***	***0.00***	***0.82***
Treat X TimePd + Prop	14	351.407	3.09	0.18
Treat + TimePd	7	370.533	22.22	0.00
Treat + TimePd + Prop	8	372.140	23.83	0.00
TimePd	4	383.036	34.72	0.00
TimePd + Prop	5	385.261	36.95	0.00
Treat	5	582.315	234.00	0.00
Treat + Prop	6	583.587	235.27	0.00
Prop	3	597.636	249.32	0.00

Fish also increased their PDBs consistent with predictions during the three disturbance time points (Supplementary Tables and Figures; Table S1). Here, the favored model (ΔAICc = 0.0) includes the interaction between treatment and disturbance type, indicating that both treatment and disturbance types differentially affect killifish behavior. First, although fish in all groups did increase PDBs during CU (when curtain was lifted and before cercariae were added, so this represents a response to pure disturbance), this increase was much lower than upon 'exposure'. Second, the PDB increase became increasingly pronounced moving between VI and CD, but was not as high as the response observed during our "exposure," which occurred 10-20 minutes later.

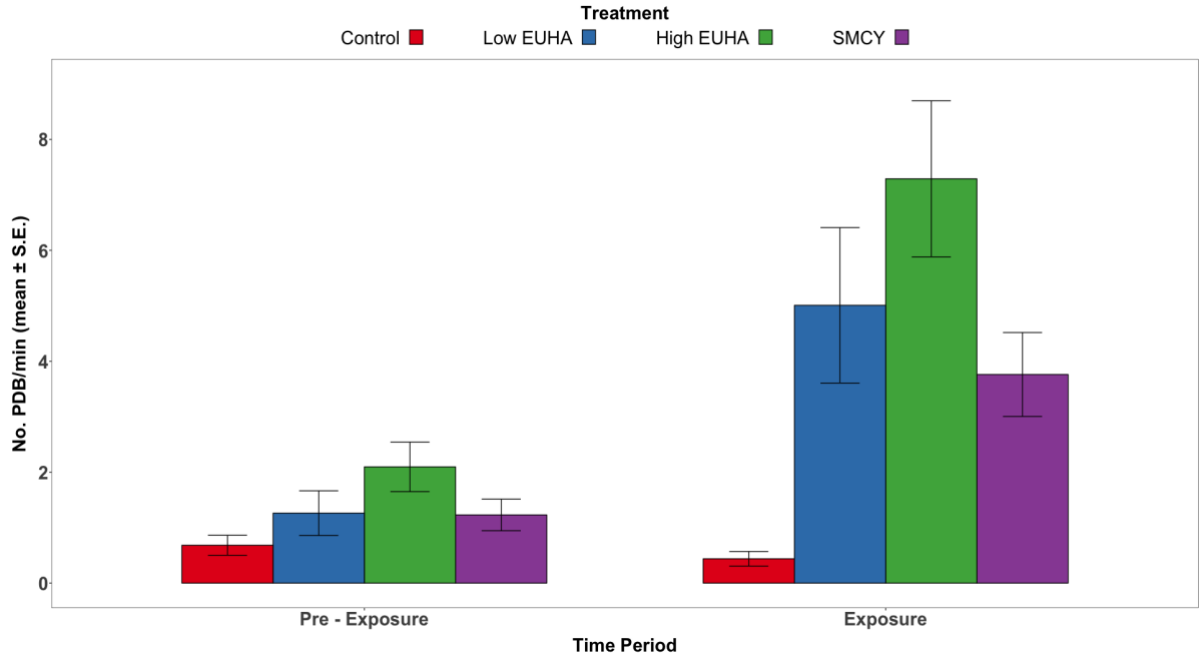

Figure 2: *Total number of potential defensive behaviors (PDBs)/1 min displayed by group-exposed lab-reared fish before and during exposure to parasites (using parameter estimates from favored model that included the interaction between treatment and time period).*

In addition to examining the average number of PDBs displayed by individually- and group-exposed fish, we were also interested in examining whether any specific PDBs increased during exposure. The multinomial logistic regression indicated that time period was a significant predictor of average number of PDB type ($p < 0.0001$, for *both* individually- and group-exposed fish). All three fish groups (LRUI, LRI and WI) displayed darts, scratches, flashes and twitches. However, darts and scratches were by far, the most common, with darts comprising the largest portion of increased behaviors (Fig. 3).

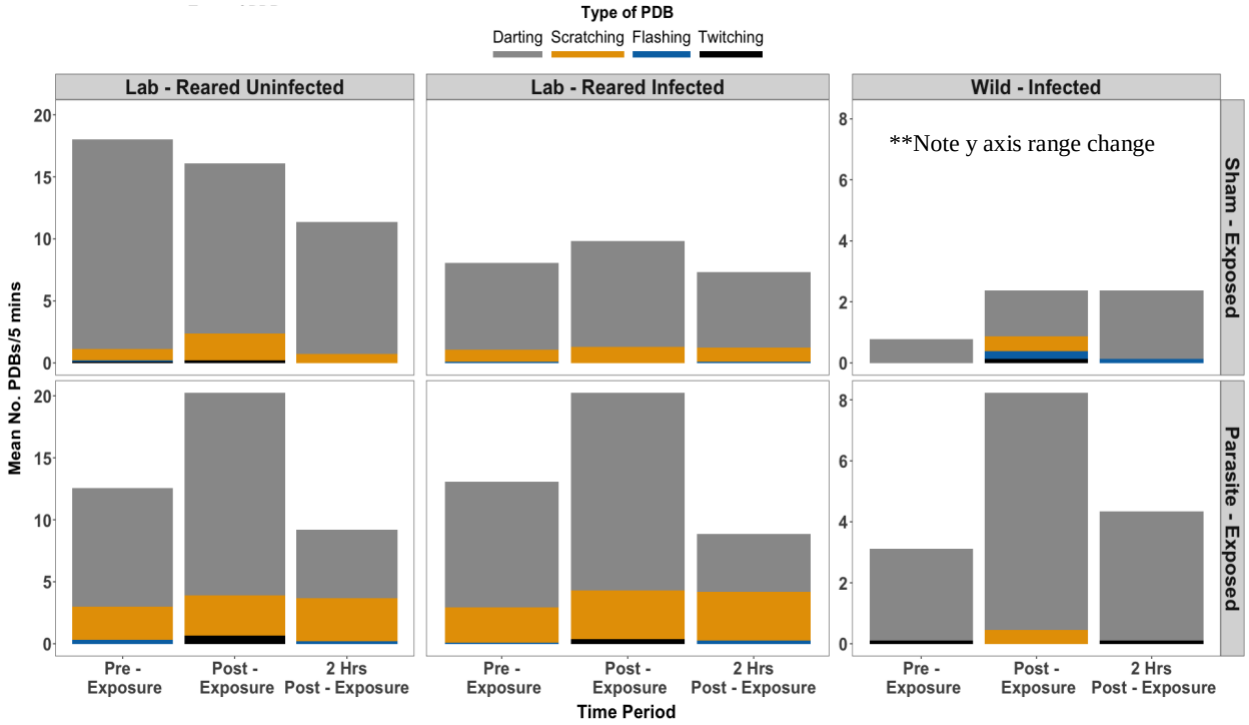

Figure 3: *Average number of each type of potential defensive behavior (PDB)/5 mins displayed by individually-exposed fish of three fish group before, immediately after and 2 Hrs after continuous exposure to parasites. The average number of PDBs/5 mins peaked during post-disturbance, with darts comprising the vast majority of behaviors displayed.*

Concerning group-exposed fish, despite exhibiting a larger variety of PDBs, darts and scratches were also the most common PDBs for group-exposed fish (Fig. 4). Individually-exposed killifish displayed a higher frequency of darts if they were exposed to parasites (from pre- to post-exposure), regardless of prior infection status. This also occurred for group-exposed fish, although in this case, the number of scratches also increased. Furthermore, this was consistent across parasite-exposed treatment groups, regardless of the parasite species (Fig. 4).

