

Supplementary material for “A brain-infecting parasite impacts host metabolism both during exposure and after infection is established”

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1. Supplementary Materials and Methods

(a) Gamete collection

Wild, adult California killifish were caught in August 2016 using a two-pole seine, from the San Elijo Lagoon Ecological Reserve (SE) in San Diego County, CA, USA (33.01°N, 117.26°W; a population naturally exposed to our focal parasite, *Euhaplorchis californiensis*). This killifish species spawns from April to September, approximately one to three days before the new and full moon (Hubbs 1965; Fritz 1975). During breeding season, mature male and female killifish can be identified by their size (typically >46 mm TL), and mature males develop a strong brassy-yellow coloration and scale tubercles (Fritz 1975). Male and female killifish were temporarily separated into five-gallon buckets filled with aerated seawater until gamete collection.

Gametes were collected following Strawn and Hubbs (1956), by gently stroking the fish ventrum from the operculum to the vent. Eggs from gravid females were placed into a petri dish, with just enough filtered seawater to cover them, and fertilised using sperm from approximately 4 – 10 males, until the seawater was a milky consistency. After ten minutes, we rinsed the eggs through a fine mesh net with filtered seawater. Eggs were returned to the laboratory at Scripps Institution of Oceanography. Eggs were housed for 17 days in glass bowls (10 cm ID) containing approximately 75 eggs each in filtered, aerated seawater containing methylene blue (3 mg/L; to minimise fungal growth on the eggs during development). After this point, we maintained the eggs in only filtered, aerated seawater until hatching at approximately 21 days post-fertilization. Throughout this period, water changes were completed daily to maintain clean conditions.

(b) Fish rearing

All fish hatched within a 22-day period (September 6-28, 2016). Killifish fry were transferred in groups of 5, iteratively as they hatched (to minimize age variation among tanks), to 16 different 38L glass holding tanks (51 x 27 x 32 cm). This process continued until each tank held 20-21 fish. Tanks were randomly assigned to one of two treatments: 1) uninfected and 2) infected by *E. californiensis*. Fish were reared in these tanks for a period of 13 months (October 2016 – November 2017) under ambient seasonal water temperature (~18°C in winter, ~21°C in summer) and light cycle conditions (from 11:13 light:dark cycle in winter to 13:11 light:dark cycle in summer). Tanks were opaque on three sides to prevent fish in adjacent tanks from seeing each other and moveable black curtains covered the front side of the tank (outside of times for feeding and infection procedures) to prevent uncontrolled visual stimuli from the surrounding area. Fish were fed a growth-promoting body-mass specific diet daily (that prevented compensatory foraging) with live-hatched *Artemia* sp. until they were approximately three months post-hatch, at which point they transitioned to a varied diet composed of frozen brine shrimp, Skretting aquaculture feed, and pea puree.

This study was part of a larger project examining the influence of *E. californiensis* on killifish physiology, neurobiology, and behaviour throughout development. The fish tested in this study were reared in four and five tanks (for the uninfected and infected treatments respectively) for the first ten months after hatching. At the end of this period, some of these fish from each

replicate tank were removed for behavioural and neurophysiological studies that are not reported here. The remaining fish were then re-housed in two and one tanks (for the uninfected and infected treatments respectively) for the final months before testing. Although sub-optimal given potential tank effects, these fish were combined into fewer tanks to avoid stress associated with social isolation at low densities (this group-housing mimicked the group sizes and densities experienced during rearing; group size = ~12 individuals, density ~0.6 fish/L). Further, this study is most interested in the change of fish metabolic rate and activity from baseline levels when exposed to cercariae (infectious parasites). Hence, each fish serves as its own control. In addition, the three group-housing tanks were all extremely similar. They were of identical construction and shared the same flow-through seawater supply. Further, they were placed on the same side of a tank rack, with one of the control tanks being immediately next to the infected tank, with the second control fish housing tank being on the lower shelf. All these factors would help preclude tank effects driving a difference between the control and infected killifish, with any differences likely to have arisen from the differences in infection regime during the preceding 10 months. Fish were not tagged prior to re-housing so their original tank of origin could not be included in subsequent analyses. At the time of testing (November 2017), on average, individuals were 13 months old, 0.59 g (mean $\pm$ SE; Control: 0.65 ± 0.07 g; Infected: 0.51 ± 0.06 g), and 28.9 mm in length (Control: 29.88 ± 0.96 mm; Infected 27.82 ± 0.74 mm). In this study, a total of 24 individuals were tested (13 uninfected and 11 infected killifish).

