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REGIONAL DISTRIBUTION OF A BRAIN-ENCYSTING PARASITE PROVIDES INSIGHT ON PARASITE-INDUCED HOST BEHAVIORAL MANIPULATION

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Brain Stem
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ABSTRACT

Some parasite species alter the behavior of intermediate hosts to promote transmission to the next host in the parasite's life cycle. This is the case for *Euhaplorchis californiensis*, a brain-encysting trematode parasite that causes behavioral changes in the California killifish (*Fundulus parvipinnis*). These manipulations increase predation by the parasite's final host, piscivorous marsh birds. The mechanisms by which *E. californiensis* achieves this manipulation remain poorly understood. As *E. californiensis* cysts reside on the surface of the killifish's brain, discerning regional differences in parasite distribution could indicate mechanisms for host control. In this study, we developed a method for repeated experimental infections. In addition, we measured brain-region specific density using a novel methodology to locate and quantify parasite infection. We show that *E. californiensis* cysts are non-randomly distributed on the fish brain, aggregating on the diencephalon/mesencephalon region (a brain area involved in controlling reproduction and stress coping) and the rhombencephalon (an area involved in controlling locomotion and basal physiology). Determining causal mechanisms behind this pattern of localization will guide future research examining the neurological mechanisms of parasite-induced host manipulation. These findings suggest that parasites are likely targeting the reproductive, monoaminergic, and locomotor systems to achieve host behavioral manipulation.

Some parasites alter their host's behavioral and physiological phenotypes to facilitate transfer to the next host in their life cycle (Holmes and Bethel, 1972; Lafferty and Morris, 1996; Barber et al., 2000; Moore, 2002, 2013; Thomas et al., 2005). For trophically transmitted parasites, such phenotype manipulation increases the probability that the intermediate host is eaten by the next host in the parasite's life cycle, a phenomenon known as parasite-increased trophic transmission (PITT) (Lafferty, 1999). Some examples of PITT-associated phenotypic alterations include altered activity, changes in microhabitat use, reduced fear responses, and altered olfactory preferences (Moore, 2002; Afonso et al., 2012; Lafferty and Shaw, 2013).

The brain-encysting trematode parasite *Euhaplorchis californiensis* (Heterophyidae) and its second intermediate host, the California killifish (*Fundulus parvipinnis*), constitute a classic example of PITT. *Euhaplorchis californiensis* has a complex, 3-host life cycle (Martin, 1950). Its first intermediate host, the California horn snail (*Cerithideopsis californica*), grazes on estuarine mudflats, where it ingests *E. californiensis* eggs. The parasite then reproduces asexually in the snail, producing free-swimming larval cercariae that search out the second intermediate host, *F. parvipinnis*. Upon encounter, the cercaria burrows through the fish's skin and presumably migrates along blood vessels or nerve tracts to the brain (McNeff, 1978; Haas et al., 2007). Once on the brain, the parasite forms an encysted larval stage (metacercaria) and resides on the meningeal surface (Martin, 1950; McNeff, 1978). Infected *F. parvipinnis* individuals exhibit 4 times more conspicuous behaviors than uninfected conspecifics, an effect that is exacerbated at higher parasite numbers (Lafferty and Morris, 1996). Some of these behaviors include surfacing (abrupt dashes to the water surface), jerking (sudden movement forward), and flashing (the fish twists dorsoventrally, causing the silver coloration on its ventral side to reflect the light above). As a result, infected fish are reported to be 10 times more susceptible to predation by the parasite's final host (marsh birds) compared to uninfected conspecifics (Lafferty and Morris, 1996). However, despite over 2 decades of work in this system, the mechanisms through which *E. californiensis* metacercaria manipulates killifish behavior remain unclear.

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Experimental infections are essential to study the causal mechanisms by which *E. californiensis* metacercaria affects the behavior of its fish host. Typically, all fish in systems where *E. californiensis* is present harbor the parasite, with intensities in adults varying from hundreds to more than 8,000 parasites (Shaw et al., 2010). Hence, to acquire parasite-free fish, researchers have collected fish from populations completely lacking the parasite and compared those to naturally infected populations (e.g., Lafferty and Morris, 1996). This introduces the confounding factor of population, meaning that any observed differences in, for example, neuroendocrine function, could potentially be due to either population effects or differences in infection status. Experimental infections remove such potential population effects, enabling research on neuroendocrine correlation to infection status. However, achieving ecologically relevant infection intensities in the laboratory can be difficult, in large part because wild fish are repeatedly infected over time and accumulate larger intensities than are readily achievable with laboratory infections. For example, while indicating clear effects on brain monoaminergic neurotransmission, a previously used experimental infection protocol employed a single laboratory infection and yielded parasite intensities that were much lower than those observed in naturally infected fish (i.e., less than 100 cysts per fish vs. thousands typically observed in a naturally infected fish) (Shaw et al., 2009). Therefore, the development of a new experimental infection protocol that achieves infection intensities comparable to those occurring in the wild is essential to uncover the causal mechanisms underpinning behavior manipulation in this host-parasite system.

Furthermore, information on whether specific brain regions are targeted by brain-infecting parasites is lacking but is necessary to understand mechanisms and specific neurological processes manipulated by parasites. In teleost fish, macro brain areas in the fore-, mid-, and hindbrain have been associated with specific behavioral and physiological outputs. Specifically, the telencephalon in the forebrain is involved in learning, memory, and approach/avoidance behavior (O'Connell and Hofmann, 2011). The midbrain contains the diencephalon and mesencephalon, which are involved in regulating reproductive physiology and behaviors as well as the interpretation of visual and motor cues (Wulliman et al., 1996). Lastly, the brain stem and the cerebellum are located within the rhombencephalon (hindbrain) and are associated with the regulation of locomotion and basal physiology (Wulliman et al., 1996).