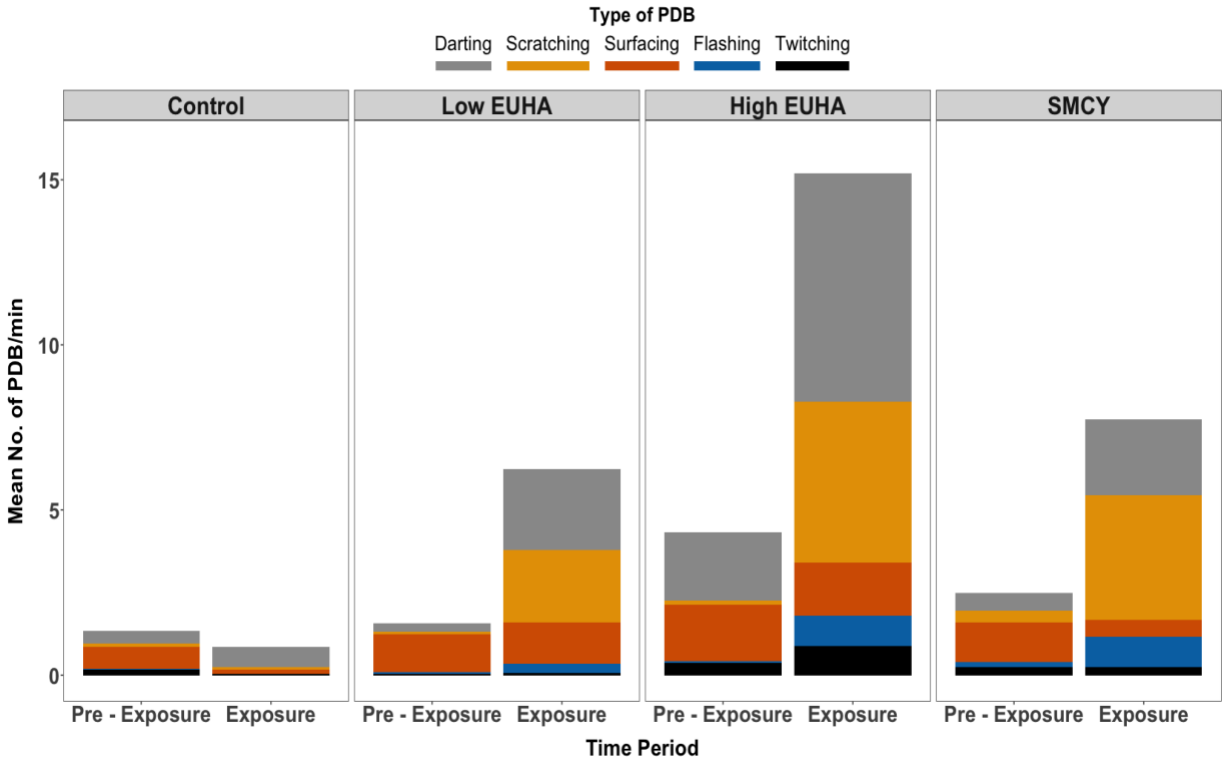

Figure 4: *Average number of each PDB type/1 min displayed by group-exposed lab-reared fish before and during exposure to parasites. Darts and scratches were the most commonly displayed behaviors.*

(b) Activity

Contrary to our predictions, the interaction between treatment and time period was not favored for individually-exposed fish. There was also was no single, strongly-favored model (Table 5), so we averaged the top three models (ΔAICc < 2), which included effects of parasite exposure treatment, time period, and prior infection status, along with the interaction between exposure treatment and prior infection status (Table 6). Although there was positive effect of parasite exposure treatment and time period (post-exposure) on activity, they were unreliable as their 95% CIs span zero. Only the effect of wild-caught fish (WI) prior infection status was a reliable predictor of activity (Table 6). This indicates that overall, WI killifish were less active

compared to lab-reared killifish (Fig. 5). For unknown reasons, baseline activity levels for LRI fish were significantly different (t = -4.2637, df = 18, p-value = 0.0005), as to-be-parasite-exposed LRI fish were already more active compared to the to-be-sham-exposed (Fig. 5, middle panel during pre - exposure).

Table 5: The 10 best LMMs describing the variation in activity of individually-exposed fish. The models listed have the lowest AICc values from the 14 estimated models. The included variables are as follows: Treat = Treatment, TimePd = Time Period and Prior Inf Stat = Prior infection status. Highlighted models in bold italics were included in model averaging.

Model	**df**	**AICc**	**△AICc**	***weights***
Prior Inf Stat X Treat + TimePd	***10***	***593.803***	***0.00***	***0.34***
Prior Inf Stat X Treat	***8***	***594.693***	***0.89***	***0.22***
Prior Inf Stat + Treat + TimePd	***8***	***595.589***	***1.79***	***0.14***
TimePd X Prior Inf Stat + Treat	12	596.156	2.35	0.11
Prior Inf Stat + Treat	6	596.543	2.74	0.09
Treat X TimePd + Prior Inf Stat	10	597.023	3.22	0.07
Prior Inf Stat X Treat X TimePd	20	599.969	6.17	0.02
Prior Inf Stat + TimePd	7	601.338	7.53	0.01
TimePd X Prior Inf Stat	11	601.525	7.72	0.01
Prior Inf Stat	5	602.276	8.47	0.00

Table 6: Average parameter estimates, standard error (ses), and 2.5th and 97.5th quantiles of the confidence intervals (CIs) for the top-ranked model and all models with △AICc values < 2 for activity of individually-exposed killifish. Pre-Exposure, sham-exposure and lab-reared uninfected are the baseline (reference) periods for the interpretation of fixed effects. Highlighted variables in bold italics have CIs that do not include zero.

Variables	**Estimate**	**se**	**2.5th Percentile**	**97.5th Percentile**
Intercept	-0.40	0.40	-1.19	0.39
Treatment				
Parasite-Exposed	0.40	0.61	-0.80	1.60
Prior Infection Status				
Lab-Reared Infected	-0.92	0.64	-2.18	0.34
Wild-Infected	***-2.21***	***0.55***	***-3.28***	***-1.14***
Time Period				
Post-Exposure	0.06	0.17	-0.26	0.39
2 Hrs Post-Exposure	-0.24	0.23	-0.68	0.21
Interaction: Treatment X Prior Infection Status				
Parasite-Exposed, Lab-Reared Infected	1.54	1.05	-0.52	3.59
Parasite-Exposed, Wild-Infected	0.27	0.75	-1.19	1.74

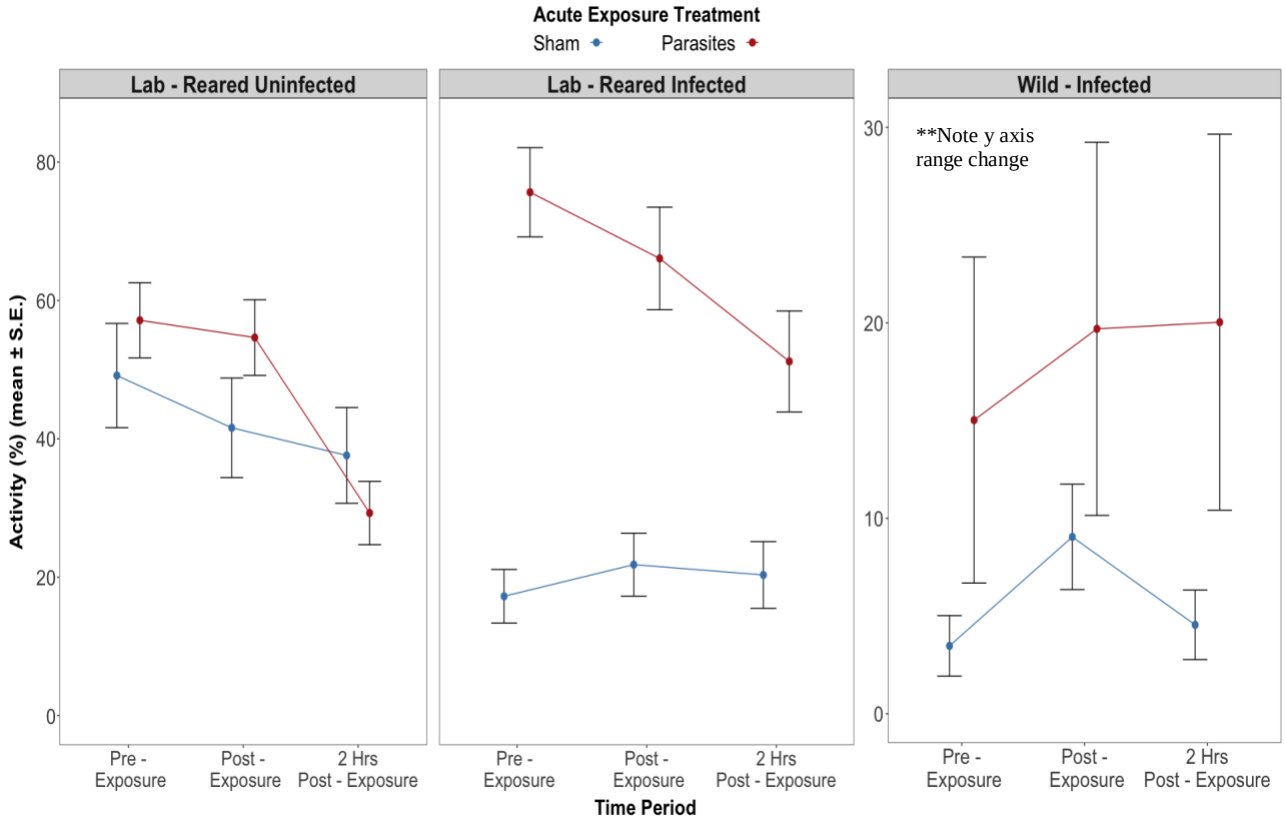

Figure 5: *Average activity levels displayed by individually-exposed killifish in three different fish groups before exposure, immediately after and 2 Hrs after continuous exposure. For unknown reasons, LRI fish in the to-be-parasite-exposed group had a significantly different baselines compared to the to-be-sham-exposed group (t = -4.2637, df = 18, p-value = 0.0005). Figure was created using predictions from the model including the three-way interaction between treatment, time period and prior infection status.*

In contrast to individual exposures, analysis of activity for group-exposed fish revealed that the interaction between treatment and time period was strongly favored (Table 7; ΔAICc = 0.0). Killifish exposed to parasites (regardless of the species) were more active than sham-exposed killifish (Fig. 6). Fish exposed to a low EUHA dose were 8x more active, while fish exposed to a high EUHA dose were 3.5x more active. Additionally, SMCY-exposed fish increased their activity by a factor of 2. Fish also increased their activity consistent with predictions during the three disturbance time points (Supplementary Tables and Figures; Table S2). Here, the favored model (ΔAICc = 0.0) included the interaction between treatment and

disturbance type, indicating that both treatment and disturbance types differentially affected killifish behavior. Killifish increased their activity beginning at CU (true response to disturbance) and increased further during VI and CD and remained at similar levels during 'exposure,' which occurred 10-20 minutes later.