(c) Long-term infection procedure

Throughout the 13-month rearing period, groups in the infected treatment were exposed to *E. californiensis* cercaria (measuring ~190 μ m long x 55 μ m wide, Martin 1950) twice weekly in their home tanks, while uninfected groups were concurrently exposed to a seawater sham. The dose of cercaria exposure per infection event increased gradually throughout the entire rearing period, in line with the fish's growth in body size. Initially, fish were exposed to a dose reflecting an average of two cercariae/fish/infection event. This exposure dose doubled every four to eight weeks throughout the rearing period, until the dose reached the maximum average of 100 cercariae/fish/infection event. As individuals were infected in groups, each individual almost certainly encountered more or less cercariae than this average. However, following a total of 97 infections over the course of 13 months, much individual variation in cercaria exposure would be averaged out. Most importantly, the difference between the treatment groups would be substantial, given the zero-level dosing of uninfected fish.

To accomplish this procedure, California horn snails (*Cerithidiopsis californica*) were collected from the University of California Kendall-Frost Natural Reserve (KF) in Mission Bay, San Diego County, CA, USA (32.80°N, 117.23°W). Individual snails were "shed" three times to confirm snail infection status (see below for the general shedding process). Infected snails were maintained in mud-flat mesocosms with a simulated tidal cycle at Scripps Institution of Oceanography.

Twenty-four hours prior to experimental fish infections, infected snails were retrieved from the mesocosms and kept out of water in a warm, moist area, to permit an accumulation of cercariae prior to shedding. Following this 24-hour period, infected snails were placed in separate glass bowls (10 cm ID; 5-6 snails per bowl), with warm (27-30°C) filtered seawater,

under an incandescent light for two to four hours. We then counted all shed cercariae and reconfirmed their species. Cercariae were then aliquoted for each tank into a tank-specific vial. Aliquoting was initially accomplished by counting parasites individually for each tank, and putting them into a 20 mL scintillation vial (24 mm diameter x 61 mm height) using a glass pipette. Once the cercaria exposure per infection event exceeded eight cercariae per fish, we aliquoted cercariae by approximating the number of cercariae per mL and aliquoting a volume of cercariae-laden water to a 120-mL polypropylene jar (56 mm diameter x 70 mm height) using a 30 mL plastic volumetric pipette (“turkey baster”). A small fishing weight affixed with non-toxic epoxy (hot glue gun) to the exterior of each jar ensured it dropped to the bottom of the tank. A piece of fishing line was also affixed to the jar and hooked onto the outside of the tank when the jar was dropped in to permit minimising disturbance to the fish when the vials were retrieved from the tanks 18-24 hours after the infection event. The final cercaria exposure occurred one day prior to the start of respirometry testing, so fish in the infected treatment experienced their final exposure one to eight days prior to testing.

The long-term infection procedure resulted in *E. californiensis* intensities ranging from 352 to 1783 metacercariae per killifish (mean $\pm$ SD: 1190 ± 308 metacercariae per fish) and densities ranging from 677 to 4100 metacercariae g⁻¹ fish (2528 ± 1040 metacercariae g⁻¹ fish) in the infected treatment, which is comparable to the infections documented in wild fish (Shaw *et al.* 2010). Dissections at the end of the experiment confirmed that none of the uninfected treatment fish had any established metacercariae. Three of the 11 fish in the infected treatment also contained 1-2 “small cyathocotylid” metacercariae, which would have originated from a rare doubly-infected snail getting through our screening process. Because they only constituted 0.02% of the total metacercariae recovered from killifish hosts, and they are smaller than *E. californiensis*, it is extremely unlikely that our experimental results were influenced by the cyathocotylid metacercariae.

