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## EXPERIMENTAL INFECTIONS WITH *EUHAPLORCHIS CALIFORNIENSIS* AND A SMALL CYATHOCOTYLID INCREASE CONSPICUOUS BEHAVIORS IN CALIFORNIA KILLIFISH (*FUNDULUS PARVIPINNIS*)

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### KEY WORDS ABSTRACT

*Fundulus parvipinnis*  
California killifish  
*Euhaplorchis californiensis*  
Parasite manipulation  
Trophic transmission  
Conspicuous behaviors  
Darting  
Dashing  
Scratching  
Flashing  
Surfacing  
Host behavior

Some parasites manipulate their host's phenotype to enhance predation rates by the next host in the parasite's life cycle. Our understanding of this parasite-increased trophic transmission is often stymied by study-design challenges. A recurring difficulty has been obtaining uninfected hosts with a coevolutionary history with the parasites, and conducting experimental infections that mimic natural processes. In 1996, Lafferty and Morris provided what has become a classic example of parasite-increased trophic transmission; they reported a positive association between the intensity of a brain-infecting trematode (*Euhaplorchis californiensis*) in naturally infected California killifish (*Fundulus parvipinnis*) and the frequency of conspicuous behaviors, which was thought to explain the documented 10–30× increase in predation by the final host birds. Here, we address the primary gap in that study by using experimental infections to assess the causality of *E. californiensis* infection for increased conspicuous behaviors in *F. parvipinnis*. We hatched and reared uninfected *F. parvipinnis* from a population co-occurring with *E. californiensis*, and infected them 1–2 times/week over half their life span with *E. californiensis* and a small cyathocotylid trematode (SMCY) that targets the host's muscle tissue. At 3 time points throughout the hosts' lives, we quantified several conspicuous behaviors: contorting, darting, scratching, surfacing, and vertical positioning relative to the water's surface. *Euhaplorchis californiensis* and SMCY infection caused 1.8- and 2.5-fold overall increases in conspicuous behaviors, respectively. Each parasite was also associated with increases in specific conspicuous behaviors, particularly 1.9- and 1.4-fold more darting. These experimental findings help solidify *E. californiensis*–*F. parvipinnis* as a classic example of behavioral manipulation. Yet our findings for *E. californiensis* infection-induced behavioral change were less consistent and strong than those previously documented. We discuss potential explanations for this discrepancy, particularly the idea that behavioral manipulation may be most apparent when fish are actively attacked by predators. Our findings concerning the other studied trematode species, SMCY, highlight that trophically transmitted parasites infecting various host tissues are known to be associated with conspicuous behaviors, reinforcing calls for research examining how communities of trophically transmitted parasites influence host behavior.

Parasite manipulation of host phenotype occurs when parasites cause changes in host physiology, morphology, or behavior in ways that enhance the parasite's fitness (Moore, 2002; Poulin, 2010; Hughes et al., 2012; Poulin and Maure, 2015; Weinersmith, 2019). Parasite-increased trophic transmission is a specific category of manipulation wherein parasites modify the host phenotype to increase the current host's risk of predation by the next

host in the life cycle (Lafferty, 1999). For example, *Gammarus pulex* amphipods infected by the trophically transmitted acanthocephalan *Pomphorhynchus tereticollis* are less likely to use a refuge in the presence of a fish predator and are more attracted to the fish predator's olfactory cues than uninfected amphipods, which likely explains why infection prevalence characterizing amphipods taken from the predator hosts' stomachs was 10 times

higher than the infection prevalence characterizing free-ranging amphipods (Perrot-Minnot et al., 2007). Such increased predation rates appear to be the typical case for trophically transmitted parasites, as indicated by a recent meta-analysis (Hasik et al., 2023). Therefore, trophically transmitted parasites may generally have important ecological effects, including strengthening predator–prey links within food webs, creating new habitats, and modifying the flow of energy among habitats (Lafferty, 2008; L efevre et al., 2008; Lafferty and Kuris, 2012).

Unfortunately, clear evidence of adaptive host manipulation remains limited because of a suite of methodological challenges. For instance, naturally infected hosts are often used to study associations between infection and host behavior (Poulin and Maure, 2015). This method can make it difficult to assign manipulation to a specific parasite, as hosts in the wild are often infected by multiple parasite species (Viney and Graham, 2013; C ezilly et al., 2014). Further, using naturally infected hosts makes it difficult to determine the direction of causality, as certain behavioral phenotypes can put hosts at greater risk for infection in the first place (e.g., Koprivnikar et al., 2012). That is, host behavior can either be a *cause* or a *consequence* of infection (Barber et al., 2017). Experimental infections are considered instrumental to confirm host manipulation, yet only 10% of recent empirical studies on trophically transmitted helminths employed experimental infections, likely because of logistical constraints in maintaining both host and parasite life cycles in the lab (Poulin and Maure, 2015).

Although experimental infections may be a vital step towards confirming parasitic manipulation of host behavior, experimental procedures can still suffer if they fail to mimic key elements of natural infection processes. For example, in many host–parasite systems, hosts acquire increasing numbers of parasites over the course of their lives. However, limited resources for research (e.g., time, funding, space, husbandry limitations) make a single, large-dose infection more tractable than repeated experimental infections throughout host ontogeny. Indeed, such single, large-dose infections are common, for example, in experimental studies examining trematode manipulation of fish behavior and physiology (e.g., Shaw et al., 2009; Klemme et al., 2016; Renick et al., 2016; Gopko et al., 2017). This may have important implications for studies examining changes in host phenotype following infection. For example, in vertebrate hosts, although infection is associated with increases in glucocorticoid stress hormones, the magnitude of this response is frequently influenced by the time since the initial infection (O’Dwyer et al., 2020). Thus, a single, large-dose infection could affect host physiology and behavior differently than the more natural accumulation of separate infections over weeks, months, or even years.

Another possible problem that besets some experimental studies occurs when infections are performed on uninfected hosts from populations that do not co-occur with the focal parasite (e.g., Shaw et al., 2009; Hernandez and Fredensborg, 2015; Renick et al., 2016), often because it can be difficult to collect uninfected hosts from populations co-occurring with the parasite. Because of local adaptation or coevolution, infecting hosts from naive populations could provide erroneous estimates of the type and magnitude of manipulation. For instance, *Gammarus pulex* hosts from populations that do not co-occur with *Pomphorhynchus laevis* are more susceptible to the acanthocephalan parasite’s manipulation of phototaxis than are hosts that co-occur with the parasite (Franceschi et al., 2010). Hence, experimental studies

should capture these basic aspects of natural infection processes to understand how parasites manipulate host behavior in the wild.

Even some of the classic and most highly cited studies in this field suffer from these limitations. One particularly well-known example of parasite manipulation of host behavior leading to increased trophic transmission involves the brain-infecting trematode *Euhaplorchis californiensis* and its California killifish (*Fundulus parvipinnis*) host (Martin, 1950). This trophically transmitted parasite appears to increase the frequency of conspicuous behaviors in its host fish to increase predation rates by final-host bird predators (Lafferty and Morris, 1996). As 1 of the few examples connecting apparent behavior modification to increased predation rates, Lafferty and Morris (1996) has accumulated over 635 citations in Google Scholar as of 2023, and this example of host manipulation is frequently featured in popular press articles and books (e.g., Zimmer, 2000; McAuliffe, 2017). However, the authors noted methodological shortcomings and urged follow-up work to confirm the results.

Here, we address the above study-design limitations and provide experimental evidence that *E. californiensis* infection causes an increased frequency of conspicuous behaviors in *F. parvipinnis*. By raising *F. parvipinnis* from fertilized eggs in the lab, we were able to conduct repeated experimental infections on fish having an evolutionary history with *E. californiensis*. To understand the unique nature of the effects of the brain-infecting *E. californiensis* on its host better, we also included experimental infection by another, naturally co-occurring trematode parasite that infects muscle and connective tissue. In addition to providing evidence for behavior modification by the newly studied parasite, our results enhance the consideration of the *E. californiensis*–*F. parvipinnis* system as a classic example of parasite manipulation of host behavior to increase trophic transmission.

## MATERIALS AND METHODS

### Study system

*Euhaplorchis californiensis* (Plagiorchiida: Heterophyidae) is a trematode parasite with a typical 3-host life cycle (Martin, 1950). Its first intermediate host is the California horn snail (*Cerithideopsis californica*), from which free-swimming stages (cercariae) emerge to seek out and infect the second intermediate host, *F. parvipinnis*. The parasite forms cysts (metacercariae) on and in the brain’s meningeal surface (Shaw et al., 2009; Helland-Riise et al., 2020a). In populations where *F. parvipinnis* co-occurs with *E. californiensis*, 100% of the adult population is often infected with the parasite, with infection intensities frequently exceeding 1,000 and composing 0.5–1.7% of the total mass of the fish (Shaw et al., 2010). *Euhaplorchis californiensis* transmits from *F. parvipinnis* when infected fish are eaten by the definitive host, a fish-eating marsh bird (Martin, 1950). The life cycle continues when parasite eggs pass with the bird’s excreta back into the marsh and are consumed by horn snails.

