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Euhaplorchis californiensis Cercariae Exhibit Positive Phototaxis and Negative Geotaxis

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ABSTRACT: Parasites often use external cues to identify and move toward environments where they are likely to encounter suitable hosts. The trematode parasite *Euhaplorchis californiensis* produces cercariae that emerge from California horn snails (*Cerithideopsis californica* [= *Cerithidea californica*]) to infect California killifish (*Fundulus parvipinnis*) as second intermediate hosts. Based upon work on a congeneric *Euhaplorchis* species from Florida, and based on the ecology of its killifish host, we hypothesized that *E. californiensis* cercariae in southern California estuaries are positively phototactic and negatively geotactic, using both sunlight and gravity to guide their movement to the upper water column. To distinguish positive phototaxis from negative geotaxis, we first quantified *E. californiensis* movement in response to light along a horizontal plane and determined they were positively phototactic. In a second experiment, we quantified *E. californiensis* movement along a vertical plane in response to an overhead light, a light from below, or no light. We found that *E. californiensis* exhibit negative geotaxis in the absence of light, but will swim in the direction of gravity to move toward a light source from below. Thus, *E. californiensis* are both positively phototactic and negatively geotactic, but cercariae prioritize phototactic cues. These results suggest that *E. californiensis* cercariae aggregate in the open water, indicating that the pelagic zone represents an area of high infection risk for California killifish hosts.

Parasites often use cues to identify and move toward microhabitats where they are likely to encounter their next host (Haas, 1992, 2003; Combes et al., 1994, 2002; Combes, 2001; Sukhdeo and Sukhdeo, 2004; Chaisson and Hallem, 2012). Trematode cercariae are free-swimming and typically have, at most, a few days to find and infect their next host (Asch, 1975; Cross et al., 2001; Pietrock et al., 2002; Koprivnikar et al., 2006, 2010). Hence, selection has likely favored behaviors that help cercariae rapidly identify and move to microhabitats where they are most likely to encounter hosts. Understanding these behaviors is crucial for understanding host–parasite dynamics.

Herein we report the response of *Euhaplorchis californiensis* cercariae to light and gravity. Fish-eating birds are used by *Euhaplorchis californiensis* as final hosts, California horn snails (*Cerithideopsis californica* [= *Cerithidea californica*]) as first intermediate hosts, and California killifish (*Fundulus parvipinnis*) as second intermediate hosts (Martin, 1950). *Euhaplorchis californiensis* appears to manipulate killifish behavior; infected fish are 10 to 30 times more likely than uninfected fish to be eaten by birds (Lafferty and Morris, 1996). California killifish are often the most abundant fish in southern California and Baja California estuaries (Allen et al., 2006; Shaw et al., 2010). Thus, any parasite that changes the behavior of the California killifish likely plays an important role in ecosystem dynamics (Lafferty, 2008).

Previous research found that cercariae of a "*Euhaplorchis* sp." from infected *Cerithideopsis scalariformis* snails in Florida exhibit positive phototaxis and negative geotaxis (Smith and Cohen, 2012; Fitzpatrick et al., 2016). DNA sequence data confirm that this *Euhaplorchis* sp. is a different species than *E. californiensis* (O. Miura, pers. comm.). Here, we sought to confirm whether these *Euhaplorchis* species from different coasts, and from different first intermediate host species, use the same cues to identify microhabitats where they are likely to encounter their pelagic second intermediate hosts.

We obtained snails from Kendall-Frost Mission Bay Marsh (San Diego County, California). *Euhaplorchis californiensis* cercariae emerge from snail hosts in response to light and submergence in seawater (Fingerut et al., 2003). We identified snails infected with *E. californiensis* by "shedding" cercariae, by placing snails into containers of filtered seawater in sunlight for 1–2 hr and identifying emerged cercariae (following Martin, 1950, 1972). We shipped *E. californiensis*-infected snails overnight to Rice University (Houston, Texas). Snails were then held in a large plastic container filled with artificial seawater (35 ppt, created using reverse osmosis filtered water and Instant Ocean®; Instant Ocean, Blacksburg, Virginia), fed boiled organic spinach, and used for experiments within 3 wk. At least 48 hr before an experiment, we removed snails from the water and held them under cool, dark conditions to permit cercaria numbers to build up in the snails.