(d) Preliminary experiment on timing of cercaria attack (attachment and penetration of killifish)

Five wild-caught killifish (30-40mm TL) were exposed to *E. californiensis* cercariae; four of these killifish were exposed immediately following euthanasia via cervical dislocation, and one was placed in cercariae-laden water for 15 mins prior to euthanasia. The latter exposure was used to confirm that cercariae infected a living fish with similar timing to the freshly euthanized fish, with a particular focus on cercariae being swept into the orobranchial chamber via respiratory pumping). Each cercaria exposure occurred in separate plastic wells (3.6 – 4.9 cm W x 3.2 cm L x 2.7 – 3.2 cm H) with 30mL of seawater containing 1000-2000 cercariae shed from three *C. californica* snails. To record where and when cercaria attachment and penetration occurred, the cervically-dislocated fish were observed and filmed (Canon EOS T6i 24MP DSLR) during the first 15 mins of exposure under a dissecting microscope (1.7x - 11.25x). We also filmed individual cercariae penetrating mounted, excised gill filaments at the compound microscope (27.6x). Observations of cervically dislocated fish confirmed that attachment occurred within 15 mins.

(e) Intermittent-flow respirometry and acute cercaria exposure

For each fish, metabolic rate was measured following an intermittent-flow respirometry methodology tailored for social fishes (Nadler *et al.* 2016a; Svendsen, Bushnell & Steffensen 2016). Briefly, each respirometry chamber was composed of two cylindrical glass tubes: an inner tube housing the focal fish (length = 10 cm, inner diameter = 3.8 cm, total volume of chamber and associated gas-impermeable tubing = 100 ml) and the outer tube housing shoal-mates (length = 8.5 cm; inner diameter = 12.0 cm, total volume of chamber minus volume occupied by inner chamber = 860 mL). This adaptation on traditional intermittent-flow respirometry provides visual and olfactory cues of shoal-mates to the focal individual in the respirometry chamber (see Nadler *et al.* 2016a for detailed methodology). The outer chamber was affixed to the exterior of the inner chamber and was used to provide visual and olfactory cues of three shoal-mates to the focal individual. This larger chamber was aerated with a continuously running flush pump. To provide olfactory cues of shoal-mates to the focal individual, the water leaving the outflow port was attached to the in-flow vent for the inner chamber's flush pump. The inner chamber was connected to a recirculating pump (to mix water in the respirometer) and a flushing pump that flushed the chamber with oxygen-saturated water for 5 min between each 17 min measurement period. Each chamber was immersed in a separate water bath (temperature: 17-19°C). Temperature was controlled by using heaters in a primary sump, which was plumbed to each individual water bath.

Dissolved oxygen concentration in the inner, focal chamber was measured every 2 s and logged using a Fire-Sting fibre-optic oxygen meter (Pyroscience, Germany), connected to a computer. The oxygen-sensing optode was placed in the recirculation loop in a flow-through cell, to ensure that flow was sufficient for a fast response time of the sensor (Svendsen *et al.*, 2016). Each chamber was immersed in a separate water bath and temperature was controlled through heaters in a primary sump, which was plumbed to each individual water bath (temperature: 17-19°C). Focal fish were fasted for 24 hours prior to experimentation to ensure that they were in a post-absorptive state and were left undisturbed in the respirometers for 20-23 hours. The measurement period used was 17 min (during which time the flushing pump was off, and the rate of oxygen uptake ($\dot{M}_{O_2}$) recorded) followed by a 5 min flushing period. The timing of this flushing-measurement cycle ensured that oxygen remained above 80% air saturation at all times (Hughes 1973). Slopes (s) were calculated from plots of oxygen concentration versus time using ordinary least squares linear regression (LabChart v6) and converted to $\dot{M}_{O_2}$. Background respiration was measured in all chambers for three measurement periods both before and after trials. We subtracted microbial respiration from all fish respiration measurements, assuming a linear increase in microbial respiration between the measured endpoints (Rodgers *et al.*, 2016).

From this data, two measures of metabolic rate were analysed: SMR and RMR. SMR was calculated as the lowest 10th percentile of all $\dot{M}_{O_2}$ measurements on an individual fish (Chabot *et al.*, 2016; Killen, 2014). Second, RMR was calculated as the mean $\dot{M}_{O_2}$ excluding the first 5 hours in the respirometer, during which time the fish is recovering from handling stress and acclimating to the respirometry chamber (Killen *et al.*, 2011).