Table 7: Model selection table showing the 9 best GLMMs describing the variation in mean fish activity levels of group-exposed lab-reared fish originating from San Elijo (SE). The models listed have the lowest AICc values from the 9 estimated models. The included variables are as follows: Prop = proportion of healthy fish, Treat = Treatment and TimePd = Time Period. Highlighted model in bold italics is the favored model.

Model	**df**	**AICc**	**△AICc**	***weights***
Treat X TimePd	***14***	***858.977***	***0.00***	***0.84***
Treat X TimePd + Prop	15	862.245	3.27	0.16
Treat + TimePd	8	983.299	124.32	0.00
Treat + TimePd + Prop	9	984.923	125.95	0.00
TimePd	5	999.216	140.24	0.00
TimePd + Prop	6	1001.502	142.53	0.00
Treat	6	2458.339	1599.36	0.00
Treat + Prop	7	2459.595	1600.62	0.00
Prop	4	2477.186	1618.21	0.00

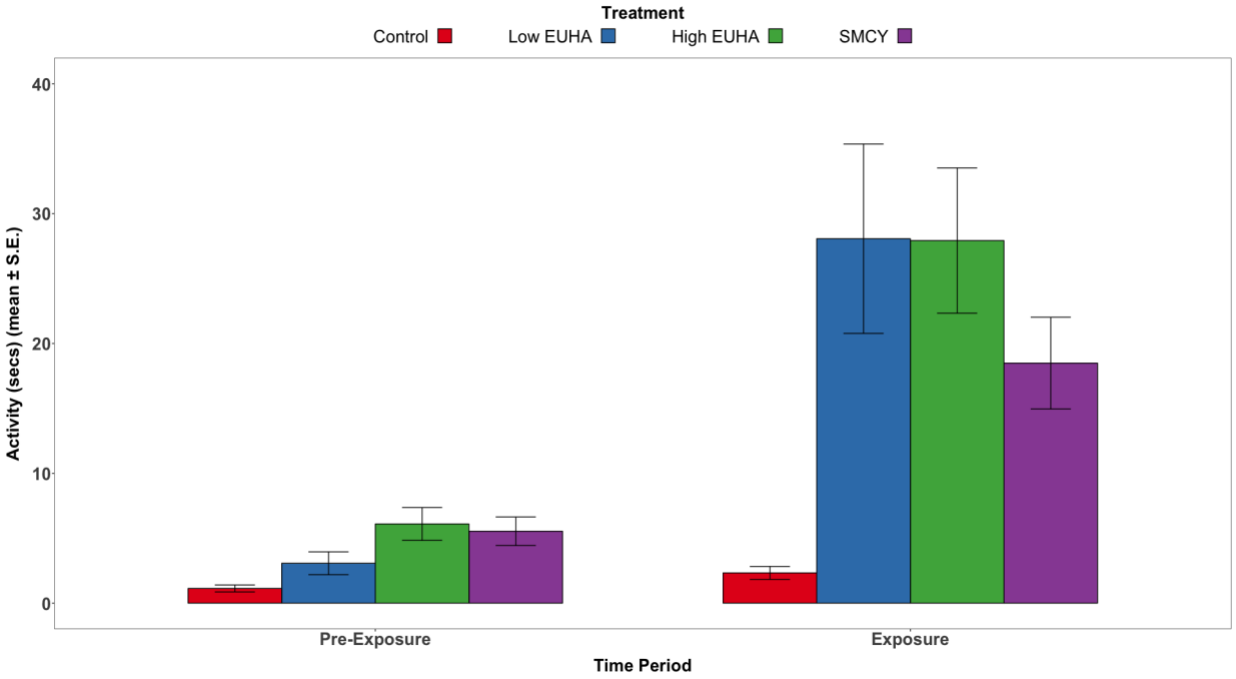

Figure 6: *Average activity/min of group-exposed lab-reared killifish before and during exposure to parasites (using parameter estimates from the favored model that included the interaction between treatment and time period).*

(c) Average vertical position

Analysis of mean vertical position in individual exposures revealed that there was a lack of treatment effect as the interaction between treatment and time period was not favored (Table 8). Since there was no favored model, we averaged our top two ranking models (ΔAICc < 2), which included the main effects of treatment and prior infection status (Table 9).

Table 8: Model selection of the 10 best LMMs describing the variation in mean vertical position in individually-exposed fish during exposure to EUHA. The models listed have the lowest AICc values from the 14 estimated models. The included variables are as follows: Treat = Treatment, TimePd = Time Period, and Prior Inf Stat = Prior infection status. Highlighted models in bold italics were included in model averaging.

Model	df	AICc	△AICc	*weights*
Prior Inf Stat + Treat	***8***	***556.456***	***0.00***	***0.37***
Prior Inf Stat	***7***	***556.637***	***0.18***	***0.34***
Prior Inf Stat + Treat + TimePd	10	559.484	3.03	0.08
Prior Inf Stat + TimePd	9	559.620	3.16	0.08
Prior Inf Stat X Treat	10	559.646	3.19	0.07
TimePd X Prior Inf Stat + Treat	14	562.365	5.91	0.02
TimePd X Prior Inf Stat	13	562.534	6.08	0.02
Treat X Prior Inf Stat + TimePd	12	562.839	6.38	0.02
Treat X TimePd + Prior Inf Stat	12	563.942	7.49	0.01
Treat	6	565.642	9.19	0.00

The effect of parasite exposure had an overall positive, but unreliable effect on the fishes' mean vertical position as its 95% CIs spanned zero (Table 9). During post-exposure LRI fish exposed to parasites decreased their vertical position, while remaining slightly higher in the water column relative to the sham-exposed group. Conversely, LRI fish moved higher in the water column regardless of exposure treatment. WI fish exposed to parasites moved slightly higher in the water compared to sham-exposed, with those exposed to parasites exhibiting greater variation in mean vertical position. Conversely, the effect of prior infection status was reliably negative for the WI fish group (Table 9) indicating that overall, wild-caught fish were closer to the tank bottom relative to lab-reared killifish (Fig. 7).

Table 9: Average parameter estimates, standard error (ses), and 2.5th and 97.5th quantiles of the confidence intervals (CIs) for the top-ranked model and all models with △AICc values < 2 for mean vertical position in individual exposures during exposure to EUHA. Pre-exposure, sham-exposure and lab-reared uninfected are the baseline (reference) periods for the interpretation of fixed effects. Highlighted variables in bold italics have CIs that do not include zero.

Variables	Estimate	se	2.5th Percentile	97.5th Percentile
Intercept	-1.92	0.30	-2.51	-1.34
Prior Infection Status				
Lab-Reared Infected	-0.03	0.31	-0.64	0.57
Wild-Infected	***-1.14***	***0.32***	***-1.76***	***-0.52***
Treatment				
Parasites	0.21	0.28	-0.33	0.76