Euhaplorchis californiensis cercariae presumably encounter the brain-stem region first on their migration from the dermal entry point to the brain, the primary region harboring important monoaminergic signaling systems (dopamine, norepinephrine, and serotonin cell clusters). These systems could be targeted by the parasite for manipulation, as they are involved in mediating sociality, risk-taking, reproductive behavior, aggression, and locomotion in fish (Winberg and Nilsson, 1993; Winberg et al., 1993; Adams et al., 1996; Mok and Munro, 1998; Smeets and González, 2000; Forlano and Bass, 2011; Lillesaar, 2011; Prasad et al., 2015; Winberg and Thörnqvist, 2016; Matsui, 2017). Shaw et al. (2009) noted that the majority of encysted *E. californiensis* metacercariae were indeed located on the brain stem (in the rhombencephalon), suggesting that *E. californiensis* individuals may target neural systems located in this brain area. Manipulation of monoaminergic activity has been reported in parasite-infected mammals (Stibbs, 1985; Gatkowska et al., 2013; Lafferty and Shaw, 2013; Herbison, 2017), fish (Øverli et al., 2001; Shaw et al., 2009; Shaw and Øverli, 2012; Lafferty and Shaw, 2013; Herbison, 2017), and invertebrates (review by Perrot-Minnot and Cézilly, 2013).

The reproductive system could be an equally suitable target for manipulations (Adamo, 2014; Adamo et al., 2014), because it regulates a myriad of physiological and behavioral responses (review by Mohammad-Zadeh et al., 2008). Notably, the reproductive system coordinates changes in physiology, behavior, and morphology that often make animals more conspicuous to better attract mates (Magnhagen, 1991; Arimune Munakata, 2010). Fish also lose normal anti-predator responses during reproduction (Lastein et al., 2008). Flashing similar to that observed in infected California killifish has been associated with mating in Atlantic killifish (*Fundulus heteroclitus*) (Newman, 1907). If the species *E. californiensis* manipulates the neuro-reproductive system, we might predict that it would prefer to infect brain areas associated with these systems, such as the diencephalon/mesencephalon region.

This study was part of a larger project examining the influence of *E. californiensis* on California killifish physiology, neurobiology, and behavior throughout development. We designed a novel protocol to achieve controlled infection intensities (total number of metacercariae) in the laboratory comparable to those seen in nature and quantified brain-region-specific parasite abundance. Discerning the regional distribution of brain-encysting *E. californiensis* metacercariae would provide valuable information on the physiological systems targeted for parasite-induced host manipulation in this model system. To achieve this goal, we designed a rigorous experimental infection regime in the laboratory to better mimic natural infections in the wild and precisely quantified the resulting regional distribution of parasites on the brain using histological techniques.

MATERIALS AND METHODS

Experimental killifish

The Institutional Animal Care and Use Committee at the University of California, San Diego, California, approved all experiments. We captured *F. parvipinnis* individuals between July and August 2016 from a naturally infected population in Kendall-Frost (KF) Marsh Reserve in San Diego, California (32°47′22″N, 117°13′56″W), by use of a 2-pole seine, and ripe (breeding condition) adults were temporarily placed in seawater in 18.9-L buckets. We applied pressure to the abdomen until eggs/milt were expelled by the fish, following methods outlined in Strawn and Hubbs (1956), and we mixed eggs and milt together in a petri dish containing a volume of seawater just large enough to cover the eggs. We released fish at their site of capture (downstream from collecting point, to avoid recapture), within 1 hr. We brought fertilized eggs back to Scripps Institution of Oceanography (SIO, 32°52′01″N, 117°15′12″W) and rinsed the eggs before placing them in a circular finger bowl (diameter: 100 mm) containing 200 ml of aerated and filtered seawater (the last batch of eggs received filtered seawater mixed with methylene blue, 0.0003%, until days 17–19 post-fertilization to increase egg survival). We kept the eggs on a light:dark cycle similar to that of natural day length (32°52′01″N, 117°15′12″W, July–August) and removed dead or unfertilized eggs daily. In addition, we did a complete water

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change every other day until hatching at approximately day 21. We transferred hatched fry to 37.8-L aquaria and maintained them in groups of 20. During the first 12 wk of life, we fed fish live brine shrimps once daily. Once the fish were ~12 wk post-hatch, we transitioned them to a more varied diet composed of blood worms, aquaculture feed (Skretting), and mashed peas.