Following the measurement of SMR and RMR, the acute response of $\dot{M}_{O_2}$ to cercaria exposure was quantified. Individual fish were first exposed to a seawater sham. This treatment

was repeated three times in sequence, with one flushing-measurement cycle (23 mins total, including 17 min measurement period plus 5 min flush cycle) between each sham treatment. Individuals were then exposed to 300 cercariae, which were obtained via shedding as outlined above. This number of cercariae was higher than the mean of 100 cercariae per fish in prior group infections (see above) to help ensure a response, but would be well within the range of what fish would have encountered during those prior infections given stochasticity around the mean. As with the seawater sham treatment, cercaria exposure treatments were also repeated three times, each separated by one flushing-measurement cycle. During each replicate cercaria exposure treatment, we quantified baseline oxygen consumption of equivalent numbers of cercariae by themselves in an empty chamber, permitting subtraction from calculations of exposed fish $\dot{M}_{O_2}$. Each of these treatments (seawater sham and cercaria) were administered through a three-way valve in the tubing between the flushing pump and the chamber. The treatment was administered by closing this valve, injecting 5 mL of treatment (sham or cercaria), and completed by injecting an additional 5 mL of seawater to flush the tubing to the chamber. A preliminary food-dye test confirmed that this procedure successfully deposited the stimulus into the chamber during each treatment. Following the final cercaria exposure treatment, each fish's post-exposure $\dot{M}_{O_2}$ was continuously recorded for an additional three measurement cycles. We also continuously recorded each fish's behaviour using a webcam (H264 Webcam Software), and quantified activity by measuring the number of 180° turns for the last 15 mins of each measurement period (see methods in Nadler *et al.* 2016a; Nadler *et al.* 2016b). The first two minutes of the measurement period were discarded for activity measurements to allow time for the cercariae to be injected into the chamber. Acute metabolic rate (MR_{acute}) was analysed during the measurement period of each replicate exposure treatment (seawater, cercaria, post-exposure).

Because there was some evidence that previously exposed fish mounted a stronger metabolic response to acute parasite exposure, we more directly tested whether this was the case by calculating each individual's change in $\dot{M}_{O_2}$ during cercaria exposure as compared to its previous sham exposures, using the following equation:

$$\Delta MR_{acute,x} = C_x - \frac{\Sigma SW_{1,2,3}}{3}$$

where C_x refers to oxygen uptake during each respective cercaria exposure ($x = 1, 2$ or 3) and SW refers to seawater sham. As there was no significant difference among the three sham exposures in either long-term treatment, the mean of these three measurements presented a baseline from which to measure changes in metabolic rate due to cercaria pre-attack, attack, and early infection.

As killifish are sensitive to handling stress (Weinersmith *et al.* 2016), MMR was measured using the chase method after the above procedures were completed in order to ensure that individuals reached SMR prior to subsequent testing, and allowed us to look at relative differences in MMR with long-term infection treatment (Roche *et al.* 2013; Rummer *et al.* 2016). This method elicits anaerobic exercise in individuals and maximal rates of oxygen uptake were measured during subsequent recovery, which is an accepted and repeatable method for determining a relative value for MMR among individuals (Killen, Norin & Halsey 2017). Fish were considered exhausted when they no longer responded to chasing with burst swimming.

Fish were then air-exposed for 30 s to further ensure that they had depleted all endogenous oxygen stores and then transferred to their respective respirometry chambers. $\dot{M}_{O_2}$ was measured for 10 mins, a time frame that ensured that oxygen saturation in the water remained > 80% air saturation (Hughes, 1973). These oxygen uptake slopes were measured at 3-min intervals, with the greatest oxygen uptake during this period taken as MMR. From an individual's SMR and MMR, we calculated their aerobic scope (AS).

Sampling of fish tissues commenced one hour following the chase procedure. Fish were taken out of respirometry chambers and euthanised using an overdose of MS-222 (250 mg/L). They were first weighed (+/- 0.01 g) and measured (standard length, SL, +/- 0.5 mm). Their brain, gills (2nd and 3rd gill arches), and white muscle were then rapidly sampled and flash frozen on liquid N₂. From infected fish, metacercariae were quickly removed from the brain tissue prior to freezing and counted to quantify infection intensity in each fish. These tissues were then stored at -80°C until enzyme activity analysis. Following tissue extraction, the remaining body tissue was compressed between two translucent plates and assessed for the number of new parasite infections that occurred during the acute exposure. This was possible because it takes at least several hours for *E. californiensis* to reach the brain (Helland-Riise et al., unpublished data) and weeks to fully encyst (Martin 1950).