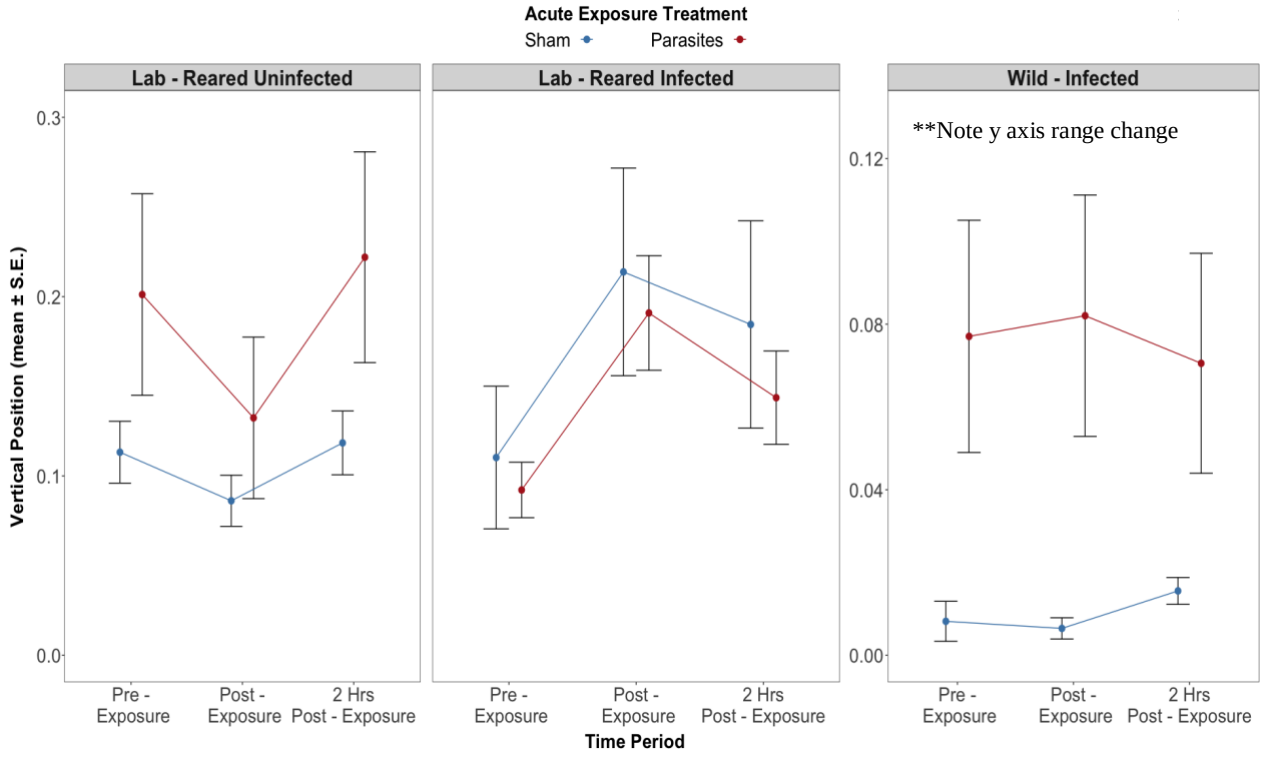

Figure 7: *Average vertical position of individually-exposed killifish in three different fish groups before exposure, immediately after and 2 Hrs after continuous exposure. A value of 0.0 for vertical position indicates the tank bottom. Figure was created using predictions from a model that included the three-way interaction between treatment, time period and prior infection status.*

Regardless of parasite dose or species, there was no change in mean vertical position of group-exposed killifish before and during exposure to parasites (Fig. 8). Given that the interaction between treatment and time period was not favored, we averaged our top 4 ranking models (ΔAICc < 2), which included an effect of treatment, time period and proportion of healthy fish (Table 10). Model averaging results showed that neither treatment, time period nor proportion of healthy fish were reliable predictors of mean vertical position, as they all have 95% CIs spanning zero (Table 11). We obtained similar results during disturbance (i.e. lack of treatment effect). In this case, fish remained even closer to the tank bottom during the entire disturbance time period, regardless of parasite dose or species (Supplementary Tables and Figures; Fig. S3).

Table 10: The 9 best GLMMs describing the variation in mean vertical position of group-exposed lab-reared fish originating from San Elijo (SE). The models listed have the lowest AICc values from the 9 estimated models. The included variables are as follows: Prop = proportion of healthy fish, Treat = Treatment and TimePd = Time Period. Highlighted models in bold italics were included in model averaging.

Model	**df**	**AICc**	**△AICc**	***weights***
TimePd	***4***	***225.681***	***0.00***	***0.30***
Treat	***5***	***226.114***	***0.43***	***0.24***
Prop	***3***	***227.237***	***1.56***	***0.14***
Treat + TimePd	***7***	***227.303***	***1.62***	***0.13***
TimePd + Prop	5	227.834	2.15	0.10
Treat + Prop	6	228.847	3.17	0.06
Treat + TimePd + Prop	8	230.372	4.69	0.03
Treat X TimePd	13	246.143	20.46	0.00
Treat X TimePd + Prop	14	250.698	25.02	0.00

Table 11: Average parameter estimates, standard error (ses), and 2.5th and 97.5th quantiles of the confidence intervals (CIs) for the top-ranked model and all models with △AICc values <2 for mean vertical position of group-exposed lab-reared fish originating from San Elijo (SE). Pre-exposure, and control treatments are the baseline (reference) periods for the interpretation of fixed effects. 95% CIs for all parameters of interest include zero. Therefore, they are not useful in predicting their effect on vertical position.

Variables	Estimate	se	2.5th Percentile	97.5th Percentile
Intercept	0.257	0.187	-0.110	0.623
Proportion Healthy	0.000	0.002	-0.003	0.003
Time Period				
Disturbance	-0.137	0.127	-0.654	0.381
Exposure	-0.034	0.077	-0.452	0.384
Treatment				
Low EUHA	0.135	0.202	-0.530	0.799
High EUHA	0.059	0.127	-0.459	0.577
SMCY	0.039	0.112	-0.449	0.527

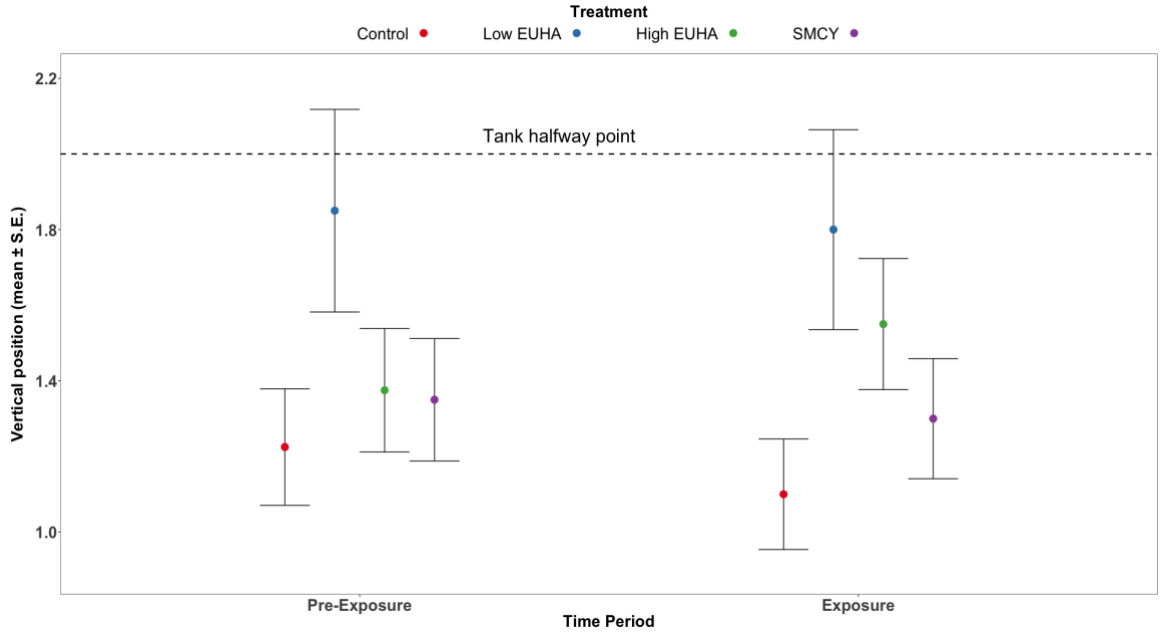

Figure 8: *Weighted average vertical position of group-exposed lab-reared killifish originating from SE before and during exposure to parasites (using predictions from model including the 2-way interaction between treatment and time period). A value of 1.0 for vertical position represents the tank bottom. Horizontal dashed line indicates the midpoint of the water level in the tank.*

(d) Group size

There was a lack of treatment effect on group size (Table 12). Model averaging results from our top two ranked models (ΔAICc < 2) included only the main effects of time period and proportion of healthy fish. Time period had a reliably positive effect on group size as their 95% CIs did not span zero (Table 13), indicating that group size increased during disturbance (Supplementary Tables and Figures; Fig. S4). Conversely, parasite exposure had a negative, yet unreliable effect on group size, which might indicate that fish might have decreased shoal cohesion (Fig. 9).

Table 12: Model selection table for the 9 best GLMMs describing the variation in mean group size of lab-reared fish originating from San Elijo (SE). The models listed have the lowest AICc values from the 9 estimated models. The included variables are as follows: Prop = proportion of healthy fish, Treat = Treatment and TimePd = Time Period. Highlighted models in bold italics were included in model averaging.

Model	**df**	**AICc**	**△AICc**	***weights***
TimePd	***5***	***328.449***	***0.00***	***0.47***
TimePd + Prop	***6***	***328.686***	***0.24***	***0.41***
Treat + TimePd	8	332.453	4.00	0.06
TimePd + Treat + Prop	9	333.523	5.07	0.04
Treat X TimePd	14	335.280	6.83	0.02
Treat X TimePd + Prop	15	337.995	9.55	0.01
Prop	4	342.083	13.63	0.00
Treat	6	345.206	16.76	0.00
Treat + Prop	7	345.908	17.46	0.00

Table 13: Averaged parameter estimates, standard error (SEs), and 2.5th and 97.5th quantiles of the confidence intervals (CIs) for the top-ranked model and all models with △AICc values < 2 for mean group size of fish originating San Elijo (SE). Pre-exposure is the baseline (reference) period for the interpretation of fixed effects. Highlighted variables in bold have CIs that do not include zero.