Experimental infections

We carried out experimental infections continuously beginning when fish were approximately 8 wk of age and throughout the life of the fish (September 2016–June 2017). Cercariae in this system emerge from snails when they are inundated with seawater, with cercariae "shedding" at the highest rates during the warm summer months (Fingerut et al., 2003). We typically infected fish twice weekly. However, on 9 occasions during the winter months (when temperatures were cooler and natural shedding rates would be lower), we were unable to complete 1 of the weekly infections due to insufficient shedding. We used California horn snails originating from KF to obtain free-swimming *E. californiensis* cercariae. We maintained the snails at SIO in mudflat mesocosms operating under an artificial tidal regime mimicking the local tidal cycle (using a modified setup from Miller and Long, 2015). For each experimental infection, we removed approximately 120 horn snails previously identified as harboring *E. californiensis* from the mesocosms and placed them in a humid environment for a minimum of 24 hr before shedding. We identified snails harboring exclusively *E. californiensis* by shedding them individually in compartment boxes on 3 separate occasions over a 2 wk period and visually inspecting the cercariae under a microscope, prior to using them in experimental infections. Two hours to 4 hr prior to an infection event, we placed groups of 7–9 snails in finger bowls (10 cm internal diameter) containing filtered seawater heated to 27 C and placed it under a fluorescent light. Parasite identity was again confirmed visually using a microscope, and we recorded the number of *E. californiensis* cercariae shed. We subjected fish to 1 of 3 infections: low parasite dose, high parasite dose, and a control receiving a sham (seawater) infection. The high-infection group received 2.5–3 times the amount of parasites as the low-infection group. We continuously increased the cercarial exposure per fish over the course of the experiment as the fish grew. For the low-infection group, the number of parasites added to the tank ranged from 1 to 124 cercariae per fish on average per exposure throughout the experiment, while the range for the high-infection group was from 2.5 to 313 cercariae per fish. A detailed overview of the infection regime is provided in Suppl. Table S1. During the first 12 wk of infections, we individually counted cercariae and placed them in scintillation vials (24-mm diameter × 61-mm height; 20-ml volume), which we topped off with warm (28 C), filtered seawater. Once infection exposures exceeded 12 cercariae per fish, we allocated parasites by a volume of parasite-laden seawater. We estimated the number of parasites in all finger bowls using sub-sampling counting methods and then pooled and aliquoted the parasite water into Qorpak jars (56-mm diameter × 70-mm height; 120-ml volume). Each tank had a designated jar, which we slowly lowered to the bottom of the tank using monofilament fishing line. We gave fish receiving a control treatment (i.e., uninfected) sham-treated jars containing cercaria-free seawater that was otherwise treated the same way as cercaria-laden water. We removed the jars from tanks 18–24 hr after infection.

Tissue processing

Following the 9-mo infection period, we euthanized the fish with a buffered solution of 250 mg/L MS-222. Immediately after, we weighed fish, decapitated them, and immediately froze the head on dry ice before being stored at –80 C for later analysis. We mounted frozen heads in Tissue-tek® (Sakura Finetek, Alphen aan den Rijn, The Netherlands) and immediately placed them on dry ice. Further, we sliced the mounted heads with a cryostat (Leica CM 3050, Leica Biosystems, Buffalo Grove, Illinois) at –20 C in serial 60 μm slices and thaw mounted the slices onto microscope slides (Menzel-gläser, Thermo Fisher Scientific, Waltham, Massachusetts); then, we refroze and stored the slices at –80 C. Before staining, we microdissected the frozen slides for target internal areas of the slice (data not included here). We microdissected the slices on a cold plate (–14 C) and did not dislocate metacercariae, as they were located on the brain surface and were not found on these internal areas. We fixated and stained the slices with Cresyl violet by thawing slides at room temperature for 1 hr, followed by immersion in: (1) 70% EtOH for 2 min, (2) Cresyl violet for 45 sec, (3) 70% EtOH for 10 sec, and (4) 100% EtOH for 10 sec, before air-drying them, mounting them with a cover glass, and sealing it in place using a film-forming polymer (IsaDora, Malmö, Sweden). We stored the slides in slide boxes at room temperature until analysis.

Image processing and parasite quantification

We digitalized the brain slices and visually analyzed the resulting images. We acquired the high-resolution images of the stained sections using an automated slide scanner system (Axio Scan Z1, Carl Zeiss Microscopy, Munich, Germany). We scanned the slides at ×10 magnification and compressed them together in 15-μm z-stacks. We visually analyzed the images using the Zen Lite Blue software (Carl Zeiss Microscopy), and we exported the scanned brain slices as TIFF files and then printed them to pdf. We used the pdf-printout for parasite quantification.

We initially screened 2 non-infected and 5 infected brains and recognized that the most reliable way of recognizing encysted *E. californiensis* metacercariae in the brain-slice scans was to identify the cyst wall and to use the scale bar in the Zen software to verify the parasite based on size (examples of parasites and non-parasite structures are included in Suppl. Fig. S1; example pictures of an infected brain slice and an uninfected brain slice are in Fig. S2). Encysted parasites are typically approximately 100 μm in diameter (Martin, 1950). In brain sections sliced at 60 μm, *E. californiensis* cysts would be potentially spread over 2–3 consecutive sections. Therefore, to avoid double counting parasites on these slices, we only counted structures with a diameter of 50 μm or more as encysted parasites. Further, we marked individual parasites on each scan to permit removal of them from counts on adjacent slices.

Subsequently, we thoroughly visually analyzed scanned brain slices from 16 experimentally infected fish using the Zen software. We identified brain regions from the brain slices using the related zebrafish stereotypic atlas by Wulliman et al. (1996). We used the 3 following general brain regions for the quantification analyses (arranged in order moving from the forebrain to the hindbrain):

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(1) telencephalon, (2) diencephalon and mesencephalon, and (3) rhombencephalon. We calculated the total number of parasites in each brain region. We roughly divided the brain into fore-, mid-, and hindbrain, and we counted parasites for these 3 entire brain regions.

Since there were individual differences in the number of brain slices corresponding to each brain area between individuals (due to differences in body size), it was necessary to correct and standardize the number of slides in each region of interest. The number of slices varied in the following manner: telencephalon 9–22 slices, diencephalon/mesencephalon 24–40 slices, and rhombencephalon 8–18 slices. To account for this inter-individual variability in brain size, we subdivided both the telencephalon and rhombencephalon into four sub-regions of interest (consisting of the mean parasites per slice for 2–6 slices per sub-region, depending on the total number of slices per brain region for each individual). Due to the larger surface area covered by the diencephalon/mesencephalon, we divided this area into 10 sub-regions of interest (consisting of the mean number of parasites per slice in 2–4 slices).