Lafferty and Morris (1996) collected uninfected *F. parvipinnis* from a population lacking *E. californiensis* and naturally parasitized *F. parvipinnis* from a different, nearby population. The fish exhibited the conspicuous behaviors noted in Table I and infected fish displayed 4 times more conspicuous behaviors overall than uninfected fish. *Euhaplorchis californiensis* intensity was most

**Table 1.** Definitions of discrete conspicuous behaviors used in our study and Lafferty and Morris (1996). Asterisks indicate the 2 behaviors most closely associated with *Euhaplorchis californiensis* infection in Lafferty and Morris (1996).

Conspicuous behaviors	Definition
Contort	An acute lateral bending of the fish's body, resulting in the formation of a C or an S shape when viewed from above. The bend involves both head and tail, and the fish's body snaps back to being unbent following the contortion. Lafferty and Morris (1996, p. 1392): "...contorting fish performed a slow, acute, dorsal-ventral [sic] bending, usually bending the head and tail in opposite directions..."
Dart	Fish suddenly and rapidly moves forward a distance of at least 1 body length, but no more than a distance equal to three-fourths the height of the tank. The fish then abruptly slows down. Directional changes middash counted as a new dash. Lafferty and Morris (1996, p. 1392): "...jerking fish moved suddenly forward 3-5 cm."
Flash/scratch	Fish moves forward quickly and turns laterally, so that 1 side of the body faces upward, often resulting in a conspicuous silvery flash of light. When a flash occurs while scraping the body against a hard surface, it is "scratching" or "chafing." As there were very few pure flashes, we combined those with scratches in our analyses, consistent with Lafferty and Morris (1996). Lafferty and Morris (1996; p. 1391-1392): "...flashing fish turned laterally so that 1 side of the body faced upward (often associated with chafing on the tank's bottom)..."
Surface*	The fish's body makes contact with the water surface. A new surfacing event is only counted if the fish moves down from the surface of the water by at least half a body length, and then returns to the surface. Lafferty and Morris (1996, p. 1391): "Surfacing fish made abrupt dashes up to the tank's surface..."
Shimmy	This behavior was observed in Lafferty and Morris (1996) and was correlated with infection by a different parasite ( <i>Renicola buchanaui</i> ). As shimmying was observed only once in our videos, it was not included in our analyses. Lafferty and Morris (1996, p. 1392): "...shimmying fish vibrated for a few seconds..."
Sum*	For both studies, "Sum" is calculating by adding up the conspicuous behaviors observed for a particular fish.

strongly associated with the frequency of surfacing, and the intensity of a liver-infecting trematode species (*Renicola buchanaui*) had a greater correlation with darting and shimmying. *Euhaplorchis californiensis* intensity is also correlated with the sum of all conspicuous behaviors (Lafferty and Morris, 1996). A key additional experiment placed naturally infected and uninfected *F. parvipinnis* from different populations into outdoor pens (1 open to predatory birds, the other not) and found that the parasitized fish were 10-30 times more likely to be consumed by the birds (Lafferty and Morris, 1996). The study also provided evidence that fish-population effects did not confound the apparent behavior modification by documenting intensity dependence within the naturally infected fish population of both the conspicuous behaviors and the probability of being eaten by a bird for *E. californiensis*.

Despite Lafferty and Morris (1996) providing compelling evidence for parasite manipulation of host behavior to facilitate trophic transmission, the use of naturally infected and uninfected fish precludes confirmation of the direction of causality for parasitism and conspicuous behaviors. The use of naturally infected fish also prevents the clear correlation of any detected behavioral effects to *E. californiensis* vs. other, nonenumerated, co-infecting parasites. These limitations are what motivated us to perform a study using experimental exposures of fish.

Further, to understand the *F. parvipinnis* response to trematode infection better, we also quantified its behavior following infection by a locally common cyathocotylid trematode. This species, small cyathocotylid (SMCY; Diplostomida: Cyathocotylidae), has a provisional description in Hechinger (2019) and is currently being more fully described (Nelson, 2021). SMCY has a very similar life cycle to *E. californiensis*, with the exception that it can infect other teleost fish in addition to *F. parvipinnis* (see known host list in Hechinger et al., 2011) and is found in skeletal muscles, fins, and connective tissue (R.F.H., unpubl. experimental exposure data).

Based on the Lafferty and Morris (1996) findings, and those of Fredensborg and Longoria (2012) and Hernandez and Fredensborg (2015) on a related *Euhaplorchis* species and its fish hosts, we

predicted that *F. parvipinnis* infected with *E. californiensis* would exhibit a greater frequency of surfacing and other conspicuous behaviors, and would spend more time near the water surface, than uninfected *F. parvipinnis*. Because *Fundulus*-infecting trematode species residing outside of the central nervous system are also associated with conspicuous behaviors (Lafferty and Morris, 1996; Bass and Weis, 2009), and because SMCY is trophically transmitted to the same hosts as *E. californiensis*, we predicted that it too would increase host conspicuous behaviors. However, we hypothesized that different infection sites and phylogenetic grouping might lead it to affect a different set of conspicuous behaviors than *E. californiensis*.

This study was part of a larger project examining the influence of *E. californiensis* on *F. parvipinnis* physiology, neurobiology, and behavior (Helland-Riise et al., 2020a, 2020b; Nadler et al., 2021). All vertebrate animal procedures were approved by the University of California, San Diego IACUC Committee (protocol S16120).

### Study population and rearing

*Fundulus parvipinnis* in this experiment were the lab-reared offspring of adults collected from a population co-occurring with *E. californiensis*. Mature adult fish were collected across multiple days in July and August 2016 by 2-pole seine from San Elijo Lagoon Ecological Reserve (33.01°N, 117.26°W) in Cardiff, San Diego County, California. Gametes were expelled from the fish by gently running a finger along the fish's abdomen, following methods in Strawn and Hubbs (1956). Eggs and sperm of over 100 males and over 30 females were mixed in 39 petri dishes in the field, rinsed in fresh seawater, and brought to Scripps Institution of Oceanography (SIO) in La Jolla, California, rinsed with filtered seawater, and placed in 100-mm-diameter glass bowls containing 200 ml filtered seawater and an aerator. Eggs were treated with methylene blue (0.0003%) as needed to prevent fungal growth. Eggs were maintained on a light:dark cycle that mirrored



local conditions. Dead or unfertilized eggs were removed daily, and water changes were done every other day.

Twenty-four, 38-L glass tanks ( $51 \times 27 \times 32$  cm) were placed on both sides of a 3-level rack in an SIO experimental aquarium room. Black curtains attached to the racks prevented activities in the room from visually disturbing the fish. Fry originating from fertilizations of the same month (i.e., in July or in August) were housed together. Because those same-month fry hatched across a range of dates, we distributed them among tanks so that each contained a roughly equal number of early-hatching and late-hatching fish until each tank housed approximately 20 fish. Fish were fed live, recently hatched brine shrimp for approximately 12 wk, and were transitioned to a diet of Skretting aquaculture feed, frozen then thawed blood worms, and mashed peas. Tanks received a continuous trickle of seawater, and once daily were also flushed with seawater, simulating the incoming tide. We maintained ambient water temperatures (18 C in winter, 21 C in summer) and light cycle (from 11:13 light: dark cycle in winter to 13:11 light: dark cycle in summer).

A subset of the tanks experienced chronic high mortality, the cause of which could not be determined. Specimens from a subset of these tanks were sent to 2 fish pathology labs, and our lab conducted examinations for metazoan and protozoan parasites, yet no pathogen could be identified as the cause of mortality. Water-quality parameters were within normal ranges, and fish in other tanks were housed in water from the same source, yet remained healthy. To avoid measuring the behavior of unwell fish, we removed from behavioral analyses any tank that experienced more than 1 fish death per month from November 2016 through April 2017. We retained 1 tank that violated this rule when fish were 6 mo old, as the mortalities all occurred on 1 night and were consistent with a 1-time, acute environmental stressor (likely a problem with the air line). Because of this issue, we had fewer tanks for each treatment than originally anticipated, and we ceased behavioral observation in May 2017 when additional tanks failed to meet our criteria for remaining in the study. To avoid overfitting statistical models, we pooled tanks of fish hatched from the July and August collections. We also pooled tanks that received low and high *E. californiensis* doses into 1 *E. californiensis* treatment group. This approach is analogous to examining a wild *F. parvipinnis* population, where birth would have occurred throughout the summer, and infection intensities would range from low to high. In March and April 2017, a total of 4, 6, and 4 tanks were included for the control, *E. californiensis*, and small cyathocotylid treatments, respectively. However, in November 2016, there were only 5 *E. californiensis* tanks and 2 small cyathocotylid tanks, as 3 cameras malfunctioned on this observation date.