Variables	Estimate	se	2.5th Percentile	97.5th Percentile
Intercept	1.145	0.44	0.642	1.826
Proportion Healthy	0.008	0.00	-0.007	0.018
Time Period				
Disturbance	***0.145***	***0.07***	***0.036***	***0.292***
Exposure	-0.002	0.07	-0.065	0.116

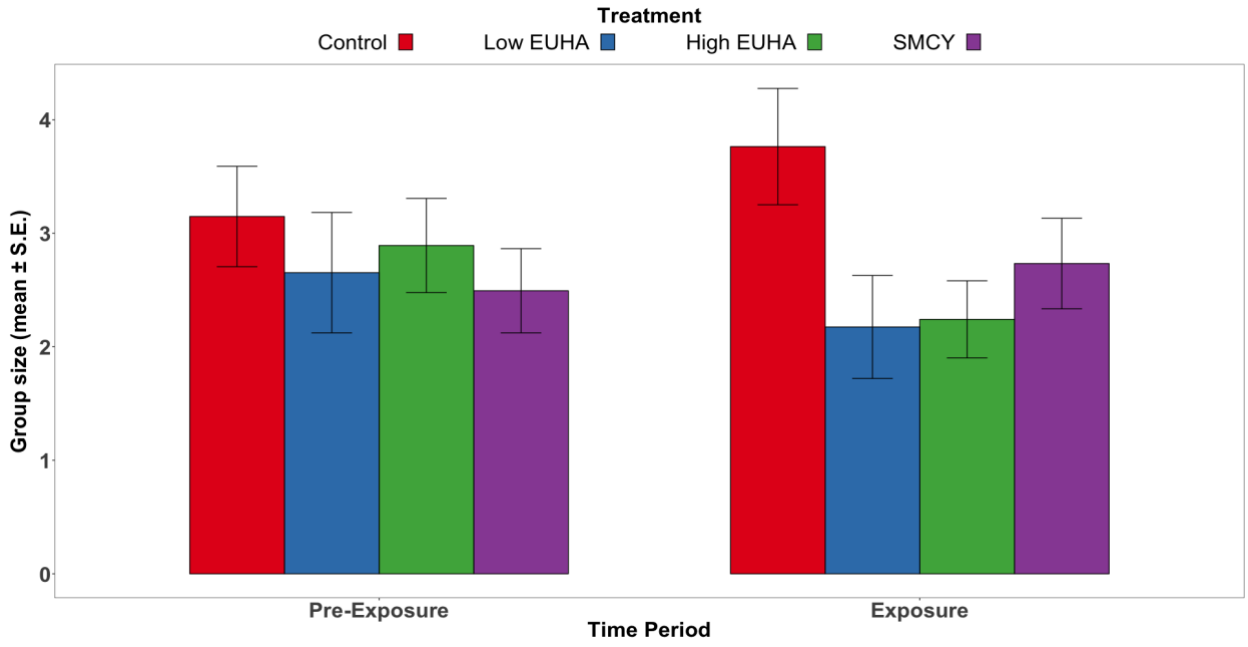

Figure 9: *Average group size of group-exposed lab-reared killifish before and during exposure to parasites. Figure was created using predictions from model that included the 2-way interaction between treatment and time period.*

Discussion

(a) Potential Defensive Behaviors

The overall increase in frequency of PDBs during parasite exposure for both individually- and group-exposed killifish suggests that killifish mount anti-parasite behavioral defenses. Concerning individual exposures, all fish groups exposed to parasites displayed more PDBs relative to sham-exposed fish. This is further noteworthy for a couple of reasons: (1) the response of WI fish indicates that they have not been desensitized to parasite exposure (i.e. have not lost ability to recognize and respond to infectious parasitic stages) despite being unexposed for approximately eight months, and (2) the response of the LRUI fish indicates an innate rather than learned response to parasite exposure. Group-exposed fish began increasing the frequency of PDBs during initial disturbance (CU) and this became more pronounced during VI and CD. This pattern is consistent with there being some initial cercariae exposure leading to an even higher response observed 10-20 mins after disturbance, when we expect the greatest exposure to parasites to have been occurring.

The increase in PDBs was mostly comprised of darts and scratches, although fish exposed to parasites also seemed to display a greater variety of PDBs (particularly group-exposed fish). Initially, we predicted that the frequency of PDBs such as darting, flashing, and twitching would increase as killifish might use them as pre-contact defensive behaviors. We also predicted that once cercariae make contact with killifish, the fish might scratch or surface in an attempt to remove them prior to successful attachment. However, the increase of only darting and scratching during parasite exposure suggests that these two PDBs might be the *primary* behaviors employed by killifish to counter parasite attack.

California killifish commonly respond to disturbance by darting (Fritz 1975). In fact, darting is a well-known fish response used for predator evasion (Helfmann et al. 2009), and we have observed California killifish darting when being hunted by herons and egrets (Hechinger, pers. obs.). Aside from predation, our data indicate that darting also serves to counter cercaria attack. Similar to the general utility of darting as an anti-predator defense among species, darting may also be an anti-parasite defense employed by many species. This idea is supported by the increase in the frequency of evasive behaviors such as 'swimming bursts' (e.g. darts) for Atlantic salmon during exposure to infectious copepods (Bui et al. 2017; Bui et al. 2018), or the extremely rapid twisting, turning and tumbling for tadpoles exposed to *Echinostoma* cercariae (Szuroczki and Richardson 2012; Thiemann and Wassersug 2000; Taylor et al. 2004). Whether darting is useful in decreasing killifish infection risk during exposure to parasites remains unknown. Therefore, future studies are necessary to examine whether it is effective in decreasing killifish risk of exposure and infection.

Our group-exposed killifish also substantially increased the amount of scratching upon cercaria exposure. In addition to being a very intuitive form of defense—likely permitting the scraping off of attacking parasites on skin—several studies have observed scratching (often referred to as 'chafing') on rough surfaces such as patches of sandy bottom (Berthe et al. 2016), sand ripples (Ritter 2011), turtle scutes (Grossman et al. 2009), the water's surface tension (Atkinson et al. 2018), and even shark skin (Papastamatiou et al. 2007). Although these reports only postulated that scratching serves as anti-parasite defense, Wyman and Walters-Wyman (1985), showed that after experimentally manipulating the external surface of two fish species by loosening a scale or inserting a small piece of charcoal under a scale, fish displayed higher chafing (scratching) rates. This was also observed in fish exhibiting a different form of external

irritation (fungal infection). In any case, the efficacy of scratching, similar to darting, has still not been directly investigated.

Although our group-exposed fish employed scratching upon cercaria exposure, the individually exposed fish did not. This difference might be explained by the presence or absence of appropriate "scratching-post" material. Our group-exposed fish tanks had sandy sediment on the bottom (where we observed most of the scratching), which likely provided enough rough substrate to permit parasite removal, and thereby elicit the observed scratching. However, tanks for individual fish exposures had no rough material available, potentially explaining why individually exposed fish did not exhibit scratching (despite increasing darting). Hence, our results further indicate that scratching is a context dependent behavior, where fish employ scratching as a defense only when it can be efficacious.

In sum, our results show that the average number of PDBs increases during parasite exposure. Out of five PDBs quantified, only darts and scratches increased during parasite exposure, suggesting an anti-parasite defensive role. Future work can directly examine the efficacy of these behaviors in countering parasite infection by examining the effect of the total number of darts and scratches displayed on host infection intensities.

(b) Activity

Contrary to our predictions and to their increase in PDBs, fish in individual exposures did not respond to parasite exposure by increasing their activity. This is likely due to the inexplicably high baseline activity levels exhibited by the LRI fish. LRI to-be-parasite-exposed fish were significantly more active compared to those in the sham-exposed treatment group. Such high baseline activities could have obscured any potential effect of treatment on activity. After examining possible causes for the unexpected difference in baselines, we were unable to find any

possible confound, experimental design flaw, or video labelling error that could explain why LRI fish to-be-parasite-exposed could be expected to have higher baseline activity levels than LRI fish in the sham exposed treatment group. Given the large size of the WI fish, it was possible that the small tank size could have hindered WI fish ability to move. Results are consistent with this as WI were significantly less active compared to lab-reared fish. Despite this, the effect of parasite exposure had a positive, yet unreliable effect on activity of WI fish, suggesting that they might be responding to parasite exposure by increasing their activity.

Despite the unexpected results in the individual exposures, consistent with the PDB results, group-exposed fish were more active when exposed to parasites. In addition and consistent with our predictions, activity began increasing during disturbance. This increase in activity first became evident during CU (when curtain goes up and before cercariae are added) and further increased during VI and CD and remained at similar levels during 'exposure'. These results suggest that similar to PDBs, activity might also function as an anti-parasite defense. Further, these results are consistent with studies examining activity during parasite exposure in a tadpole-trematode system (Koprivnikar et al. 2006; Koprivnikar et al. 2012; Rohr et al. 2009; Taylor et al. 2004). These studies showed that tadpole activity increases during parasite exposure (Taylor et al. 2004), and that more active tadpoles acquired lower infection burdens (Koprivnikar et al. 2006). Thus, it is reasonable to assume that killifish displaying these high activity levels during parasite exposure might be attempting to dodge cercariae swimming in the water column in an effort to reduce infection burdens. Future work should be directed at assessing the efficacy of heightened activity as an anti-parasite defense for California Killifish.

(c) Vertical Position

Changes in spatial distribution of hosts during parasite exposure have been shown to be effective anti-parasite behavioral strategies that may be useful in decreasing infection risk by avoiding areas harboring infectious parasite stages (Wisenden et al. 2009). Contrary to this, our study showed that killifish did not alter their vertical position in the water column during exposure to parasites. Concerning fish in individual exposures, there was no consistent trend in mean vertical position of either fish group. Mean vertical position of fish exposed in groups also did not change in response to parasite exposure. They remained close to the tank bottom regardless of parasite dose or species. Furthermore, during disturbance, fish seemed to move even closer to the tank bottom, which is to be expected as fish probably became more startled with each subsequent visual or mechanical disturbance and perhaps considered the tank bottom as a refuge. Overall, this lack of treatment effect was not surprising, because originally, we were skeptical about observing a change in vertical position, particularly in the very small individual exposure tanks. Despite this, we had decided to quantify this behavioral trait in case there might have been an effect even at this small scale.