(f) Energy metabolism enzyme assays

Tissue samples were homogenised and analysed for activity of the key metabolic enzymes lactate dehydrogenase (LDH) and citrate synthase (CS) according to published protocols (Thibault, Blier & Guderley 1997; Seebacher *et al.* 2003; McClelland *et al.* 2005). Frozen brain, white muscle, and the left second gill arch from uninfected and infected killifish were rapidly weighed, placed in 10 vol of ice-cold homogenization buffer (50 mM HEPES, 1mM EDTA, 0.1% Triton X-100, pH 7.4), and immediately homogenized on ice using a hand pestle. All summary information on the weight of tissues collected for enzyme assays can be found in Table S1. The homogenate was pulse centrifuged (< 3 s) to remove larger debris and the resulting supernatant was divided into aliquots for analysis of LDH and CS. Enzyme activity was determined spectrophotometrically (SpectraMax iD3, Molecular Devices, San Jose, CA). LDH activity was measured through the depletion of nicotinamide adenine dinucleotide (NADH) at 340 nm. The LDH assay buffer contained 50 mM Tris, 5mM NADH and 25 mM sodium pyruvate (pH 7.4). CS activity was determined through the increase in absorbance at 412 nm due to the reaction between free co-enzyme A (CoA) and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). The CS assay buffer contained 50 mM Tris, 1 mM DTNB, 12 mM acetyl CoA and 20 mM oxaloacetate. For LDH assays, a 1:50 (tissue:homogenization buffer) dilution was used. A 1:10 dilution was maintained for CS assays. For the LDH assay, 10µL of appropriately diluted tissue homogenate were mixed with 190µL of LDH assay buffer (total well volume = 200µL). For the CS assay, 10µL of appropriately diluted tissue homogenate were mixed with 205µL of CS assay buffer (total well volume = 215µL). These dilutions were optimized during preliminary studies to ensure $R_{sq} > 0.95$ in the change in absorbance through time in all tissues and assays. All assays were performed in triplicate at 27°C for a 10 min incubation period. Control samples were assayed without sodium pyruvate and oxaloacetate for LDH and CS respectively to control for background activity. Procedures for LDH and CS assays were modified from McClelland et al.

(2005) and Dalziel et al. (2012). Relative anaerobic potential was calculated by dividing LDH activity over CS activity for each sample (Hochachka *et al.* 1982). Tissue samples from 13 uninfected and 11 infected killifish were analysed. Gills did not provide enough material for conducting both assays so only LDH activity was measured. Although all fish were exposed to cercaria prior to tissue sampling, it is unlikely that cercaria attack influenced the measurements of enzyme activity (either LDH or CS activity) or protein abundance (relative NKA abundance, as described below). This is largely because plasticity in enzyme activity typically occurs over the course of days or weeks (e.g., Rogers, Seebacher & Thompson 2004), and we sampled all tissues within ~3 hours of first exposure. Hence, the enzyme activity data should be a reliable indicator of the effects of long-term treatment

(g) Na^+/K^+ -ATPase abundance and immunolocalization

Na^+/K^+ -ATPase (NKA) was immunodetected using the $\alpha 5$ monoclonal antibody (Developmental Studies Hybridoma Bank Iowa City, USA) against the α -subunit of chicken NKA (Lebovitz, Takeyasu & Fambrough 1989) using the protocols described in Kwan et al. (2019). Additionally, monoclonal α -tubulin antibody (catalog number: A11126, ThermoFisher Scientific, Waltham, USA) was also immunodetected to normalize potential differences in protein.