### Experimental infections

We conducted experimental infections in the housing tanks beginning in September 2016 (when the fish were ~1 mo old) and continuing throughout the experiment. We obtained cercariae from naturally infected *C. californica* from the University of California Kendall–Frost Natural Reserve in Mission Bay, San Diego County, California (32.80°N, 117.23°W). This locality is nearby (~25 km) from the source locality of our experimental fish, San Elijo Lagoon. The parasites and fish occur at both localities, and we confirmed that Kendall–Frost *E. californiensis* could readily infect San Elijo *F. parvipinnis* (Nadler et al., 2021). We note that

we had originally set about conducting our study using fish and infected snails from both localities. However, the substantial mortality of the Kendall–Frost experimental fish (described previously) and access limitations at San Elijo precluded us from obtaining a large enough supply of infected snails for experimental infections.

The details of our recently developed experimental infection protocol are available in Helland-Riise et al. (2020a), and in the Suppl. Data. Briefly, fish were infected with 1 of 4 parasite treatments: sham (seawater control), low-dose *E. californiensis*, high-dose *E. californiensis*, or SMCY. Tanks were numbered consecutively along the rows, and each of the 4 parasite treatments was randomly assigned to a tank in each set of 4 consecutively numbered tanks.

As the fish grew we increased the parasite exposure dose per fish. In the *E. californiensis* tanks, the low-dose treatment increased from ~1 to 100 cercariae per fish, and the high-dose treatment increased from ~3 to 310 cercariae per fish (Table S1). A range of 15–160 infected snails were shed on each infection day to acquire these cercariae. We completed 52 experimental infections with *E. californiensis* from the time when *F. parvipinnis* were ~1 mo old to the time when the fish were 8 mo old (the time of our last behavioral observations). The fish were not euthanized and dissected at this time because they were slated for use in additional experiments. However, use of this infection protocol on *F. parvipinnis* dissected at 10 mo old whose parents were captured at Kendall–Frost Natural Reserve yielded intensities of 815.8 ( $\pm 93.7$ ) for the low-dose treatment and 1,454.3 ( $\pm 214$ ) for the high-dose treatment, which is fairly close to the 1,880.8 ( $\pm 164.9$ ) observed in wild fish from this population (Helland-Riise et al., 2020a). Because of slow fish growth in the lab, the parasite densities achieved (i.e., the number of parasites per gram of fish mass) were high relative to similarly aged wild fish from a sample taken from the same wetland, with high-dose fish having an average density of 2,379 parasites/g whereas wild fish had an average density of only 382.9 parasites/g (Helland-Riise et al., 2020a). However, these densities are consistent with densities observed in other wetlands where *F. parvipinnis* and *E. californiensis* co-occur (R.F.H., unpubl. data). Additionally, Nadler et al. (2021) dissected 11 of the low-dose *E. californiensis*-infected fish used in this study at 13 mo old and found an average infection intensity of 1,190 metacercariae and an average density of 2,528 metacercariae per gram of fish. These values are similar to levels seen in comparably aged wild fish (Shaw et al., 2010). Nadler et al. (2021) also noted that 3 of the 11 *E. californiensis*-treatment fish harbored 1–2 SMCY metacercariae, whose cercariae had likely ended up in vials because of rare double-infected snails that went undetected during our screening procedures. It is implausible that such low levels of accidental infection could have had detectable impacts on our results because SMCY metacercariae are smaller than those of *E. californiensis*, they accounted for a negligible portion (<0.02%) of the metacercariae in those treatment fish, they only occurred in ~25% percent of the *E. californiensis*-treatment fish, and because those accidental intensities were ~2% of the average intensity of SMCY treatment fish (which themselves did not have strikingly strong behavioral changes—see Results).

Cercariae from the SMCY were less likely than *E. californiensis* cercariae to shed from the snails during the winter months, which is consistent with observed lower cercaria production during the winter months in the wild (Fingerut et al., 2003). We completed

21 experimental infections with this parasite, with doses that increased over time from ~3 cercariae per fish to 225 cercariae per fish as the fish grew (Table S1). Between 15 and 138 snails infected by the SMCY were shed during each experimental infection to ensure the cercariae used in all experimental infections maintained genetic heterogeneity. Dissections of 12 SMCY-infected fish, when the fish were 9 mo old, revealed infection intensities ranging from 0 to 120, with a mean of 52, which is well within the range of values observed in wild fish sampled throughout southern California and Baja California (R.F.H., unpubl. data).

The report on the dissection of 13 of the control fish used in this study at 13 mo by Nadler et al. (2021) found no trematode infections in these fish, which is consistent with the results of our necropsies performed on 22 control fish following lab mortality.

### Behavioral data collection

Behavior videos were recorded using Amcrest (Houston, Texas) ProHD 1080P (2.1MP) WiFi Security Cameras IP2M-841B (Black) mounted in front of each tank. Amcrest Pro software automatically recorded 30-min videos at predetermined times, allowing video recording without disturbing the fish. Videos were analyzed from 3 separate days, with 1 day each in November 2016, March 2017, and April 2017 (“observation dates”), when the fish were approximately 3, 7, and 8 mo old, respectively. For fish infected by *E. californiensis*, the most recent infection event preceding an observation day occurred 7, 4, and 1 day prior for the November, March, and April observation days, respectively. Infection timing was the same for SMCY-infected fish, except in April the prior infection event occurred 10 days before videos were analyzed; these fish had received sham infections 7 and 1 day before behavioral videos because the number of SMCY cercariae shed from snails on these days was insufficient to permit experimental infections. In the analyses described below, video analyzers were blind to parasite treatment.

At a young age, some of the fish across all treatment groups developed abnormalities associated with their swim bladders, in which bladders either filled but did not subsequently deflate or bladders deflated and did not subsequently refill. These fish could not stay neutrally buoyant but were otherwise healthy. They remained in the tanks to minimize disturbances to the other fish and to maintain consistent tank densities but were excluded from behavioral analyses.

### Discrete conspicuous behaviors quantification

Conspicuous behaviors were quantified in the videos taken 1 hr before feeding in the morning at 0830 hr on 1 day each in November 2016, and 0800 hr in March and April 2017. We tracked individual fish in a tank on each observation day by numbering them on an image-still created from the first frame of each video. We used Random.org to pick 10 fish randomly for behavior quantification. If a tank had 10 or fewer fish, all fish were analyzed. We were unable to track individuals across observation dates because these juvenile fish were too small to be fitted with individual tags large enough to read in a video. In addition to excluding the fish that were unable to stay neutrally buoyant, we excluded any fish that had a strand of feces that was not detached at the start of the

video, as fish often engaged in conspicuous behaviors in an effort to detach the strand.

We quantified the number of conspicuous behaviors displayed by each of the up to 10 fish per tank over 5 min. We tracked 4 discrete conspicuous behaviors: contorting, darting, flashing/scratching, and surfacing. Our definitions for these behaviors differ slightly from Lafferty and Morris (1996) (Table I) and reflect the most frequently observed conspicuous behaviors our fish displayed during preliminary observations.

All fish were analyzed twice to confirm behavioral quantification. The first time a set of trained student observers watched the videos and noted the times when they observed conspicuous behaviors. To ensure consistency, K.L.W. then repeated the analysis, using the student observations as a check against missing conspicuous behaviors, and assigned the category for each observed conspicuous behavior. During this second round of analysis, videos were analyzed by picking 1 tank at random from November 2016, 1 tank at random from March 2017, and then 1 tank at random from April 2017. The cycle was then repeated until all videos were reanalyzed. We completed 376 5-min observations. For control fish, 40 fish were analyzed for each observation date. For *E. californiensis* fish, data were collected on 50, 57, and 56 fish, and for SMCY data were collected on 20, 38, and 35 fish in November 2016, March 2017, and April 2017, respectively. Each combination of treatment and date encompassed a sample size that included 2–6 tanks (mean 4.3) and 20–57 fish (mean 41.8).

### Proportion near the water surface

To explore how infection impacts the vertical position of fish in a tank, we quantified the proportion of *F. parvipinnis* within 8 cm of the water surface. The top 8 cm region was marked by a line of white tape placed along the sides and back of the tank (another line of white tape was placed 10 cm below the first and was not used in this analysis). We collected image stills from times 0, 2.5, and 5 min. At each time point, we counted all fish in the tank and those in the top compartment. The number of fish in each tank that were unable to stay neutrally buoyant was determined during daily health checks and video recordings. These fish were identified at each time point, and the number residing in the top compartment was recorded as well.