Unlike our study, a different fish-parasite system was successful in demonstrating how changes in spatial distribution can function as efficient anti-parasite behavioral defenses (Poulin and Fitzgerald 1988). In parasite-free aquaria, sticklebacks, *Gasterosteus aculeatus*, preferred to be near the tank bottom. In aquaria containing the ectoparasite, *Argulus canadensis*, sticklebacks shifted up in the water column to move away from the source of infection, as *A. canadensis* prefers to be near the tank bottom. Whereas Poulin and Fitzgerald (1988) conducted a thorough assessment of the parasite's spatial distribution prior to the host's introduction into the tanks, we did not. Our prediction regarding killifish spatial distribution during parasite exposure was

founded on our understanding regarding cercarial behavior. We predicted that killifish would move closer to the tank bottom in an effort to avoid contact with EUHA cercariae, which might become aggregated near the water's surface as they are positively phototactic (move towards the light) and negatively geotactic (move against gravity) in laboratory water bottles with still water (Weinersmith et al. 2018). However, we are uncertain whether the cercariae would have been aggregated near the top in the short amount of time they had in the tanks or given the tanks' water flow. SMCY's movement in response to light and gravity has yet to be examined. However, we predict that it will be similar to that of EUHA. In sum, our results suggest that altering their position in the water column might not be an anti-parasite behavioral defense employed by California killifish, but an examination of cercarial distribution in the water column under realistic conditions is required to confirm this.

(d) Shoal size

Despite the potential benefits of forming larger groups to dilute out parasite attacking stages (Stumbo et al. 2012; Mikheev et al. 2013), our results do not support this prediction as fish exposed to parasites did not increase their group size. On the contrary, group cohesion seems to have experienced a slight, yet non-significant, decrease during parasite exposure. The high activity levels and larger number of PBDs (mostly darting and scratching) displayed during parasite exposure could help explain this anomaly, as fish could have become dispersed while attempting to dodge parasites in the water or remove them after making contact. Conversely, the only increase in group size observed occurred during disturbance, which is not surprising given that fish increase group size and shoal cohesion during some situations perceived as threatening (Krause and Ruxton 2002; Stumbo et al. 2012), such as during a predation attack. In this study,

we did not simulate a predation attack, but it appears as though the visual disturbance of our curtain and the mechanical disturbance provided by the vial were sufficiently large to alter group size. Thus, our results suggest that compared to increases in activity and number of darts and scratches, shoal size might not be as effective in defending against cercariae exposure and attack (at least not at the scale that we examined).

(e) Interactions of anti-parasite behaviors and established parasite behavioral modification

Although the focus of this study was to examine killifish anti-parasite behavioral defense during parasite exposure, we have also provided the first direct experimental evidence of behavioral manipulation. Before exposure to parasites, killifish already infected with parasites (high EUHA treatment group) displayed a higher number of PDBs compared to control fish (not infected with parasites). In this context, these behaviors are usually referred to as 'conspicuous behaviors' that could facilitate predation (and successful parasite transmission), as opposed to 'potential defensive behaviors,' as there are no parasites in the water column to defend against. These results are consistent with the previous research concerning behavioral manipulation by EUHA (see introduction). Aside from displaying an increased number of PDBs, previously EUHA-infected killifish were also more active prior to exposure to parasites compared to controls. Interestingly, so were SMCY-infected killifish, providing the first evidence of behavior modification and potential parasite increased trophic transmission for this parasite species. Further, previously-infected killifish (of both species) responded differently to the visual disturbance of the curtain being lifted. This is particularly important given that lifting the curtain provides a disturbance untainted by the presence of cercariae. Whereas only high EUHA- and SMCY-infected killifish displayed a higher number of 'conspicuous behaviors' compared to

controls, all previously-infected killifish were more active when the curtain was lifted providing more evidence of behavioral modification. This differential response to disturbance seems to suggest a suppression of the normal stress response in previously-infected killifish, consistent with the results of experimental work documenting changes in serotonin activity in EUHA infected fish (Shaw et al. 2009). This effect also appears to be occurring with SMCY infection, possibly via a different mechanism than that of EUHA.

Despite the altered behavior of previously-infected killifish, our results suggest that killifish behavioral defenses remain intact. During exposure to parasites, they increased both the average number of PBDs and activity levels, presumably in an effort to decrease the risk of acquiring new infections. These types of behavioral mechanisms of defense are especially important in evasion of parasite species capable of imposing large fitness costs on their hosts. In addition, the efficacy of such behavioral defense will depend on the number of simultaneous threats an organism is faced with. In this study, we examined how killifish responded behaviorally solely to the threat of parasitism. However, in the wild, they also experience the threat of predation. Thus, we expect responses to these threats to conflict with each other. During parasite exposure, fish exposed in groups displaying high activity levels would at times become distracted, causing them to accidentally bump into another fish (pers. obs.). A similar situation occurred when quantifying killifish behavior in the wild (Nelson et al. unpublished results). On several occasions, killifish darting and scratching in the wild have been observed to accidentally bump into one another, presumably during anti-parasite defense, as killifish are exposed to parasites on a daily basis. This observation presents an interesting ecological scenario. For instance, killifish mounting anti-parasite behavioral defenses during parasite exposure might lower their infection risk (Atkinson et al. 2018; Koprivnikar et al. 2006) while simultaneously

increasing their risk of predation (due to a lack of vigilance) as they become more conspicuous to bird predators. Thus, creating a trade-off. To date, the behavioral response of killifish to a simultaneous threat of parasitism and predation has yet to be examined. However, a few studies have examined this in a tadpole-trematode system (Koprivnikar and Penalva 2015; Szuroczi and Richardson 2012). During simultaneous exposure to predators and parasites, tadpoles responded more strongly to the threat of predation regardless of whether they were presented with a live predator (Szuroczi and Richardson 2012) or predator cues (Koprivnikar and Penalva 2015). Therefore, it is quite possible our killifish-trematode system might yield similar results. However, it appears that our system provides an added level of complexity as the parasites and their killifish host are engaged in an intense tug-of-war. On one hand, EUHA (and possibly SMCY via a different mechanism) is actively manipulating killifish behavior as it attempts to reach its final bird host. On the other hand, our results suggest that killifish actively attempt to defend against these parasites. This begs the question, Will anti-parasite behavioral defense supersede the effects of behavioral manipulation under the threat of predation, or is the effect of behavioral manipulation stronger than the will to defend against parasites? Either way, this scenario underscores the importance of examining anti-parasite behavioral mechanisms of defense as they have the potential to interact with other important aspects of a host's ecology.

Conclusion

Our results showed that killifish individually exposed (both previously infected and naïve) to parasites increase their average number of PDBs, but not their activity. It is well established that parasitic infection can modify host behavior (Moore 2002). Therefore, it was possible that the increase in PDBs displayed by previously infected killifish during parasite

exposure was due to behavior modification as opposed to response to exposure. However, the increase in PDBs displayed by naïve fish indicated that *exposure* to parasites and not *infection* was driving this increase. Further, this also indicated that it was an innate response rather than learned. In groups, parasite-exposed killifish increased both their number of PDBs and activity. Furthermore, darts (both individual and group exposures) and scratches (group exposures) were by far, the most commonly displayed PDBs and were also the only ones to increase during exposure to parasites, suggesting that they might be the most effective behaviors at decreasing killifish exposure and infection risk. In fact, the rapid swimming behavior (here termed darting) is commonly employed during predator evasion (Helfmann et al. 2009). Therefore, it may also be employed to dodge parasites swimming in the water. Given the increase in frequency of scratching, it is likely employed during removal of parasites before successful attachment. Thus, we predict fish exhibiting high frequencies of darting and scratching during exposure to parasites should harbor lower parasite burdens. Future studies should directly test this prediction.

Vertical position (in either experiment) was not influenced by exposure to parasites. We predicted that killifish would move towards the tank bottom to avoid cercariae that might have become aggregated near the surface. However, the small volume of water used in the tanks for individual exposures could have prevented cercariae from becoming stratified in the water, which could help explain why killifish did not alter their vertical position. Despite the larger volume of water in the tanks used for group exposures (potentially allowing for stratification of cercariae in the water), killifish also did not alter their vertical position during parasite exposure. This suggests that this might not be an effective behavioral defense against parasites. In addition, we were unable to examine cercarial distribution in the water. Therefore, before dismissing

vertical position as an anti-parasite behavioral defense of killifish, a thorough examination of cercarial distribution in the water is warranted.

Contrary to our prediction, group size did not increase during exposure to parasites despite several studies documenting its efficacy in decreasing an individual's risk of infection. The fishes' heightened activity levels and the increased frequency of PDBs displayed during parasite exposure could have caused fish to become dispersed. Thus, leading to smaller groups.