To assess the reliability of quantifying total parasite intensity (i.e., counts of the total number of parasites in a host) using this brain-slice quantification method, we quantified parasite numbers by means of brain squashing in 50 experimentally infected fish that died within ±1 mo of our sampling period. We weighed these fish and quantified parasites by removing the brains and squashing them between 2 glass slides, following Shaw et al. (2010). We also counted any parasites that fell off the brain during dissection.

We sought to assess whether our experimental, repeated infection protocol generated parasite intensities comparable to those observed in wild, naturally infected fish. To do this, we collected a sample of 25 wild late juvenile and adult killifish from the same marsh (KF) in April 2016. We sliced the brains as described above, but at 150 μm. For this analysis, we used this thicker slicing dimension due the larger body size in the wild fish. We quantified *E. californiensis* cysts in these fish using the description in Shaw et al. (2009) (counting the number of parasites on each slide using a microscope) and compared their mean intensities to those of our experimental fish.

Brain surface area

Because the *E. californiensis* metacercaria encysts on the brain meninges, the brain-region-specific surface areas influence the available space for parasites. We therefore measured the circumference of each brain slice in ImageJ (v.1.52K) in micrometers and multiplied this number by slice thickness to calculate the total surface area of each brain region, and then we transformed the brain surface area in square micrometers into brain surface area in square millimeters. Total surface area per region was then calculated as the sum of its individual slices. We then calculated surface area parasite density (metacercariae/mm^2 brain surface area) by brain region by dividing the total number of parasites in each brain region by its total surface area (i.e., number of parasites/mm^2 brain surface area).

Statistics

We used 2-way ANOVA tests to compare parasite intensity (total number of parasites in an infected individual) and surface area parasite density (parasites/mm^2 brain surface area) among the 3 brain regions and the 2 infection treatments (low and high). We performed Tukey post-hoc comparisons to determine differences among the 3 brain regions. We found no differences in fish body mass between the low-infection (mean ± SE = 0.61 ± 0.05 g) and high-infection (0.61 ± 0.03 g) groups, so fish body mass was not included in these analyses. We used 1-way ANOVA tests to compare parasite number and mass parasite density (parasites/g body mass) among the low-infection group, high-infection groups, and the naturally infected group. We used a Mann–Whitney U-test to compare the body mass of experimentally infected and naturally infected fish. To determine the relationship between the total number of metacercariae found on the diencephalon/mesencephalon and the total number of metacercariae on the brain, we used Spearman's rho test. We performed a Student's *t*-test on parasite counts to compare slicing and squashing methods. We checked assumptions for each statistical test through visual inspection of residual and qqnorm plots. We square root or log 10 transformed data as needed to meet assumptions of normality. We conducted all statistical analysis using JMP (JMP Pro 14.1.0).

RESULTS

Counting method

Quantification of parasite intensity (total parasite numbers in an infected individual) from brain slices (mean ± SE = 1,885.7 ± 171.1) yielded values comparable to counts from the traditional, brain-squashing quantification method (1,716.1 ± 145.9, $t_{(39)} = 0.75$, $p = 0.46$) in experimentally infected fish.

Experimental infections

Repeated experimental infections yielded infection intensities exceeding those obtained by previous experimental infection studies in both the low-infection (815.8 ± 93.7) and the high-infection (1,454.3 ± 214) treatment groups (Fig. 1A). The high-infection treatment group achieved infection intensities similar to naturally infected fish (1,880.8 ± 164.9). Both the high-infection and naturally infected groups exhibited significantly higher parasite intensities than the low-infection treatment group (1-way ANOVA: $F_{(2,40)} = 11.76$, $p < 0.001$; Tukey post-hoc: high exposure–naturally infected: $p = 0.34$, low exposure–high exposure: $p = 0.02$, low exposure–naturally infected: $p < 0.001$).

However, the body mass of the experimentally infected (laboratory-reared) fish (0.62 ± 0.05 g; Fig. 1B) was significantly lower than wild-caught fish (5.41 ± 0.4 g; Mann–Whitney U-test: $p < 0.001$). Consequently, mass parasite density (parasites/g body mass) in experimentally infected fish (1,392.3 ± 84.1 parasites/g body mass [low], 2,379.0 ± 221.0 [high]) exceeded the values observed in naturally infected fish (382.9 ± 36.0; Fig. 1C; 1-way ANOVA: $F_{(2,40)} = 55.6$, $p < 0.001$; Tukey post-hoc: high infection treatment–naturally infected $p < 0.001$, low infection treatment–naturally infected $p < 0.001$, low infection treatment–high infection treatment $p = 0.039$).