This quantification was done for the March and April 2017 recording dates at 3 times of day: at midnight (“Night”; 0000 hr), sunrise (“Sunrise”; 0645 hr in March when the fish were 7 mo old, and 0615 hr in April when the fish were 8 mo old), and in the morning before feeding (“Morning”; as described for the conspicuous behavior quantification). We focused on these dates based on the assumption that behavioral changes would be more pronounced as fish acquired more parasites through repeated experimental infections relative to the November 2016 date. The lights in the room were off during the Sunrise and Night videos, and a red light on the cameras permitted seeing the fish. Shadowed corners in the bottom of the tank prohibited us from tracking individual fish and measuring conspicuous behaviors at these times (although we rarely observed conspicuous behaviors at night). However, because we could quantify vertical position after dark, we did so, as it seemed plausible that the trematodes could increase the likelihood of transmission to nocturnal predatory birds (e.g., black-crowned night herons, *Nycticorax nycticorax*) by manipulating its host into remaining near the water surface.

We calculated the proportion of fish near the water surface in the 3 image stills per tank at each of the 3 times of day on each of the 2 observation dates. This provided 72 image stills total for both the control and SMCY treatments (which had 4 tanks each) and 108 image stills total for the *E. californiensis* treatment (which had 6 tanks).

### Statistical analysis

We performed statistical analyses in RStudio (version 2022.07.2.576) (RStudio Team, 2022) running R (version 4.2.2) (R Core Team, 2022). To assess model adequacy, we evaluated q-q plots of observed against expected residuals from the global models using DHARMA (Hartig and Lohse, 2020), and by examining plots of predicted values from the top model overlaid on the raw data.

Initial data exploration revealed that discrete conspicuous behavior frequencies were highly overdispersed. We therefore used negative binomial generalized linear mixed models using `glmer.nb` in the `lme4` package (Bates et al., 2020). The package `glmmTMB` (Brooks et al., 2017) permitted confirmation that zero-inflated models did not better fit the data. For each response variable defined in Table I, we examined 5 models: the global model with observation date, parasite treatment, and their interaction; 3 simpler models with either or both main effects; and the intercept-only model. All models included a random intercept for tank to account for tank effects and the repeated-measures study design. Because the number of fish varied among and within tanks over time (given differential mortality), we initially included tank density in each global model because density could have impacted behavior. If density was not significant in the global model for a particular response variable, it was dropped. Otherwise, density was maintained in all 5 models for a particular response variable.

Models examining the proportion of fish near the water surface were generated using the `glmer` function from `lme4` (Bates et al., 2020), with a binomial error distribution and a logit link. For each time of day, we examined 5 models as described previously for the discrete conspicuous behaviors. However, because *F. parvipinnis* is social (Fritz, 1975), we hypothesized that nonneutrally buoyant fish near the water surface could attract focal fish. We therefore initially included the number of nonneutrally buoyant fish near the water surface in a global model. If the variable was significant, it was retained in all 5 models for each time point, and if it was not significant it was dropped.

We evaluated the models for each response variable using corrected Akaike Information Criterion (AICc) values (Burnham and Anderson, 2002) using the `model.sel` function in package `MuMIn` (Bartoń, 2020). Because AICc is not always appropriate for models using overdispersed data (Anderson et al., 1994), we also compared models using corrected quasi-AIC (qAICc). This procedure used the `Ictab` function in the `bbmle` package (Bolker and R Core Development Team, 2021), where the dispersion parameter supplied originated from the global models.

We separately evaluated the importance of parasite treatment on each conspicuous behavior. Our general approach was to first ask whether parasitism occurred in any or all of the “favored models” for each conspicuous behavior. We defined favored models as those with  $\Delta\text{AICc}$  or  $\Delta\text{qAICc} \leq 2$ , as such models have substantial evidence for being the best model or models out of the group of candidate models (Burnham and Anderson, 2002). We then calculated the predicted fold increases in conspicuous behaviors caused

by infection relative to the appropriate control (the overall change relative to control, or the date-specific control when the treatment by observation date interaction was favored). The predicted effects (expected marginal means) from the models were obtained using the R package “effects” (Fox et al., 2022).

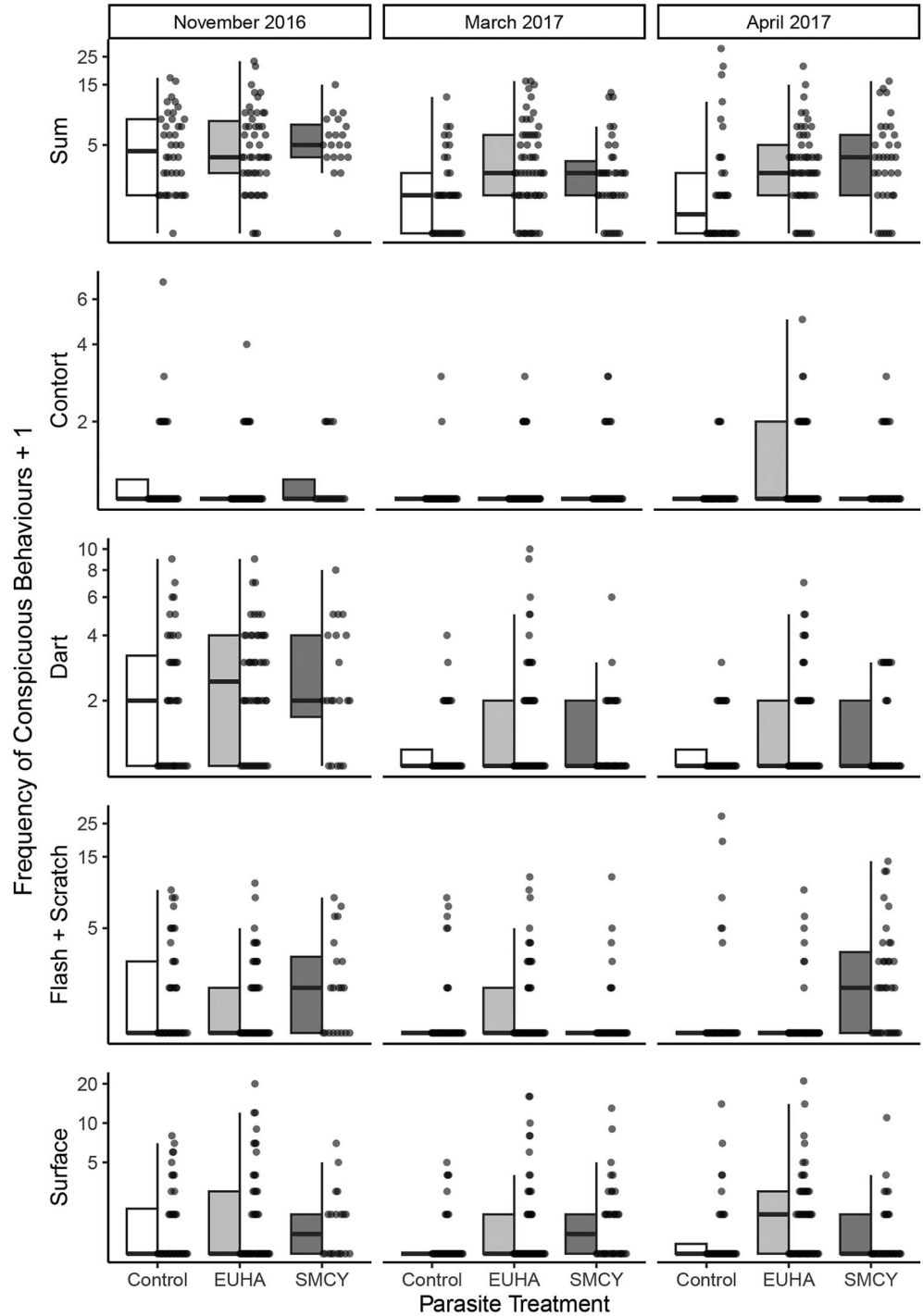
We focus on the predicted effects, their 95% confidence intervals, and the fold differences. But we also conducted planned contrasts to provide *P* values for the effect of parasitism on each behavior. When there was no treatment-by-date interaction, there was a single contrast for each species comparing the parasite species to control concerning the examined behavior. When a favored model had the treatment by observation date interaction, there were 3 contrasts for each parasite species, for which we compared the examined behavior to the control on the same date. We used the `multcomp` package (Hothorn et al., 2022) in R to conduct the planned contrasts. We conducted 1-tailed tests, consistent with expectations from prior evidence and theory that parasites would increase the expression of the behaviors. Further, we present and focus on observed *P* values evaluated at a critical value (alpha) of 0.05. However, to permit additional caution when interpreting individual tests, we note when significant *P* values did not withstand holding the family-wide false discovery rate (FDR) to 0.05. We used the Benjamini and Hochberg (1995) FDR procedure, which is appropriate for tests that are independent or positively dependent (Benjamini and Hochberg, 1995; Benjamini and Yekutieli, 2001). We treated as a single “family” the entire set of contrasts conducted for each parasite species (15 contrasts when using the main effects model, or 17 when using the cofavored model that included the treatment by observation date interaction).