Although our primary goal was to examine the effect of parasite exposure on killifish behavior, we also have evidence of behavior manipulation. Previous infection by EUHA led to a higher frequency of 'conspicuous behaviors' and activity before exposure to parasites. Interestingly, so did SMCY-infected killifish, providing the first evidence of behavior modification and potential parasite increased trophic transmission for this parasite species. Our results also show that despite this behavior manipulation, killifish behavioral defenses remain intact. Hence, *exposure* to parasites and not just *infection* can influence killifish behavior and such behavioral changes can have significant implications for ecological interactions. For instance, energy allocated for behavioral defense against parasites is energy that killifish might otherwise have used for other functions such as foraging, mating or anti-predator vigilance. In fact, killifish displaying larger numbers of PDBs and higher activity levels could potentially increase their conspicuousness to bird predators and situations as this pose interesting ecological scenarios for future killifish research.

REFERENCES

Atkinson, E. M.., Bateman, A.W., Dill, L.M., Krkošek, M., Reynolds, J.D., and Godwin, S.C. 2018. "Oust the Louse: Leaping Behaviour Removes Sea Lice from Wild Juvenile Sockeye Salmon *Oncorhynchus nerka*." *Journal of Fish Biology*, 93 (2): 263-71. doi:10.1111/jfb.13684.

Baker, R.L., and Smith, B.P. 1996. "Conflict between antipredator and antiparasite behaviour in larval damselflies. *Oecologia*, 109: 622–628.

Barber, I., and Huntingford, F.A. 1996. "Parasite Infection Alters Schooling Behaviour: Deviant Positioning of Helminth-Infected Minnows in Conspecific Groups." *Proceedings of the Royal Society B: Biological Sciences*, 263 (1374): 1095–1102. https://doi.org/10.1098/rspb.1996.0161.

Barber, I., Downey, L.C., and Braithwaite, V.A. 1998. "Parasitism, Oddity and the Mechanism of Shoal Choice." *Journal of Fish Biology*, 53 (6): 1365–68. https://doi.org/10.1006/jfbi.1998.0788.

Bartoń, K. 2019. MuMIn: Multi-Model Inference. R package version 1.43.6. https://CRAN.R-project.org/package=MuMIn.

Bates, D., Maechler, M., Bolker, B., Walker, S. 2015. "Fitting Linear Mixed-Effects Models Using lme4". *Journal of Statistical Software*, 67 (1), 1-48. doi:10.18637/jss.v067.i01.

Behringer, D.C., Karvonen, A., and Bojko, J. 2018. "Parasite Avoidance Behaviours in Aquatic Environments." *Philosophical Transactions of the Royal Society B: Biological Sciences*, 373 (1751). https://doi.org/10.1098/rstb.2017.0202.

Berthe, C., Lecchini, D. and Mourier, J. 2017. "Chafing Behavior on a Patch of Sandy Bottom by Ocellated Eagle Ray (*Aetobatus Ocellatus*)." *Marine Biodiversity*, 47 (2): 379–80. https://doi.org/10.1007/s12526-016-0463-8.

Birkeland, K., and Jakobsen, P.J. 1997. "Salmon Lice, *Lepeophtheirus salmonis*, Infestation as a Causal Agent of Premature Return to Rivers and Estuaries by Sea Trout, *Salmo trutta*, Juveniles." *Environmental Biology of Fishes*, 49 (1): 129–37. https://doi.org/10.1023/A:1007354632039.

Bolker, B.M., Brooks, M.E., Clark, C.J., Geange, S.W., Poulsen, J.R., Stevens, M.H.H., and White, J.S.S. 2009. "Generalized Linear Mixed Models: A Practical Guide for Ecology and Evolution." *Trends in Ecology and Evolution*, 24 (3): 127–35. https://doi.org/10.1016/j.tree.2008.10.008.

Brooks, M.E., Kristensen, K., van Benthem, K.J., Magnusson, A., Berg, C.W., Nielsen, A, Skaug, H.J., Maechler, M, and Bolker, B.M. 2017. "Modeling Zero-Inflated Count Data With GlmmTMB." *BioRxiv*, 132753. https://doi.org/10.1101/132753.

Brooks, M.E., Kristensen, K., van Benthem, K.J., Magnusson, A., Berg, C.W., Nielsen, A., Skaug, H.J., Maechler, M., and Bolker, B.M. 2017. "glmmTMB Balances Speed and Flexibility Among Packages for Zero-inflated Generalized Linear Mixed Modeling". *The R Journal*, 9 (2), 378-400.

Bui, S., Halttunen, E., Mohn, A.M., Vågseth, T., and Oppedal, F. 2018. "Salmon Lice Evasion, Susceptibility, Retention, and Development Differ amongst Host Salmonid Species." *ICES Journal of Marine Science,* 75 (3): 1071–79. https://doi.org/10.1093/icesjms/fsx222.

Bui, S., Oppedal, F., Samsing, F., and Dempster, T. 2017. "Behaviour in Atlantic Salmon Confers Protection against an Ectoparasite." *Journal of Zoology*, 304 (1): 73–80. https://doi.org/10.1111/jzo.12498.

Burnham, K.P., and Anderson, D.R. 2002. "Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach (2nd Ed)". *Ecological Modelling,* Vol. 172. https://doi.org/10.1016/j.ecolmodel.2003.11.004.

Cox, D. R., and Snell, E. J. 1968. "A General Definition of Residuals." *Journal of the Royal Statistical Society*, 30 (2): 248–75.

Delcourt, J., and Poncin, P. 2012. "Shoals and Schools: Back to the Heuristic Definitions and Quantitative References." *Reviews in Fish Biology and Fisheries,* 22 (3): 595–619. https://doi.org/10.1007/s11160-012-9260-z.

Dunn, P.K., and Smyth, G.K. 1996. "Randomized Quantile Residuals." *Journal of Computational and Graphical Statistics,* 5 (3): 236–44. https://doi.org/10.1080/10618600.1996.10474708.

Fritz, E.S. 1975. "The Life History of the California Killifish (*Fundulus Parvipinnis* Girard) in Anaheim Bay, California." *Fish Bulletin,* 165 (165): 91–106. http://www.escholarship.org/uc/item/5vt2q1wk.

Genna, R.L., Mordue, W., Pike, A.W., and Mordue (Luntz), A.J. 2005. "Light Intensity, Salinity, and Host Velocity Influence Presettlement Intensity and Distribution on Hosts by Copepodids of Sea Lice, *Lepeophtheirus Salmonis*." *Canadian Journal of Fisheries and Aquatic Sciences,* 62 (12): 2675–82. https://doi.org/10.1139/f05-163.

Grossman, A., Sazima, C., and Sazima, I. 2009. "Rub and Move: Barracudas (*Sphyraena Barracuda*) Use Swimming Turtles as Scraping Surfaces in the South-Western Atlantic." *Marine Biodiversity Records,* 2: 2–4. https://doi.org/10.1017/S1755267209001237.

Hart, B.L. 1992. “Behavioral Adaptations to Parasites: An Ethological Approach.” *The Journal of Parasitology,* 78 (2): 256–65. https://doi.org/10.1016/S0149-7634(05)80038-7.

Hartig, F. 2019. “*DHARMa: “*Residual Diagnostics for Hierarchical (Multi-Level / Mixed) Regression Models.” R package version 0.2.4. https://CRAN.R-project.org/package=DHARMa.

Hechinger, R.F., Lafferty, K.D., Huspeni, T.C., Brooks, A.J., and Kuris, A.M. 2007. “Can Parasites Be Indicators of Free-Living Diversity? Relationships between Species Richness and the Abundance of Larval Trematodes and of Local Benthos and Fishes.” *Oecologia,* 151 (1): 82–92. https://doi.org/10.1007/s00442-006-0568-z.

Helfman, G.S., Collette, B.B., Facey, D.E., and Bowen, B.W. 2009. “*The Diversity of Fishes*.” Chichester: Wiley-Blackwell.

Hubbs, C., and Strawn, K. 1956. "Interfertility Between Two Sympatric Fishes, *Notropis Lutrensis* and *Notropis Venustus*." *Evolution,* 10 (4): 341-44. doi:10.2307/2406995.

James, C. T., Noyes, K.J., Stumbo, A.D., Wisenden, B.D., and Goater, C.P. 2008. “Cost of Exposure to Trematode Cercariae and Learned Recognition and Avoidance of Parasitism Risk by Fathead Minnows *Pimephales Promelas*.” *Journal of Fish Biology*, 73 (9): 2238–48. https://doi.org/10.1111/j.1095-8649.2008.02052.x.

Kiesecker, J.M., Skelly, D.K. 2000. “Choice of Oviposition Site by Gray Treefrogs : The Role of Potential Parasitic Infection.” *Ecology,* 81 (10): 2939–439.

Klemme, I., and Karvonen, A. 2016. “Learned Parasite Avoidance Is Driven by Host Personality and Resistance to Infection in a Fish-Trematode Interaction.” *Proceedings of the Royal Society B: Biological Sciences*, https://doi.org/10.1098/rspb.2016.1148.

Koprivnikar, J., and Penalva, L. 2015. “Lesser of Two Evils? Foraging Choices in Response to Threats of Predation and Parasitism.” *PLoS ONE,* 10 (1): 1–11. https://doi.org/10.1371/journal.pone.0116569.

Koprivnikar, J., Forbes, M.R., and Baker, R.L. 2006. “On the Efficacy of Anti-Parasite Behaviour: A Case Study of Tadpole Susceptibility to Cercariae of *Echinostoma Trivolvis*.” *Canadian Journal of Zoology,* 84 (11): 1623–29. https://doi.org/10.1139/z06-158.