Parasite distribution

Surface area parasite density (parasites/mm^2 brain surface area) was higher in the high-dose compared to low-dose treatments (49.4 ± 6.0 vs. 30.0 ± 1.7 parasites/mm^2 brain surface area). The

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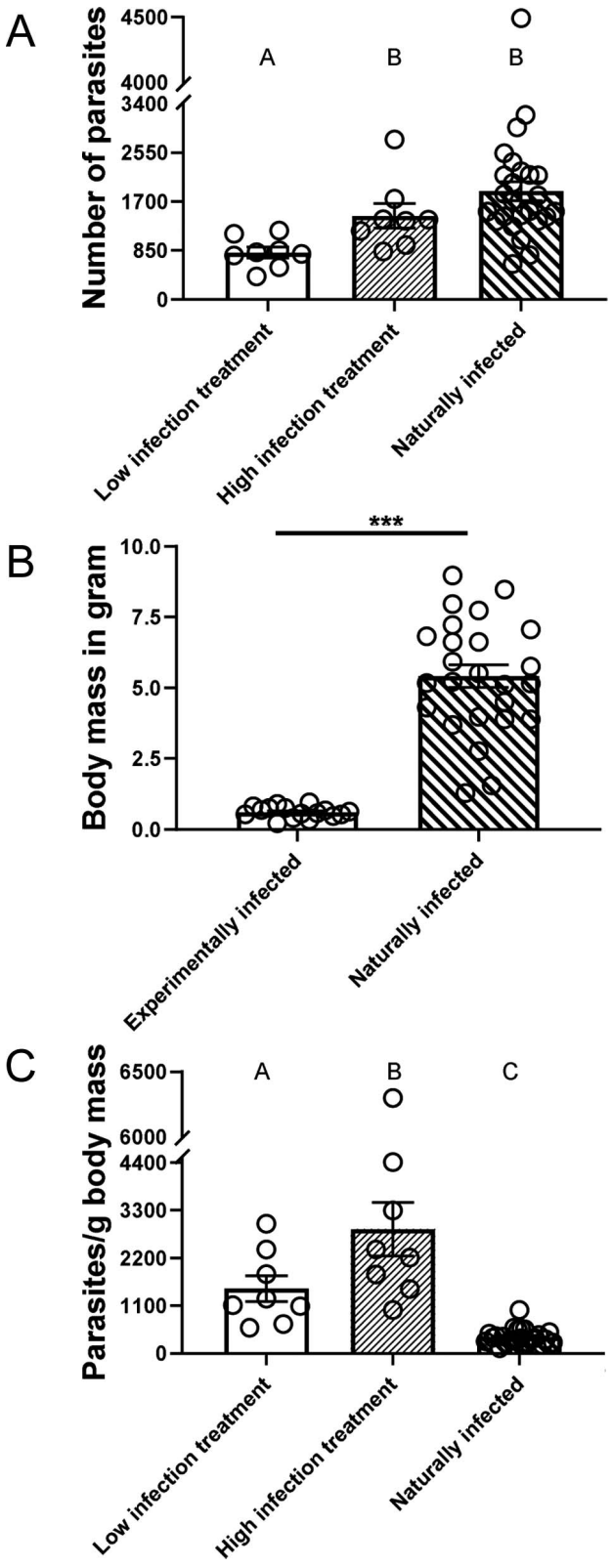


Figure 1. Figure displaying the number of *Euhaplorchis californiensis* parasites (**A**), weight (**B**), and mass parasite density (parasites/g body mass) (**C**) in experimentally infected and naturally infected fish. Different letters indicate differences in Tukey post-hoc test performed after a 1-way ANOVA; stars indicate significant difference in the Mann–Whitney U-test. On the *y*-axis, we show the number of parasites (**A**), body mass in grams (**B**), and parasite density (parasites/g body mass) (**C**), and on the *x*-axis, we show the different treatment groups: low-infection treatment (n = 8), high-infection treatment (n = 8), experimentally infected (n = 16), and naturally infected (n = 25).

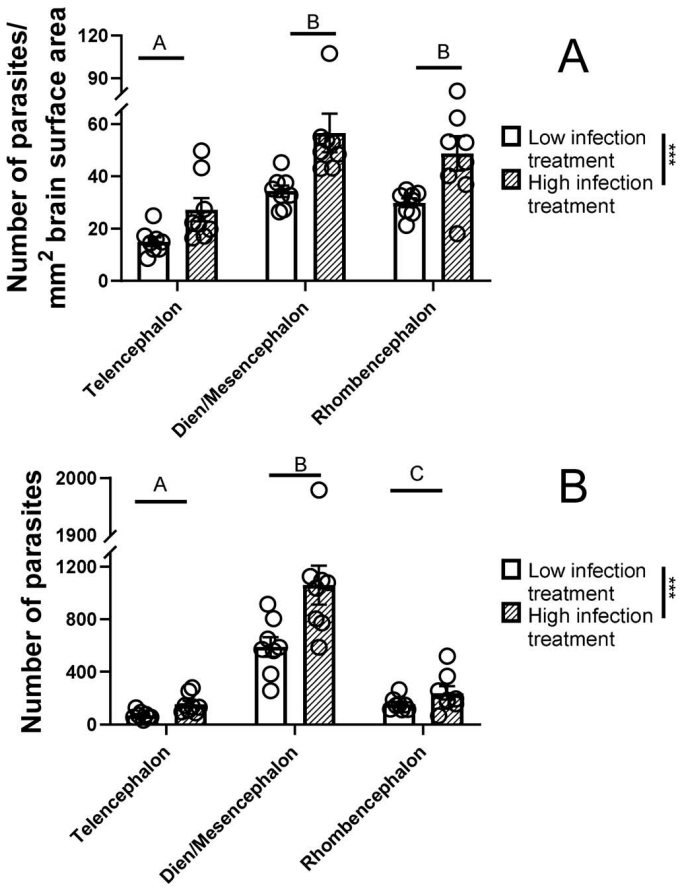


Figure 2. Contrasting *Euhaplorchis californiensis* brain surface parasite density (**A**) and parasite numbers (**B**) in 3 brain regions in both high- and low-infection treatment groups. Different letters indicate the statistical difference between the different brain regions given by a Tukey post-hoc test, and the stars indicate a statistical difference between the 2 infection treatments (low and high). In all groups, n = 8. Brain surface parasite density is defined as the number of parasites per square millimeter of brain surface area.

parasites achieved higher densities in the diencephalon/mesencephalon and the rhombencephalon compared to the telencephalon (Fig. 2A; 2-way ANOVA: $F_{(5,42)} = 14.2$, $p < 0.001$, brain area: $F_{(2,42)} = 22.3$, $p < 0.001$, treatment: $F_{(1,42)} = 24.5$, $p < 0.001$, treatment × brain area: $F_{(2,42)} = 0.12$, $p = 0.89$; Tukey post-hoc comparisons: telencephalon–diencephalon/mesencephalon $p < 0.001$, telencephalon–rhombencephalon $p < 0.001$, diencephalon/mesencephalon–rhombencephalon $p = 0.34$, low–high infection regime $p < 0.001$).