We conducted 3 experiments to quantify *E. californiensis* responses to light and gravity cues (see Suppl. Data, Fig. 1S for diagrams of experimental apparatuses). Two of the experiments are described here. The Horizontal Experiment ran over 2 consecutive days in March 2015 and the Vertical Experiment ran on 1 day in March 2016. A Supplemental Vertical Experiment yielded results qualitatively similar to the Vertical Experiment described herein (see Suppl. Material). All data collected during experiments and the R code used to analyze the data are available as Supplemental Materials. Experiments were run in rooms lacking windows, and water temperatures ranged from 21.6 to 23.8 C.

To collect cercariae for behavioral trials, we placed 15 to 20 snails in 300 ml of artificial seawater under full-spectrum lighting (Coralife Actinic 39W T5 H0 and 10,000K 39W T5 H0 bulbs). Snails were aggregated because we were not interested in phototaxis differences among *E. californiensis* genotypes but, rather, in obtaining population-average characteristics. After 1 hr, water containing the snails and emerged cercariae was gently mixed, and 1 ml of this water was added to 5 ml of artificial seawater. This procedure was repeated, and these 6-ml aliquots of cercariae-infested seawater were stored for use in trials to be run

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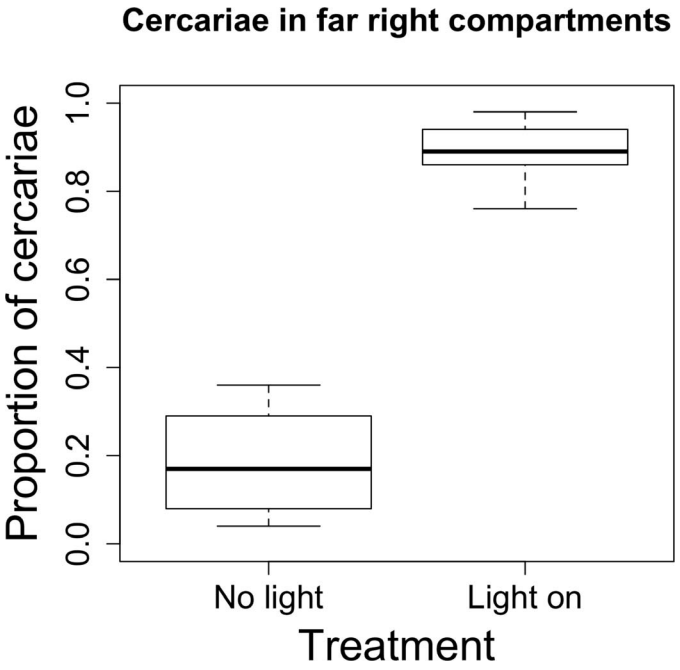

FIGURE 1. Results from the Horizontal Experiment. Box-and-whisker plots present the median (thick black line), lower and upper quartile (bottom and top of box, respectively), and minimum and maximum (bottom and top of whiskers, respectively) proportions of *Euhaplorchis californiensis* cercariae in the 3, far-right compartments near the lamp in the "no light" (control) and "light on" treatments. The difference between treatments was statistically significant (Table 1S).

that day. All experiments within a day were completed $\leq$7 hr post-emergence to generally coincide with ecologically relevant timeframes of tidal inundation. While the cercariae of some trematode species respond differently to light depending on its intensity (Haas, 1992), Smith and Cohen (2012) found that their *Euhaplorchis* sp. swims toward light when light intensities exceed 4.0×10^{15} photons m^{-2} s^{-1}. Light intensity in our experiments was measured using an Apogee MQ-200 Series (Apogee Instruments Inc., Logan, Utah) quantum meter and exceeded the threshold light intensity identified for the closely related congener (Smith and Cohen, 2012) (see below for light intensity values for each experiment). Our light source for experiments was a 122-cm Aqueon Freshwater T5 Dual Lamp Fixture (Aqueon Products, Central Aquatics, Franklin, Wisconsin), which contains one 6,700 K Daylight bulb and 1 Colormax Color Enhancing bulb.