We also examined the *overall* effect of each parasite species on fish behavior as evident in our entire data set. We did so by examining the mean effect size from the entire pool of separate behavioral effects for each species. We extracted the fold differences (response ratios) for each parasite treatment relative to the control treatment from the same day and time (from the models including the observation date by treatment interaction, also controlling for tank density in the analysis of flash/scratch and the number of nonfocal fish that did not stay neutrally buoyant in the night analysis of the proportion of fish near the water surface). This provided us with a total of 21 total fold differences for each parasite species (i.e., [5 discrete conspicuous behaviors \* 3 observation dates] + [proportion of fish near the water surface measured at 3 times of day \* 2 observation dates] = 21 fold differences total). To approximate 95% confidence limits for the pool of behavioral effects, we use Efron’s percentile method (Manly, 1997) on 5,000 bootstrap samples. We did this procedure on the actual fold differences, which has the advantage of being based on the scale, range, and true asymmetry of the fold differences that we present and discuss. However, to permit negative effects to have equal weighting, we also used the logged fold differences (log response ratios) and backtransformed those to actual fold differences.

## RESULTS

Data and R code are available on Dryad ([doi.org/10.5061/dryad.4mw6m90g2](https://doi.org/10.5061/dryad.4mw6m90g2)). Figures 1 and 3 depict the transformed raw data alongside box plots. To clarify the patterns relating to observation date and parasite treatment, Figures 2 and 4 display the estimated marginal means for treatment effects on each behavior by observation date. Model rankings are presented in Table II. Table III provides the effect sizes for parasite treatment from the top models, and





**Figure 1.** Box plots alongside raw data for the frequency of conspicuous behaviors (defined in Table I) exhibited by California killifish (*Fundulus parvipinnis*) over 5 min on each of the 3 observation days. One was added to all observations, and the y-axes are log<sub>2</sub> transformed to allow for easier visualization of these highly variable data. Fish were either sham infected (Control), infected by *Euhaplorchis californiensis* (EUHA), or infected by a small cyathocotylid (SMCY) trematode species.

Table S2 presents the detailed results from the top-ranked model for each behavior. Table S3 lists the entire set of planned contrasts and the results from these contrasts.

**Overall effect**

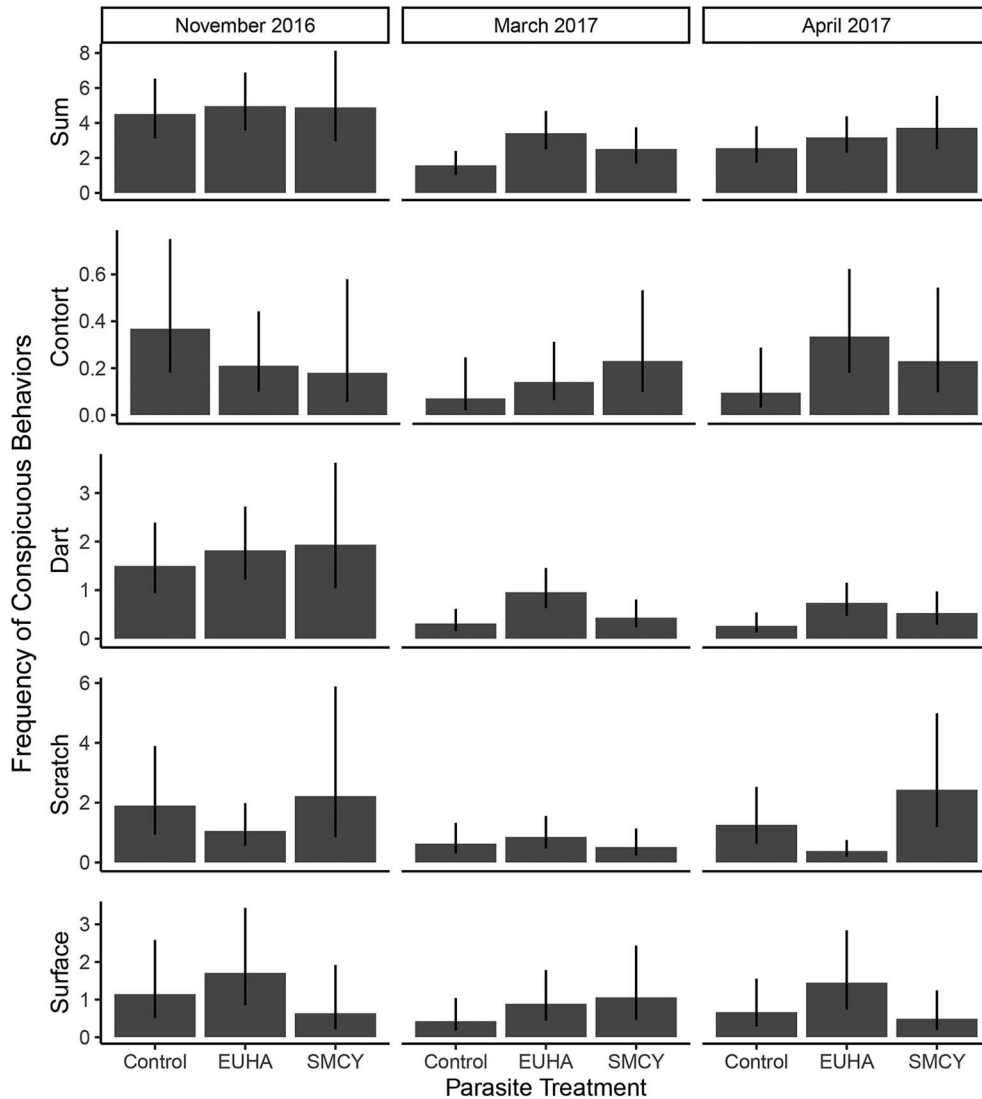
When examining the entire set of observed effects each parasite had on host behavior, we found that *E. californiensis* increased conspicuous behaviors 1.8-fold (95% confidence interval [CI]: 1.4–2.3) using actual fold differences and 1.5-fold (1.1–2.0) using

log fold differences, and that SMCY increased conspicuous behaviors 2.5-fold (1.4–4.0) using actual fold differences and 1.7-fold (1.2–2.4) using log fold differences.

**Discrete conspicuous behavior analysis**

There was evidence for parasites influencing the sum of discrete conspicuous behaviors, as parasite treatment was a main effect (along with observation date) in 1 of the 2 models favored by both AICc and qAICc. That model indicates that *E. californiensis*





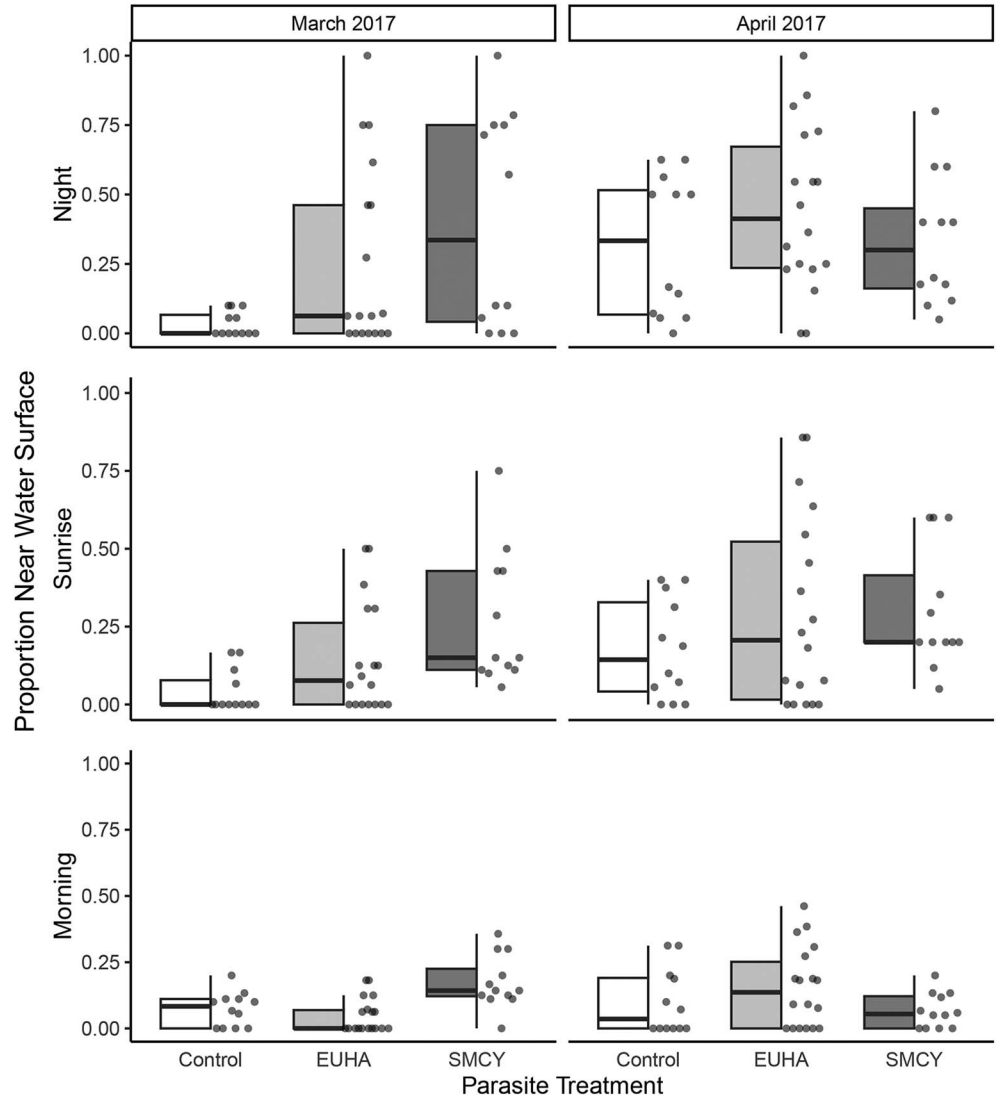
**Figure 2.** Estimated mean frequencies ( $\pm 95\%$  confidence intervals) of conspicuous behaviors (defined in Table I) exhibited by California killifish (*Fundulus parvipinnis*) in different treatment groups over 5 min on each observation day, as calculated from the global models. Fish were either sham infected (Control), or infected by *Euhaplorchis californiensis* (EUHA) or the small cyathocotylid (SMCY).