Parasite intensity was approximately 7× greater on the diencephalon/mesencephalon than the telencephalon, and approximately 3.5× greater than on the rhombencephalon. (Fig. 2B; 2-way ANOVA $F_{(5,42)} = 43.4$, $p < 0.001$, brain area: $F_{(2,42)} = 96.9$, $p < 0.001$, treatment: $F_{(1,42)} = 21.0$, $p < 0.001$, treatment × brain area: $F_{(2,42)} = 1.0$, $p = 0.31$; Tukey post-hoc comparisons: telencephalon–diencephalon/mesencephalon $p < 0.001$, telen-

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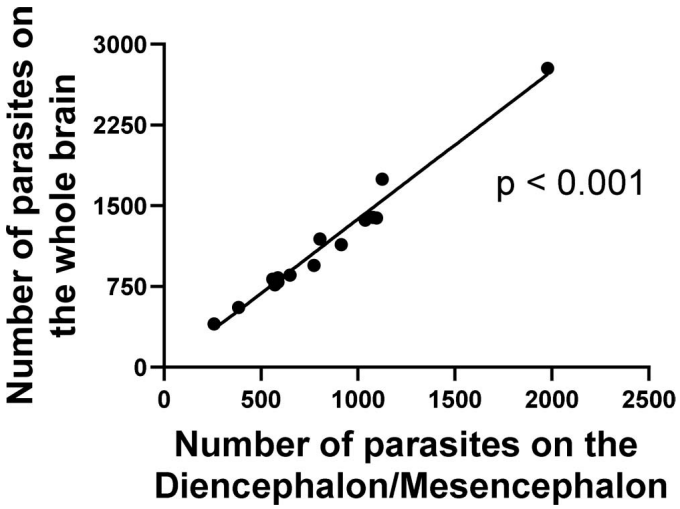


Figure 3. Correlation between the number of encysted *Euhaplorchis californiensis* metacercariae on the whole brain of California killifish (*Fundulus parvipinnis*) and the number of parasites on the diencephalon/mesencephalon region in experimentally infected *F. parvipinnis* individuals. The correlation was checked using Spearman's rho (= 0.98).

cephalon–rhombencephalon $p < 0.001$, diencephalon/mesencephalon–rhombencephalon $p < 0.001$). There was a strong correlation between the number of parasites in the diencephalon/mesencephalon and the total number of parasites on the entire brain (Spearman's rho = 0.98, $p < 0.001$; Fig. 3).

Brain surface area pattern was equal for both infection groups, and the diencephalon/mesencephalon had >3× the surface area compared to either the telencephalon or rhombencephalon (Fig. 4A). Examining *E. californiensis* surface area parasite density and intensity at a higher spatial resolution revealed specific peaks in abundance in different parts of the brain regions, specifically in the diencephalon/mesencephalon, for both the low- and the high-infection groups (Fig. 4B–E).

DISCUSSION

Using an experimental infection protocol capable of reproducing natural infection intensities, we were able to show that *E. californiensis* metacercariae occur at the highest surface area densities on the diencephalon/mesencephalon and the rhombencephalon brain regions, with few parasites aggregating on the telencephalon. Examining surface area parasite density at higher resolution revealed specific peaks in abundance within these brain regions, specifically in the anterior of the diencephalon/mesencephalon and the posterior mesencephalon/anterior rhombencephalon. These density peaks suggest that the parasites may primarily target the mid- and hindbrain areas to manipulate their host's behavior.

There are a number of mechanistic reasons that this behavior-modifying parasite would preferentially settle in these brain regions. The rhombencephalon (which contains serotonergic cell bodies of the raphe region) is associated with locomotion and basal physiology (Wulliman et al., 1996). Infected fish exhibit increased frequency of surfacing (Lafferty and Morris, 1996) as well as altered serotonergic/dopaminergic activity (Shaw et al., 2009; Shaw and Øverli, 2012), indicating that *E. californiensis* individuals might indeed target areas within the rhombencephalon, for instance, the serotonergic system. Neuronal networks in the diencephalon/mesencephalon areas, such as the hypothalamus and optic tectum, have been associated with stress coping, reproductive physiology, and associated behavioral outputs (Wulliman et al., 1996). *Euhaplorchis californiensis* cysts may aggregate in this area to better manipulate systems associated with stress and/or reproduction. With respect to concentrating in the diencephalon/mesencephalon, it is interesting to note that some of the behaviors typical of infected California killifish (Lafferty and Morris, 1996) are similar to mating behaviors of a closely related killifish species (Newman, 1907). Because mating behavior is largely controlled by the hypothalamic–pituitary–gonadal (HPG) axis (which is within the diencephalon/mesencephalon brain area), it could be interesting to investigate if *E. californiensis* cysts specifically influence components of this system. That is, by stimulating this system, the parasite could promote the expression of conspicuous reproductive behaviors (e.g., flashing) under non-relevant ecological situations (e.g., while feeding), rendering fish more prone to predation by the parasite's final host birds. In this context, Weinersmith et al. (2016) measured water-borne levels of 11-ketotestosterone (11-KT), the primary end point in the male teleost HPG axis, in both male and female infected California killifish, but they found no effect of infection. However, it is important to note that the fish in that study were not sexually mature, and changes in 11-KT may only occur in sexually mature fish. More work is thus needed to explore the role of the HPG axis in manipulation of killifish behavior by *E. californiensis* cysts.