In the Horizontal Experiment, we placed cercariae in a shallow-water apparatus to quantify their horizontal movement in response to light. The apparatuses were plastic boxes (internal dimensions: 24.0 × 17.2 × 3.5 cm) with plastic dividers that created 4 rows of equal size. Each row was divided into 9 compartments using clear, removable dividers. A central compartment in each row measured 4.8 × 4.3 cm and the 8 remaining compartments measured 2.4 × 4.3 cm. The 2 outer rows in each box were filled with 100 ml of artificial seawater (~1.5 cm deep) and were used for experiments. Black fabric was wrapped around the box to block light and covered all but one end of the box. We pipetted cercaria-infested water into the central compartment and dividers were then removed. The box was then closed and, after 5 min, we either turned on a light on the right side of the box ("light on" treatment) for 10 min or left the room dark as a control ("no light" treatment). We also ran trials with the light on the left side of the box and achieved similar results (see Suppl. Material for data and results from analysis), thus the cercariae exhibited no directional bias. Light intensity where the light encountered the box was 1.9×10^{19} photons m^{-2} s^{-1} (range 1.9 to 1.9×10^{19} photons m^{-2} s^{-1}). Each set of trails included a "light on" treatment and a "control" treatment, and the order of these treatments was randomized. Two or 3 plastic boxes were used in each trial.

Following the 10-min treatment periods, we reinserted row dividers and counted cercariae in each compartment using a dissecting microscope. Individuals counting cercariae were blind to treatment type. We rinsed boxes with artificial seawater between trials and used new cercariae for each trial. Six trials were run for a total sample size of 13 light on and 13 control treatments. These sample sizes reflect the removal of one row from each treatment prior to cercaria counts when human error was believed to have influenced the distribution of cercaria in the row (e.g., because mishandling of a divider during reinsertion may have created a current and changed the distribution of cercariae). We conducted all statistical analyses in R version 2.15.3 (R Development Core Team, 2015) using general linear models. Residuals were distributed normally according to Anderson–Darling tests.

For the Horizontal Experiment, we first examined whether there was a greater proportion of cercariae in the 3 far right compartments when the light was on compared to the no light control. Time (minutes) between the end of the cercaria shedding period and the start of each particular trial was added as a covariate, as was an interaction between "time since emergence" and treatment to explore potential changes in cercaria behavior over time.

The average number of cercariae placed in the experimental boxes was 109 (range: 31–203). In the Horizontal Experiment, cercariae exhibited positive phototaxis, and neither time since emergence nor the interaction between time since emergence and treatment influenced their behavior ($F_{3,22} = 122.4$, $R^2 = 0.94$, $P < 7.1 \times 10^{-14}$; see Supp. Data Table 1S; Fig. 1). In the no light control treatments, 70% of cercariae remained in the 3 center compartments and only 19% moved to the right side of the box. In contrast, in the light treatments an average of only 10% of cercariae stayed in the center while 89% moved toward the light.

In the Vertical Experiment, we examined both phototactic and geotactic cercaria behavior. We quantified whether the cercariae moved toward a light shining from above or below in a vertical apparatus comprised of 3 (7-cm long) white polyvinyl chloride (PVC) tubes (1.3 cm inner diameter). Four of these apparatuses were used in each trial. The tubes were connected with 2 PVC ball valves (2.0 cm inner diameter), and a clear piece of PVC (2 mm thick) was adhered to the bottom of the apparatus using aquarium silicon. The apparatus was wrapped in duct tape to block light. The ball valves were open during experiments. Artificial seawater was added to the apparatus and then parasites were pipetted into the center of the apparatus. If necessary, additional water was added to top off the apparatus. We then initiated 1 of 3 treatments (each of which lasted for 15 min): the light was turned on above the apparatus, the light was left off, or the light was turned on below the apparatus. When the light was on, light intensity at the tube entrance was 2.2×10^{19} photons m^{-2}