Koprivnikar, J., Gibson, C.H., and Redfern, J.C. 2012. “Infectious Personalities: Behavioural Syndromes and Disease Risk in Larval Amphibians.” *Proceedings of the Royal Society B: Biological Sciences,* 279 (1733): 1544–50. https://doi.org/10.1098/rspb.2011.2156.

Koprivnikar, J., Redfern, J.C., and Mazier, H.L. 2014. “Variation in Anti-Parasite Behaviour and Infection among Larval Amphibian Species.” *Oecologia,* 174 (4): 1179–85. https://doi.org/10.1007/s00442-013-2857-7.

Krause, J., and Godin, J.G.J. 1994. “Influence of Parasitism on the Shoaling Behaviour of Banded Killifish, *Fundulus Diaphanus*.” *Canadian Journal of Zoology,* 72 (10): 1775–79. https://doi.org/10.1139/z94-240.

Krause, J., and Ruxton, G.D. 2002. *Living in Groups*. Oxford: Oxford University Press.

Kuznetsova A., Brockhoff, P.B., Christensen, R.H.B. 2017. “lmerTest Package: Tests in Linear Mixed Effects Models.” *Journal of Statistical Software*, 82 (13): 1-26. doi: 10.18637/jss.v082.i13.

Lafferty, K.D., and Morris, K.A. 1996. “Altered Behavior of Parasitized Killifish Increases Susceptibility to Predation by Bird Final Hosts.” *Ecology*, 77 (5): 1390–97. 10.2307/2265536

Lowenberger, C.A., and Rau, M.E. 1994. “Selective Oviposition by *Aedes Aegypti* (Diptera: Culicidae) in Response to a Larval Parasite, *Plagiorchis Elegans* (Termatoda: Plagiorchiidae).” *Environmental Entomology*, 23 (5): 1269-76.

Martin, W. E. 1972. An annotated key to the cercariae that develop in the snail *Cerithidea californica*. *Bulletin of the Southern California Academy of Sciences,* 71: 39–43.

Martin, W. E. 1950. "*Euhaplorchis californiensis* N.g., N. Sp., Heterophyidae, Trematoda, with Notes on Its Life-Cycle." *Transactions of the American Microscopical Society,* 69 (2): 194-209. doi:10.2307/3223410.

McCullagh, P, and Nelder, J.A. 1989. *Generalized Linear Models*. Chapman and Hall.

Mikheev, V.N., and Pasternak, A.F. 2006. “Defense Behavior of Fish against Predators and Parasites.” *Journal of Ichthyology*, 46: S173–79. https://doi.org/10.1134/S0032945206110063.

Mikheev, V.N., Pasternak, A.F., Taskinen, J., and Valtonen, T.E. 2013. "Grouping Facilitates Avoidance of Parasites by Fish." *Parasites & Vectors*, 6 (1): 1–8 doi:10.1186/1756-3305-6-301.

Mohammed, R.S., Reynolds, M., James, J., Williams, C., Mohammed, A., Ramsubhag, A., van Oosterhout, C. and Cable, J. 2016. “Getting into Hot Water: Sick Guppies Frequent Warmer Thermal Conditions.” *Oecologia*, 181 (3): 911–17. https://doi.org/10.1007/s00442-016-3598-1.

Moore, J. 2002. *Parasites and the Behavior of Animals.* Oxford: Oxford University Press.

Papastamatiou, Y.P., Meyer, C.G., and Maragos, J.E. 2007. “Sharks as Cleaners for Reef Fish.” *Coral Reefs*, 26 (2): 277. https://doi.org/10.1007/s00338-007-0197-y.

Poulin, R., and FitzGerald, G.J. 1989. “Risk of Parasitism and Microhabitat Selection in Juvenile Sticklebacks.” *Canadian Journal of Zoology* 67, (1): 14–18. https://doi.org/10.1139/z89-003.

Poulin, R., and FitzGerald, G. J. 1989. “Shoaling as an Anti-Ectoparasite Mechanism in Juvenile Sticklebacks (*Gasterosteus* Spp.).” *Behavioral Ecology and Sociobiology,* 24 (4): 251–55. https://doi.org/10.1007/BF00295205.

Poulin, R., Rau, M.E., and Curtis, M.A. 1991. “Infection of Brook Trout Fry, *Salvelinus Fontinalis*, by Ectoparasitic Copepods: The Role of Host Behaviour and Initial Parasite Load.” *Animal Behaviour,* 41 (3): 467–76. https://doi.org/10.1016/S0003-3472(05)80849-8.

Quinn, G.P. and Keough, M.J. 2002. *Experimental Design and Data Analysis for Biologists.* Cambridge University Press.

Richards, E.L, van Oosterhout, C., and Cable, J. 2010. “Sex-Specific Differences in Shoaling Affect Parasite Transmission in Guppies.” *PLoS ONE,* 5 (10): 1–6. https://doi.org/10.1371/journal.pone.0013285.

Ritter, E.K. 2011. “Use of Sand Ripples to Enhance Chafing in Caribbean Reef Sharks (*Carcharhinus perezi*) and Blacktip Sharks (*Carcharhinus limbatus*).” *Bulletin of Marine Science,* 87 (3): 413–19. https://doi.org/10.5343/bms.2010.1082.

Rohr, Jason R., Autumn Swan, Thomas R. Raffel, and Peter J. Hudson. 2009. “Parasites, Info-Disruption, and the Ecology of Fear.” *Oecologia* 159 (2): 447–54. https://doi.org/10.1007/s00442-008-1208-6.

Shaw, J.C, Korzan, W.J., Carpenter, R.E., Kuris, A.M., Lafferty, K.D., Summers, C.H., and Overli. O. 2009. “Parasite Manipulation of Brain Monoamines in California Killifish (*Fundulus Parvipinnis*) by the Trematode *Euhaplorchis Californiensis*.” *Proceedings of the Royal Society B: Biological Sciences,* 276 (1659): 1137–46. https://doi.org/10.1098/rspb.2008.1597.

Stumbo, A.D., James, C.T., Goater, C.P., and Wisenden, B.D.. 2012. “Shoaling as an Antiparasite Defence in Minnows (*Pimephales Promelas*) Exposed to Trematode Cercariae.” *Journal of Animal Ecology,* 81 (6): 1319–26. https://doi.org/10.1111/j.1365-2656.2012.02012.x.

Symonds, M.R.E., and Moussalli, A. 2011. “A Brief Guide to Model Selection, Multimodel Inference and Model Averaging in Behavioural Ecology Using Akaike’s Information Criterion.” *Behavioral Ecology and Sociobiology,* 65 (1): 13–21. https://doi.org/10.1007/s00265-010-1037-6.

Szuroczki, D., and Richardson, J.M.L. 2012. “The Behavioral Response of Larval Amphibians (Ranidae) to Threats from Predators and Parasites.” *PLoS ONE,* 7 (11). https://doi.org/10.1371/journal.pone.0049592.

Taylor, C.N, Oseen, K.L., and Wassersug, R.J. 2004. "On the Behavioural Response of *Rana* and *Bufo* Tadpoles to Echinostomatoid Cercariae: Implications to Synergistic Factors Influencing Trematode Infections in Anurans." *Canadian Journal of Zoology,* 82 (5): 701–6. https://doi.org/10.1139/z04-037.

Thiemman, G.W., and Wassersug, R.J. 2000. "Patterns and Consequences of Behavioral Responses to Predators and Parasites in Rana Tadpoles." *Biological Journal of the Linnaean Society,* 71 (3): 513-28. Doi: 10.1111/j.1095-8312.2000.tb01272.x.

Wagenmakers, EJ.,, and Farrell, S. 2004. "AIC Model Selection Using Akaike Weights." *Phychonomic Bulletin and Review,* 11 (1): 192–96. https://doi.org/10.1021/ef300604q.

Warton, D.I., and Hui, F.K.C. 2011. "The Arcsine Is Asinine: The Analysis of Proportions in Ecology." *Ecology,* 92 (2): 276–81. https://doi.org/10.1016/0021-9797(67)90004-5.

Weinersmith, K.L., Brown, C.E., Clingen, K.B., Jacobsen, M.C., Topper, L. B., and Hechinger, R.F. 2018. "*Euhaplorchis Californiensis* Cercariae Exhibit Positive Phototaxis and Negative Geotaxis." *Journal of Parasitology,* 104 (3): 329–33. https://doi.org/10.1645/17-80.

Wisenden, B.D., Goater, C.P. and James, C.T. 2009. "Behavioral defenses against parasites and Pathogens." In *Fish Defenses: Pathogens, Parasites and Predators,* (eds. C. Zaccone, A. Mathis, and G. Kapoor). USA: Science Publisher.

Wyman, R.L., and Walters-Wyman, M.F. 1985. "Chafing in Fishes: Occurrence, Ontogeny, Function and Evolution." *Environmental Biology of Fishes,* 12 (4): 281–89. https://doi.org/10.1007/BF00005458.

Zuur, A.F., Ieno, E.N., and Elphick, C.S. 2010. "A Protocol for Data Exploration to Avoid Common Statistical Problems." *Methods in Ecology and Evolution,* 1 (1): 3–14. https://doi.org/10.1111/j.2041-210X.2009.00001.x.