To our knowledge, only a few previous studies have found that trophically transmitted parasites tend to cluster in specific brain regions (Hendrickson, 1979; Shirakashi and Goater, 2002; Berenreiterová et al., 2011; McConkey et al., 2013). Such aggregation implies that the parasites are targeting specific brain regions for host behavior modification, versus the behavioral effects arising as a simple outcome of general pathology. Interestingly, a few of these cases also indicate that parasites aggregate in specific brain areas to more efficiently induce behavioral changes. For example, the brain-infecting trematode *Ornithodiplostomum ptychocheilus*, found in fathead minnows (*Pimephales promelas*) , aggregates mainly in the mesencephalon, specifically the optic tectum, which encodes visual stimuli (Shirakashi and Goater, 2001). Notably, infected minnows exhibit a reduction in visually mediated behaviors and spend more time at the water surface, presumably increasing their risk of predation (reviewed in Barber et al., 2000). Additionally, a closely related killifish species (*Fundulus similis*) infected by another *Euhaplorchis* species (*Euhaplorchis* species A) spends more time near the water surface as the intensity of infection increases (Fredensborg and Longoria, 2012). Because *E. californiensis* cysts also aggregate in the diencephalon/mesencephalon (this study), and infected California killifish do more abrupt dashes to the surface (Lafferty and Morris, 1996), it is possible that they use some of the same host-manipulating mechanisms. Further studies should, therefore, examine whether *E. californiensis* metacercariae manipulate processes driven by the optic tectum.

An alternative explanation for why the parasites aggregate in the posterior regions of the brain is that the parasites simply encyst in whatever region of the brain they first encounter, and they move to the next closest brain region to avoid crowding. While this idea is sensible because the brain stem is thought to be

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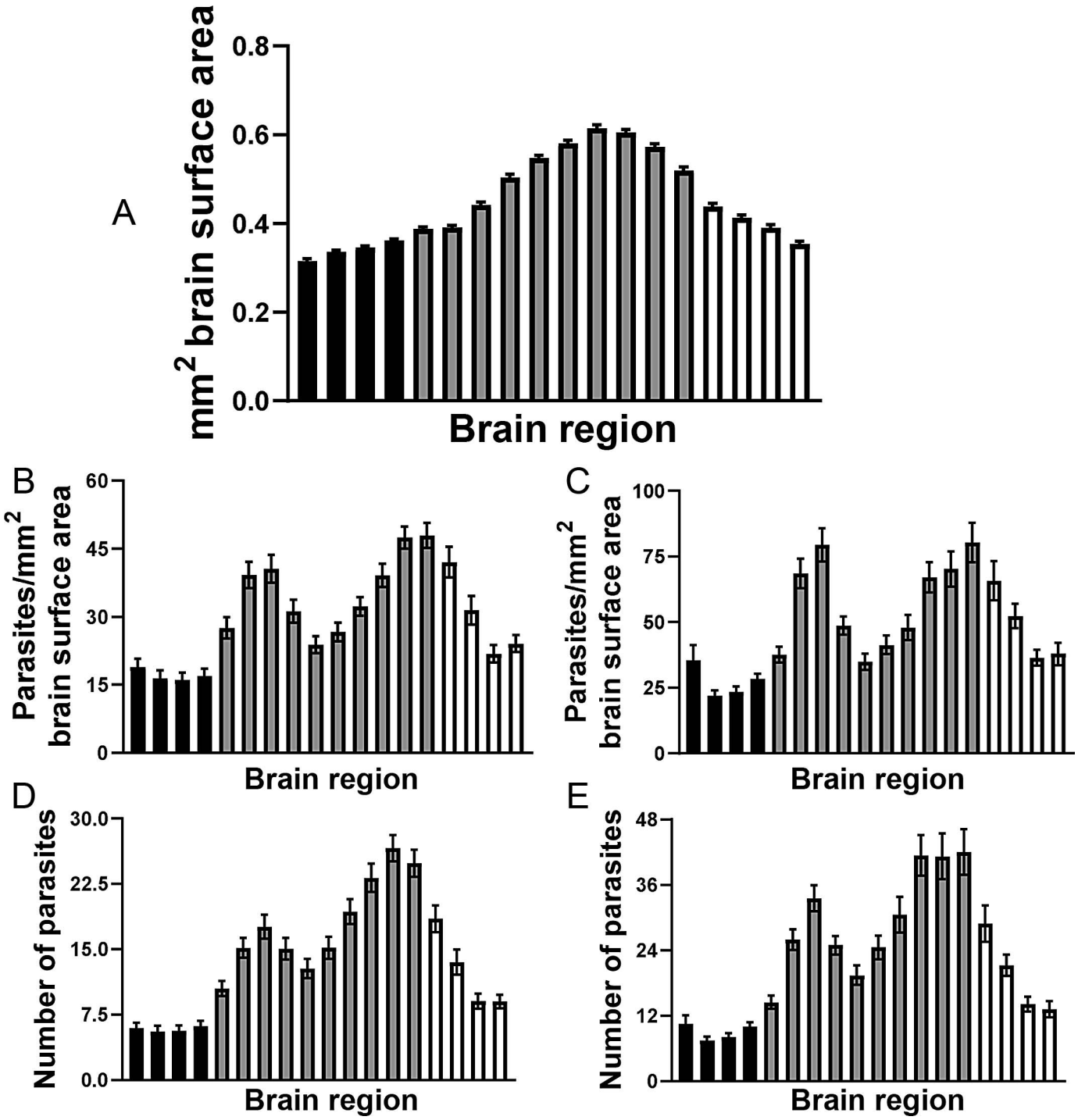