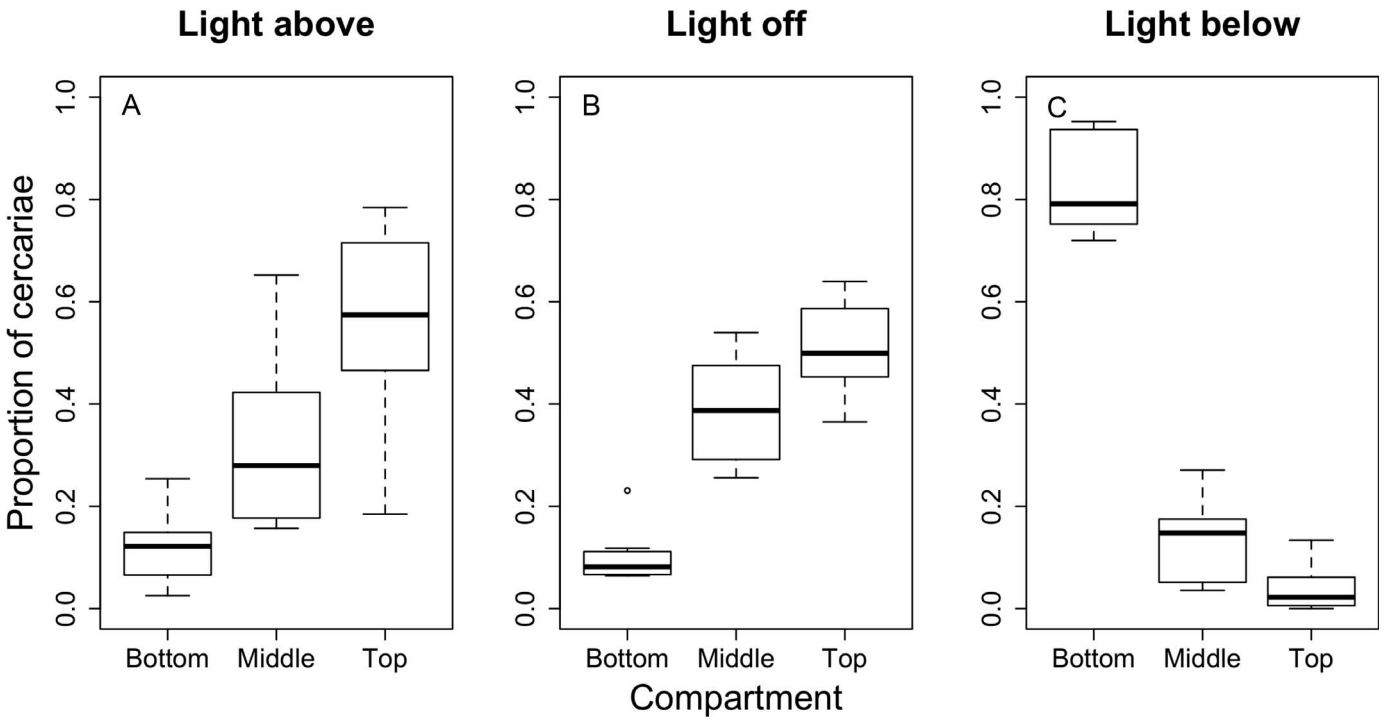

FIGURE 2. Results from the Vertical Experiment. Box-and-whisker plots present the median (thick black line), lower and upper quartile (bottom and top of box, respectively), and minimum and maximum (bottom and top of whiskers, respectively) proportions of *Euhaplorchis californiensis* cercariae that were found in the bottom, middle, and top compartments of the vertical apparatus (**A**) when a light was turned on above the apparatus ("light above"), (**B**) when the light was turned off ("light off"), and (**C**) when the light was turned on below the apparatus ("light below"). The proportion of cercariae in the top compartment is significantly higher in the light above and light off treatments relative to the light below treatment, and there were significantly more cercariae in the bottom compartment when the light was turned on from below relative to the 2 other treatments (Table 2S).

s^{-1} (range 1.6 to 2.5×10^{19} photons m^{-2} s^{-1}). For the "light above" treatment, a piece of clear plexiglass was placed on top of the apparatus and a dark fabric was wrapped around the bottom of the apparatus to prevent light from entering from below. For the "light below" treatment, we did the reverse. Ball valves were closed following the treatment period. Water from the top, middle, and bottom compartments was subsequently poured into separate containers, and each compartment was rinsed with seawater prior to pouring out the next compartment. We counted cercariae using a dissecting scope. We rinsed the apparatus in artificial seawater between trials, cercariae were used only once, and each treatment was run twice (for a total of 8 samples per treatment).