and SMCY infection both consistently increased  $\sim 1.4$ -fold the sum of conspicuous behaviors. Only the planned contrast for the effect of *E. californiensis* was significant ( $z = 1.8$ ,  $P = 0.033$ ), but it did not withstand false-discovery-rate (FDR) control. Further reflecting ambiguity in the effect of parasitism, the 95% confidence intervals for the effects of the parasites in the model included 0, and the closely scored, top-ranked model only included date. The top-ranked model indicated that fish, regardless of infection status, displayed less ( $\sim 0.5$  and  $\sim 0.6\times$ ) conspicuous behaviors in March and April 2017, respectively, compared to November 2016.

Parasite treatment had ambiguous effects on contortions. Parasite treatment was in 1 of the 3 favored models using AICc, but not qAICc. That favored model indicated that the effect of parasitism was inconsistent among days (i.e., it included the parasite treatment by observation day interaction). Although each parasite species tended to increase contortions 2.0- to 3.5-fold on the March and April dates, only the 3.5-fold increase caused by *E. californiensis* in April was significant in the planned contrasts ( $z = 2.0$ , and  $P = 0.025$ ) and this effect was not significant after FDR control. Further indicating the unreliability of the effect of

parasites on contortions, the top 2 favored models did not include parasitism. Those models retained only observation date or the intercept. The model containing only observation date indicates that *F. parvipinnis* contorted about half as frequently on the March 2017 observation date compared to November 2016.

Parasitism appeared to influence the frequency of darting, as treatment was included in the top 2 of 3 models favored by both AICc and qAICc. The top model included the main effect of parasitism (along with observation date) and indicated that *E. californiensis* infection consistently caused fish to dart 1.9-fold more than uninfected fish and that SMCY infection consistently increased darting 1.4-fold. Here too, only the planned contrast for *E. californiensis* was significant ( $z = 2.5$ ,  $P = 0.006$ ) and it was not so after FDR control. This top model indicated that infection-induced increases in darting consistently occurred across observation dates on top of the 30–34% decrease in darting seen in March and April 2017 relative to November 2016. The second-best model indicated that each parasite species increased darting to varying extents (1.2- to 3.1-fold) among dates (as the model included the treatment by observation date interaction). The 3.1-



**Figure 3.** Box plots alongside raw data for the proportion of California killifish (*Fundulus parvipinnis*) within 8 cm of the water surface in March and April 2017 at midnight (Night), sunrise (Sunrise), and in the morning before feeding (Morning). Fish were either sham infected (Control), infected by *Euhaplorchis californiensis* (EUHA), or infected by a small cyathocotylid (SMCY) trematode species.

and 2.8-fold increases in darting caused by *E. californiensis* in March and April were significant in the planned contrasts ( $z = 2.8, P = 0.003$ ;  $z = 2.4, P = 0.008$ , respectively, with the former standing up to FDR control).

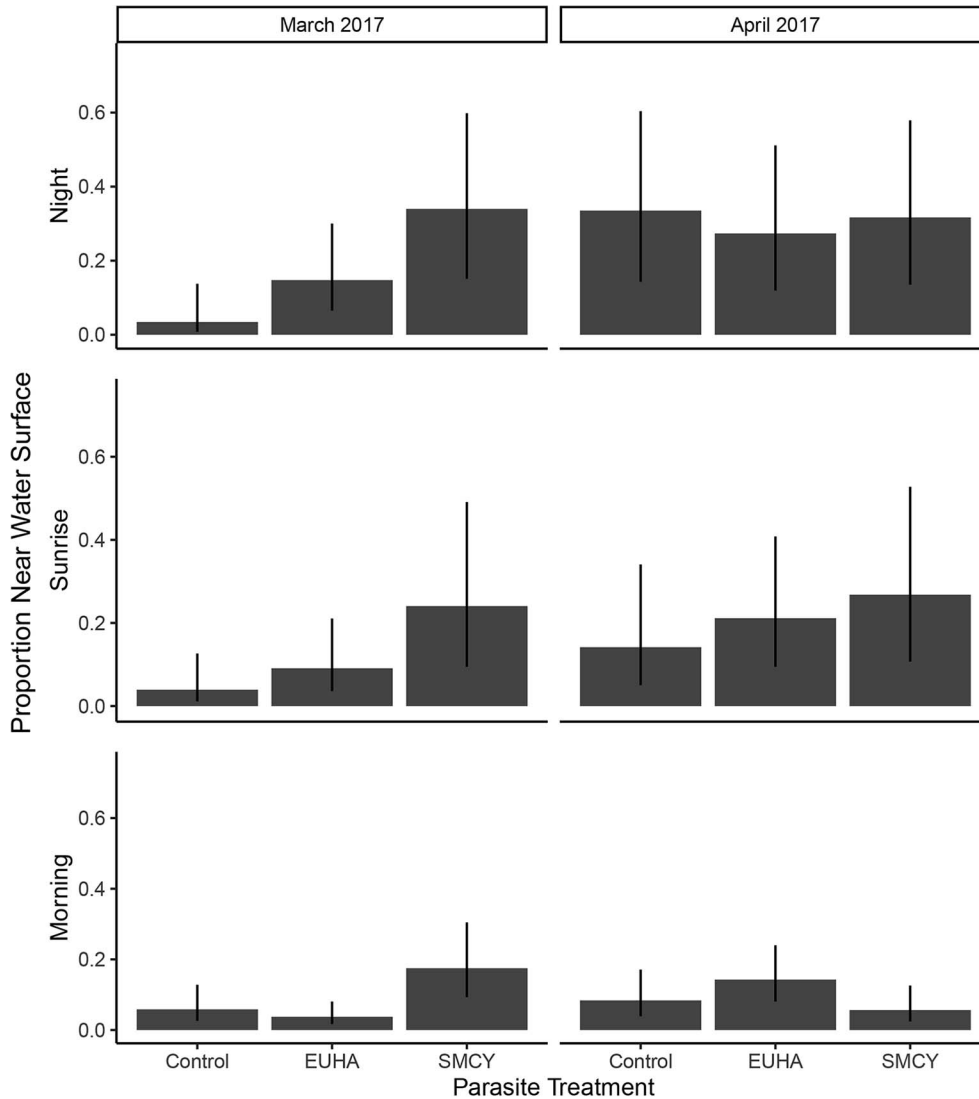
There was weak evidence that parasitism sometimes influenced flashing and scratching, as the single model favored by both AICc and qAICc included parasitism and its interaction with observation day. However, the effect was inconsistent and weak over time, and no planned contrasts were significant. Fish in higher-density tanks tended to flash and scratch less frequently.

There was some evidence that infection by *E. californiensis* consistently increased surfacing among the 3 observation days, as the main effect of parasitism was the sole predictor in 1 of the 3 models favored by AICc or qAICc. This model indicates that *E. californiensis* increased surfacing 1.8-fold and that SMCY was roughly equivalent to controls on each observation day. However, the 95% confidence intervals for the effect of parasites in this model included 0 (paralleled by insignificant contrasts). Also, parasitism was not included in the other 2 (and more highly scored) favored models, which retained only the intercept

or observation date, reflecting the weak trend for fish in March and April 2017 to surface 63 and 71% as often, respectively, as in November 2016.