Figure 4. Mean (± SE) total brain surface area (**A**), parasites found per square millimeter of brain surface area (i.e., parasite density) for low-infection (**B**) and high-infection (**C**) treatment groups, and**,** total parasite numbers found in each brain region for low-infection (**D**) and high-infection (**E**) treatment groups, respectively. The brain regions of interest, the telencephalon (black bars), diencephalon/mesencephalon (gray bars), and the rhombencephalon (white bars), are located on the *x*-axis. In order to make comparisons among individuals, it was necessary to standardize each brain region into sub-regions. That is, both the telencephalon and rhombencephalon were divided into 4 sub-regions of interest. Due to the large surface area covered by the diencephalon/mesencephalon, this area was divided into 10 sub-regions of interest. Note that the figure in (**A**) illustrates the total brain surface area in each sub-region, which, when compared to the brain surface parasite densities in both treatment groups, shows that the parasite aggregation numbers are not dependent upon available surface area.

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the first region encountered by the parasite, and it has some support from the low infections achieved in Shaw et al. (2009), our higher-intensity experimental infection data suggest that this is not the case. First, *E. californiensis* surface area parasite densities were consistently highest in the rhombencephalon and diencephalon/mesencephalon in *both* our high- and low-infection fishes. Hence, there is no evidence that parasites first obtained high surface area parasite densities in the rhombencephalon and then infected more anterior regions as crowding increased. Second, our higher-resolution spatial mapping of surface area parasite density clearly demonstrates that the parasites do not aggregate in the posterior regions; surface area parasite density exhibited 2 distinct peaks, 1 in the anterior diencephalon/mesencephalon and 1 in the posterior mesencephalon/anterior rhombencephalon. Even the most posterior of these peaks is anterior of the brain stem, showing that *E. californiensis* cysts do not simply infect the brain at the uncrowded areas they find anterior to the spinal cord.

However, the notion that *E. californiensis* cercariae enter the brain via the spinal cord is not well documented (it rests on inference from qualitative observations in an unpublished master's thesis on a congeneric parasite). Although it seems very plausible that the parasites do enter via the spinal cord, they may also follow major nerve tracts to the brain. For instance, the 2 distinct density peaks could be caused by *E. californiensis* cercariae following the optic nerve (for the anterior peak) and cranial nerves (for the posterior peak). Although we were unable to observe the nerves in our brain slices, the peaks are positioned at the approximate locations where the optic and cranial nerves enter the brain (Wulliman et al., 1996). Interestingly, even if parasites aggregated in brain regions near their points of entry, this could still set the stage for the parasites evolving to manipulate neurological processes specific to those brain regions. Hence, clearly distinguishing the routes that *E. californiensis* cercariae follow to enter the brain presents an exciting line of future research.

Through this work, we designed a new experimental infection protocol that achieved ecologically relevant parasite infection intensities and created a method for counting parasites from brain slices. Our experimental infections yielded parasite intensities greatly surpassing those achieved in previous research (e.g., >815 vs. <100 in Shaw et al., 2009). Our high-infection group exhibited infection intensities within the range typically found in populations of wild-infected California killifish (Shaw et al., 2010), and those we collected from the same source population (KF) as our experimentally infected fish. However, the mass parasite densities surpassed those in our wild sample (i.e., $\sim$6 times higher for the high-infection group and $\sim$3 times higher for the low-infection group). Based on the natural history of *F. parvipinnis* (Fritz, 1975), we expect that our experimental fish were similar in age to many of those in the wild sample. However, it appears that our experimental fish did not grow as fast as they would have in the wild, as they were smaller than those in the wild sample. As mass parasite density is likely an important factor modulating the parasites' ability to manipulate their host (Shaw et al., 2009; Shaw and Øverli, 2012; Weinersmith et al., 2016), further refinement of the fish husbandry procedures is likely required to better replicate the body size observed in nature.

Our results demonstrate that counting parasites from slices provides comparable counts to those from brain squashes. While the brain-squashing method permits rapid and reliable counts of the total number of parasites, it does not permit region-specific physiological, neurochemical, molecular, or surface-area parasite density data (as obtained here) to be obtained. Further, there was a strong correlation between the number of parasites in the diencephalon/mesencephalon and the total number on the brain. This is not surprising, given that the highest parasite numbers were found on the diencephalon/mesencephalon (given its greater total surface area and higher parasite densities). Nevertheless, it is clear that we can use this strong correlation to estimate total intensity on the brain by exclusively counting parasites from the diencephalon/mesencephalon (for sub-sampling techniques, see Shaw et al., 2005). Hence, using the slice counting method permits both a wide range of brain-region-specific sampling while also permitting quantification of the individual host's total parasite intensity.

In short, our experimental infection regime and brain-slice parasite counting technique permitted a higher-resolution understanding of how brain parasites interact with their host. We revealed that *E. californiensis* cysts concentrate most in 2 areas of the brain, potentially providing clues to the route of parasite entry to the brain and the specific neurological mechanisms underlying the parasites' manipulation of host behavior.

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