On average, 100 cercariae (range from 30 to 375) were placed in the apparatus for the Vertical Experiment. The percent of cercariae in the top of the apparatus during the light above, light off, and light below treatments was 56%, 51%, and 4%, respectively (Fig. 2). The proportion of cercariae in the top compartment was statistically different for the light below treatment, but there was no statistical difference between the light above and light off treatments (overall model: $F_{5,18} = 24.01$, $R^2 = 0.83$, $P < 2.3 \times 10^{-7}$; Table 2S; Fig. 2). Because there were similar numbers of parasites in the top compartment whether the light above was on or off, it appears that *E. californiensis* cercariae exhibit both positive phototaxis and negative geotaxis. There was a significant effect of time since emergence that was consistent among treatments (interactions not significant; Table 2S). The effect size was positive, suggesting that the parasites are more likely to swim upward with increasing time after leaving the snail host. Substantially more cercariae aggregated in the bottom of the apparatus when the light was turned on from below compared to the other treatments, and neither time since emergence nor the interaction between time since emergence and treatment were significant predictors (overall model: $F_{5,18} = 108.5$, $R^2 = 0.96$, $P < 8.6 \times 10^{-13}$; Table 2S; Fig. 2). The percent of cercariae in the apparatus bottom during the light above, light off, and light below treatments was 12%, 10%, and 83%, respectively. This indicates that positive phototaxis overrides negative geotaxis for *E. californiensis*, as the parasites swam in the direction of gravity to move toward a light cue.

The results indicate that *E. californiensis* shows positive phototaxis and negative geotaxis, which is consistent with the behavior of the *Euhaplorchis* sp. found in Florida (Smith and Cohen, 2012; Fitzpatrick et al., 2016). When faced with conflicting cues, *E. californiensis* cercariae prioritize light cues and will move with gravity toward a light source. Future studies should confirm that the positive phototaxis exhibited by *E. californiensis* cercariae results in aggregation at the water surface in the field (where factors such as angular light, different light intensities, and water currents could alter parasite distributions) and examine how this interacts with various other aspects of the ecology and behavior of the parasite and the host (e.g., predation on cercariae, transmission success, distribution of parasites among host individuals, and host spatial distribution).

Killifish individuals may vary from one another concerning how much time they spend at the surface (as documented for

other fish, e.g., Wilson et al., 2011). Fish that spend more time near the surface should be most likely to be eaten by predatory birds, the final hosts of *E. californiensis*. Hence, even in the absence of manipulation, *E. californiensis* cercariae may benefit by aggregating at the water surface and infecting those killifish that are most likely to transmit the parasite to its final host. Interestingly, *E. californiensis* metacercariae also appear to induce killifish to make more-frequent trips to the water surface (Lafferty and Morris, 1996). Thus, infection may induce positive feedbacks where fish that were initially more likely to spend time at the water surface acquire parasites that make them increasingly more likely to spend time at the water surface and therefore further increase their risk of *E. californiensis* infection. California killifish adults are frequently infected by thousands of *E. californiensis* metacercariae (Shaw et al., 2010) and such positive feedbacks may underlie these high infection intensities.

Furthermore, *E. californiensis* parasites may benefit from such positive feedbacks between infection and killifish behavior. High-intensity infections increase the probability that a killifish is eaten by a final host bird (Lafferty and Morris, 1996). Therefore, *E. californiensis* metacercariae living in hosts with higher-intensity infections have greater transmission success. Moreover, there appear to be no drawbacks for *E. californiensis* metacercariae to live in high-intensity infections, as they show no sign of crowding in their killifish host; their body size may even be slightly larger in high-density relative to low-density infections (Weinersmith et al., 2014). Thus, positive feedbacks between infection and behavior may result in high-intensity *E. californiensis* infections that benefit the parasites by increasing their likelihood of transmission while carrying no costs associated with resource limitation.

Our findings that *E. californiensis* cercariae exhibit positive phototaxis and negative geotaxis combine with previous results to suggest the occurrence of positive feedbacks between behavior and infection and such feedbacks may benefit *E. californiensis*.

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