**Proportion near the water surface**

Parasitism appeared to modify the amount of time spent near the surface at variable levels among observation days, as, for each of the examined times of day, the top-ranked model by both AICc and qAICc included the interaction between parasite treatment and observation day. Specifically, the effects on time spent near the surface ranged from 0.6- to 4.9-fold for *E. californiensis* and 0.9- to 14.4-fold for SMCY among days and times. For *E. californiensis*, only the 4.9-fold increase at night in March was significant in the planned contrasts ( $z = 1.7, P = 0.042$ ), though the effect was no longer significant after FDR control. For SMCY in March, the 14.4-fold increase at night ( $z = 2.9, P = 0.002$ ), the 7.8-fold increase at sunrise ( $z = 2.4, P = 0.009$ ), and the 3.4-fold increase in the morning ( $z = 2.1, P = 0.016$ ) were significant in the planned contrasts. However, out of these 3 contrasts, only the contrast for night withstood FDR control. No contrast from April was significant.



**Figure 4.** Estimated mean proportions ( $\pm 95\%$  confidence intervals) of California killifish (*Fundulus parvipinnis*) near the water surface on the March and April 2017 observation days at midnight (Night), sunrise (Sunrise), and in the morning before feeding (Morning), as calculated from the global models. Fish were either sham infected (Control), or infected by either *Euhaplorchis californiensis* (EUHA) or the small cyathocotylid (SMCY).

The number of fish that were unable to stay neutrally buoyant was only significant in the global model for Night, when each non-neutrally buoyant fish near the water surface made focal fish 1.6 times more likely to be near the water surface.

## DISCUSSION

Lafferty and Morris (1996) provided clear evidence that *F. parvipinnis* fish with greater levels of infection by *E. californiensis* expressed more conspicuous behaviors and that those fish were eaten at higher rates by final host birds in the field. The 2 most important gaps from their study were (1) experimental documentation of the direction of causality—namely that infection caused increased conspicuous behaviors—and (2) clear assignment of any effects specifically to *E. californiensis*, versus some other co-infecting parasite. These gaps are what we fill with this study. Further, we set up the study design to avoid problems that typically beset experimental studies (e.g., by repeatedly infecting fish hatched from a population that is normally exposed to the parasite, and by including treatments by a second possible behavior modifying

parasite—SMCY). Our results indicate that *E. californiensis* and SMCY do, indeed, modify host behaviors. For instance, *E. californiensis* infection caused an overall 1.8-fold increase in conspicuous behaviors, whereas the small cyathocotylid caused a 2.5-fold increase in these behaviors. Hence, our work combines with the original study of Lafferty and Morris, and subsequent physiological work (Shaw et al., 2009; Shaw and Øverli, 2012; Renick et al., 2016; Weinersmith et al., 2016; Helland-Riise et al., 2020b; Nadler et al., 2021) to further solidify the position of *E. californiensis* and *F. parvipinnis* as a classic example of parasite-increased trophic transmission with likely importance for food-web dynamics.

However, on the whole, the effects of infection on behavior in our study were weaker or more ambiguous than those documented in the laboratory-based behavior assays in Lafferty and Morris (1996). Table III summarizes our parasite-related results. For example, both parasites were associated with a 1.4-fold increase in the sum of conspicuous behaviors, whereas Lafferty and Morris (1996) documented a 4-fold increase. Similarly, our documented 2-fold increase in darting for *E. californiensis* and 1.4-fold increase for SMCY contrasts with the 29-fold increase documented in Lafferty

**Table II.** Model rankings for generalized linear mixed models for the conspicuous behaviors (defined in Table I) and the proportion of fish near the water surface across 3 times of day. All models included a random intercept for tank and could also include terms for the observation date (Date), parasite treatment (Treat), and their interaction (Int). A “+” indicates that a term was included in the model. Each row represents a different model, and models are presented in the order they were ranked by corrected quasi-Akaike information criterion (qAIC). The change in the corrected AICs ( $\Delta\text{AICc}$  and  $\Delta\text{qAICc}$ ) are presented, and models with  $\Delta\text{AICc} \leq 2$  in bold. Models for which both AICc values are greater than or equal to 25 are not shown.

	Date	Treat	Int	$\Delta\text{AICc}$	$\Delta\text{qAICc}$
<b>Conspicuous behaviors</b>					
Sum				<b>0.0</b>	<b>0.0</b>
	+			<b>0.8</b>	<b>1.0</b>
	+	+		3.4	3.7
			+	17.7	17.0
		+		19.6	19.0
Contort				<b>0.0</b>	<b>0.0</b>
	+			<b>0.0</b>	<b>0.4</b>
	+	+	+	<b>1.8</b>	<b>3.4</b>
		+		3.7	3.8
	+	+		3.5	4.1
Dart				<b>0.0</b>	<b>0.0</b>
	+	+		<b>1.2</b>	<b>0.3</b>
	+		+	<b>1.3</b>	<b>2.0</b>
Flash/scratch				<b>0.0</b>	<b>0.0</b>
	+	+	+	3.0	6.8
		+		3.5	8.8
	+			3.5	8.8
				4.5	11.4
Surface				<b>0.0</b>	<b>0.0</b>
	+			<b>0.5</b>	<b>0.4</b>
		+		<b>1.7</b>	<b>1.6</b>
	+	+		2.4	2.1
	+	+	+	4.0	3.3
<b>Proportion near water surface</b>					
Night				<b>0.0</b>	<b>0.0</b>
	+	+	+	18.4	19.1
	+			20.4	21.2
Sunrise				<b>0.0</b>	<b>0.0</b>
	+	+	+	3.8	12.5
	+			<b>1.7</b>	12.9
		+		19.1	46.1
				17.2	46.6
Morning				<b>0.0</b>	<b>0.0</b>
	+	+	+	22.7	21.7
	+			24.0	23.1

and Morris (1996). Further, the effects of conspicuous behaviors in our study appeared to be more ambiguous in the sense that the effect was not consistently apparent among the 3 observation days or because the specific behavior was not consistently retained as a predictor in every favored model. Below, we discuss several factors that may account for the less strong or consistent effects that we observed compared to Lafferty and Morris (1996). These factors are likely important to understand how parasite manipulation of host behavior is manifested in the wild.

### The degree of fish stress may explain the strength of *E. californiensis* behavior modification

Trophically transmitted parasites may benefit from manipulating their hosts' responses to stressors. For example, rodents infected by *Toxoplasma gondii* lose their innate aversion to the smell of urine from feline final host predators (Berdoy et al., 2000), which is thought to increase predation risk. Additionally, the intermediate-host amphipod *Gammarus fossarum* is less likely to hide following exposure to an electric shock when they are infected by cystacanths of the fish acanthocephalan *Pomphohynchus tereticollis* (Cozzarolo and Perrot-Minnot, 2022). In the *Euhaplorchis-Fundulus* system, differences in experimental design between studies hint that behavior modification is accentuated in the presence of a stressor. We suspect our behavioral data represent the behaviors of unstressed fish, as the tanks were kept behind a curtain, human observers were not in the room while videos were captured, and video cameras were activated remotely. In contrast, in prior studies examining the behavior of *Fundulus* hosts infected by *Euhaplorchis* parasites (Lafferty and Morris, 1996; Fredensborg and Longoria, 2012; Hernandez and Fredensborg, 2015), fishes were almost certainly stressed during behavioral observations. In these studies, observers were in the room and at least partly visible to the fish while behavioral observations were made. Much of the time, the fish were likely further stressed by the way researchers would track the behavior of an individual fish, then collect that fish from the tank with a dip net (a stressful event), followed by tracking the behavior of another individual fish in that tank. These studies documented relatively stronger effect sizes than we did. This association between the magnitude of behavioral change and the presence of an obvious stressor suggests the possibility of context-specific manipulation in the wild; predators are initially attracted to the low-level increase in conspicuous behaviors exhibited by infected fish (our study), and, once in the presence of a predator, this stressor induces fish to increase conspicuous behaviors substantially (evidenced in the above-cited prior work) rather than, for example, freezing or hiding.

Prior physiological work further supports the above contextual scenario for behavior manipulation and trophic transmission. Acute stress usually increases brain serotonergic activity in fish (Winberg and Nilsson, 1993), including *F. parvipinnis* (Shaw et al., 2009; Helland-Riise et al., 2020b). Increased serotonergic activity can suppress spontaneous behaviors and induce “freezing behavior” in fish (Höglund et al., 2005). Indeed, *E. californiensis* infection blunts the typical serotonergic response of both unstressed and stressed fish (Shaw et al., 2009). The suppression of poststress serotonergic activity should therefore tend to increase spontaneous, conspicuous, and risk-taking behaviors, say, when an infected fish is stressed by the presence of a predator. Hence, a hypothesized 2-pronged method, where *E. californiensis* first modifies host behavior at some baseline level (as reflected in our results) to draw the attention of predators and then, upon predator attack, more substantially induces inappropriate antipredator responses, may explain the greatly increased trophic transmission observed in Lafferty and Morris (1996). Future work should explore how the frequency of conspicuous behaviors in infected *F. parvipinnis* changes in response to predator presence and attack.

### Does manipulation depend on host age, length, or reproductive state?

The reproductive state of the fish may have influenced the parasites' ability to modify host behavior. The fish used in our



**Table III.** Summary of the effect sizes (fold differences relative to controls) of *Euhaplorchis californiensis* (EUHA) and the small cyathocotylid (SMCY) on discrete conspicuous behaviors of California killifish (*Fundulus parvipinnis*) or the proportion of fish near the water surface. Model indicates the terms included in the favoured model (i.e., a model for which Akaike information criterion [ $\Delta\text{AICc}$ ] or quasi-Akaike information criterion [ $\Delta\text{qAICc}$ ]  $\leq 2$ ), and Treatment refers to parasite treatment. Effect sizes are fold changes (response ratios) in the behavior of infected fish relative to control fish overall or on the same date for models with a treatment by observation date interaction. Effect sizes in bold are associated with significant group differences in planned contrasts using a per comparison  $\alpha$  of 0.05, and those with an asterisk withstood false discovery rate control. Model selection presents the model's  $\Delta\text{AICc}$  and  $\Delta\text{qAICc}$  values, and the model's ranking relative to all of the favored models for each behavior.

Model	Behavior	Fold differences (by date when interaction)		Model selection		
		EUHA	SMCY	$\Delta\text{AICc}$	$\Delta\text{qAICc}$	Ranking
Conspicuous behaviors						
~ Treatment * Date (Nov, Mar, Apr)						
	Contort	0.6, 2.0, <b>3.5</b>	0.5, 3.3, 2.4	1.8	3.4	Third of 3
	Dart	1.2, <b>3.1*</b> , <b>2.8</b>	1.3, 1.4, 2.0	1.2	0.3	Second of 3
	Flash + scratch	0.6, 1.4, 0.3	1.2, 0.8, 1.9	0	0	First of 1
~ Treatment + Date						
	Sum	<b>1.4</b>	1.4	0.8	1	Second of 2
	Dart	<b>1.9</b>	1.4	0	0	First of 3
~ Treatment						
	Surface	1.8	1.1	1.7	1.6	Third of 3
Proportion near water surface						
~ Treatment * Date (Mar, Apr)						
	Night	<b>4.9</b> , 0.7	<b>14.4*</b> , 0.9	0	0	First of 1
	Sunrise	2.4, 1.6	<b>7.8</b> , 2.2	0	0	First of 2
	Morning	0.6, 1.8	<b>3.4</b> , 0.7	0	0	First of 1

study were juveniles, whereas the fish sizes and inclusion of sex in statistical models in Lafferty and Morris (1996) and Hernandez and Fredensborg (2015), respectively, suggest that those studies used sexually mature fish. The conspicuous behavior “flashing” is like a mating behavior observed in other *Fundulus* species upon reaching sexual maturity (Newman, 1907; Shute and Lindquist, 1983). Manipulation of reproductive behaviors like flashing may require that physiological pathways associated with reproduction are established, which was not the case in the juvenile fish used in this study. Recent studies in other species also suggest that reproductive physiology is a pertinent target for the manipulation of host phenotype (Johansen et al., 2019).

Further, expression of the manipulated phenotype may not be a viable strategy for *E. californiensis* until *F. parvipinnis* have outgrown the many gape-limited nonhost fish predators. Manipulation may only be favored if it increases the parasite's transmission to host over nonhost predators (Parker et al., 2009), and conspicuous behaviors in small *F. parvipinnis* may also draw the attention of common nonhost fish predators, including the staghorn sculpin (*Leptocottus armatus*), yellowfin goby (*Acanthogobius flavimanus*), and long-jawed mudsucker (*Gillichthys mirabilis*). Like fish in general (e.g., Scharf et al. 1998), these fish are nearly certainly gape limited, and the sizes typically found in southern California estuaries will preclude them from eating larger *F. parvipinnis*. The *F. parvipinnis* used in Lafferty and Morris (1996) were an average of 6 cm long, and the authors note that there are “. . . typically no predatory fish large enough to eat killifish of the size we used in the experiment.” Our fish at 7–8 mo of age were only an average of 2.2 cm total length based on dissections of a subset of fish at that time, which likely makes them susceptible to many of the nonhost fish predators. Thus, *E. californiensis* may invest less in manipulation until fish pass a

certain age or size threshold, and our young, small fish may have been below this threshold.

### Population differences

Could the discrepancy in the strength of results have arisen from the use of different populations of hosts and parasites across studies? In particular, might we have observed less strong evidence for manipulation because *E. californiensis* from Kendall–Frost are locally adapted to their killifish hosts, and are thus poorer manipulators of the *F. parvipinnis* from San Elijo? We cannot rule out this possibility, as it is theoretically expected and empirically supported that parasites will often be better adapted to local hosts than to allopatric hosts (e.g., Lively, 1989; Ballabeni and Ward, 1993; Ebert, 1994; Morand et al., 1996). However, even under conditions of local host–parasite coevolution, it is possible to detect the opposite sometimes: that allopatric parasites perform *better* than sympatric parasites—it depends on the specific phases of local coevolutionary interactions (Morand et al., 1996). Such cycling may explain why Franceschi et al. (2010) and Hafer (2018) did not detect evidence for local adaptation concerning the ability to manipulate host behavior in 2 different trophically transmitted parasites (the tapeworm, *Schistocephalus solidus*, and the acanthocephalan, *Pomphorhynchus laevis*, both in crustacean intermediate hosts). However, both of those studies did report strong effects of the parasite source population concerning the degree of manipulation of host behavior. Hence, despite our Kendall–Frost parasites being able to infect San Elijo fish readily (Nadler et al., 2021), it is possible that the weaker or less consistent behavioral effects that we observed relative to what Lafferty and Morris (1996) documented for a sympatric host–parasite pair from a different population could be explained by such coevolutionary cycling or other “population effects.” A ripe target for future work would be to quantify the extent to

which behavioral manipulation by *E. californiensis* from different localities varies among *F. parvipinnis* populations.

### Behavior modification and real-world communities of manipulators

In our study, we found that the SMCY, which resides in muscle and connective tissue, was associated with an increase in several conspicuous behaviors, and with increased activity near the water surface throughout the day on 1 of the observation days. Further, Lafferty and Morris (1996) documented that the intensity of the liver-dwelling trematode *Renicola buchanani* was positively associated with darting and shimmying. In a closely related *Fundulus* species, populations with a greater intensity of several gill-infecting trematode metacercariae exhibited more conspicuous behaviors than populations with lower intensities (Bass and Weis, 2009). Thus, multiple trematode parasite species that infect *Fundulus* species are associated with behavioral changes that we suspect increase predation by final host predators, and these behavioral effects may overlap or be parasite specific. The observation that multiple trophically transmitted parasites residing in different host tissues induce similar conspicuous behaviors highlights at least 3 important points. First, it highlights the difficulty in studying the behavioral impacts of only 1 parasite species in hosts naturally infected by multiple parasites. *Fundulus parvipinnis* is commonly infected by at least 14 metazoan parasites (Lafferty, 2008), and the results in Lafferty and Morris (1996) may be stronger than ours given the presence of additive behavior manipulation by unquantified, covarying trophically transmitted parasites. Second, to understand the distribution of manipulated phenotypes in nature better, studies are needed that employ experimental infections and examine how multiple parasite species impact host behavior when in isolation and when co-infecting a host (see Cézilly et al., 2014 for a review on cooperation and conflict between multiple behavior-manipulating parasites). Finally, it has potential implications for our search for the mechanisms through which parasites change host behavior. Much attention has been focused on *E. californiensis* because intuition suggests a brain-infecting parasite is uniquely poised to impact behavior. However, if parasites residing in gills, the liver, connective tissue, skeletal muscle, and fins are associated with the same conspicuous behaviors, this suggests either the existence of multiple physiological paths to manipulate the same behaviors or that the same strategy may be used by parasites residing in various tissues and that a brain infection is not necessary for exploiting this path.

### CONCLUSIONS

Our study employed a relatively robust experimental infection protocol to provide direct evidence that *E. californiensis* infection increases conspicuous behaviors in *F. parvipinnis*. Additionally, we provide evidence that a muscle-dwelling trematode species also modifies the behavior of *F. parvipinnis*. Our findings further firm up the role of this host–parasite system as a classic example of parasite-increased trophic transmission. However, we encourage follow-up work to quantify associations between experimental infection, behavior, and predation by final host predators. The documented behavioral changes were less strong and consistent than in prior work, generating new research questions about the context dependency of behavioral manipulation, the role of

parasite and host population in determining the magnitude of manipulated phenotypes, and the need to quantify the effects of co-infecting parasites to understand better the role of parasites in controlling host behavior and hence increased trophic transmission in ecosystems.

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