Measurement of NKA protein abundance was quantified by Western blot. Three gill samples ($n = 2$ control gills, $n = 1$ infected gill) were used to optimize the homogenization protocol used in these assays so were not included in final analyses, which included 11 uninfected and 10 infected killifish gills (all from 2nd gill arch, right side). Immediately after dissection, gill samples were flash frozen in liquid N_2 and stored at -80°C until processed. Frozen gill tissue was pulverized with porcelain mortar and pestle and mixed in an ice-cold protease inhibiting buffer (250 mmol l^{-1} sucrose, 1 mmol l^{-1} EDTA, 30 mmol l^{-1} Tris, 10 mmol l^{-1} benzamidine hydrochloride hydrate, 200 mmol l^{-1} phenylmethanesulfonyl fluoride, 1 mol l^{-1} dithiothreitol, pH 7.5). Debris was removed by low-speed centrifugation (3000 g for 10 minutes, 4°C), and the resulting solution's protein concentration was determined by Bradford protein assay. Immediately prior to western blotting, homogenized samples (5 μg of protein/lane) were denatured by mixing with an equal volume of loading buffer comprised of 2x Laemmli buffer (90%) and 2-mercapaethanol (10%). After heating at 70°C for 5 minutes, proteins were separated in a 7.5% polyacrylamide mini gel (15 minutes at 60 volts, then 45 minutes at 200 volts). Proteins were then transferred onto a polyvinylidene difluoride (PVDF) membrane using a Bio-Rad (Hercules, CA, USA) wet electroblotting transfer cell (90 mAmps; 8 hours). After transfer, the PVDF membrane was incubated in blocking buffer (Tris-buffered saline, 1% tween, 10% skim milk) at room temperature (RT) for 1 hour, then incubated with the primary antibody (anti-NKA antibody: 1.5 $\mu\text{g}/\text{mL}$; anti- α -tubulin antibody: 1 $\mu\text{g}/\text{mL}$) overnight at 4°C . On the following day, the PVDF membrane was washed in Tris-buffered saline + 1% tween (TBS-T) at RT 3 times (10 minutes each), incubated in goat anti-mouse HRP-linked secondary antibodies (1:10,000, Bio-Rad) at RT for 1 hour, and washed again in TBS-T at RT 3 times (10 minutes each). Protein bands were revealed with ClarityTM Western ECL Substrate (Bio-Rad, Hercules, CA, USA), and the abundance of NKA (100 kDa) and α -tubulin (50 kDa) protein bands were imaged and quantified with ImageLabTM software (BioRad). Data is presented as "relative NKA abundance",

which was obtained by calculating the ratio of NKA / α -tubulin abundance, and normalizing all data by the uninfected treatment's average value.

The localization of NKA-rich cells (ionocytes) was examined using immunofluorescence in gills from three uninfected and three infected killifish. All procedures were performed at RT. Dissected gills (3rd gill arch, right side) were fixed in 4% paraformaldehyde in phosphate buffer saline (PBS; catalog number: 46-013-CM, Corning Incorporated) for 6 hours, washed in 50% ethanol for 6 hours, and stored in 70% ethanol until processing. For whole-mount immunostaining, gills were washed with sodium borohydride (1 mg/mL in PBS) to reduce background autofluorescence 6 times (10 minutes each), kept in blocking buffer (PBS, 0.02% normal goat serum, 0.0002% keyhole limpet hemocyanin, 1 hour), then incubated with α 5 monoclonal antibody (0.08 μ g/mL) overnight. On the following day, samples were washed with PBS with 0.1% Tween (PBS-T) 3 times (10 minutes each), incubated in goat anti-mouse Alexa FluorTM 546 (catalog number: Z25004, ThermoFisher) secondary antibody (1:500) and DAPI (1 μ g/mL) for 1 hour, followed by PBS-T washes for 3 more times (10 minutes each). For imaging, gills were individually placed onto a concave slide and secured with a cover slip. Images generated with an inverted confocal microscope (Zeiss LSM 800) using the 20X lens. Z-stack images of 89 to 197 slices (45 - 102 μ m) were scanned from the leading and trailing edges of killifish gill filaments, and compiled to create maximum intensity projections using Zeiss imaging software, ZEN (version 2.6).

2. Supplementary results for cercaria attachment and penetration timing

In both treatments (exposure following euthanasia and exposure prior to euthanasia; n = 5 killifish total), cercarial attachment occurred within 15 mins. In all cases of epithelial attachment to the outer body, epithelial penetration remained ongoing after 15 mins. However, the process for epithelial penetration was completed within 15 mins in some cases of attachment to the gill filament.

3. Supplementary Figures

Figure S1. Effect of long-term infection treatment (uninfected or infected with the trematode parasite *Euhaplorchis californiensis*) and body size on the acute metabolic rate (measured as rate of oxygen uptake, MR_{acute}) of the California killifish (*Fundulus parvipinnis*; $n = 13$ uninfected and $n = 11$ infected) during (A) exposure to a seawater sham, (B) exposure to cercaria (infectious parasites) and (C) one-hour post-exposure. Shading represents 95% confidence intervals for the best fit lines. The data represent a repeated measure, with each individual exposed to seawater and cercaria three times, and measured three times post-exposure. A moderately significant three-way interaction among body mass, exposure treatment, and long-term infection treatment was detected (linear mixed-effects model, $p = 0.046$).

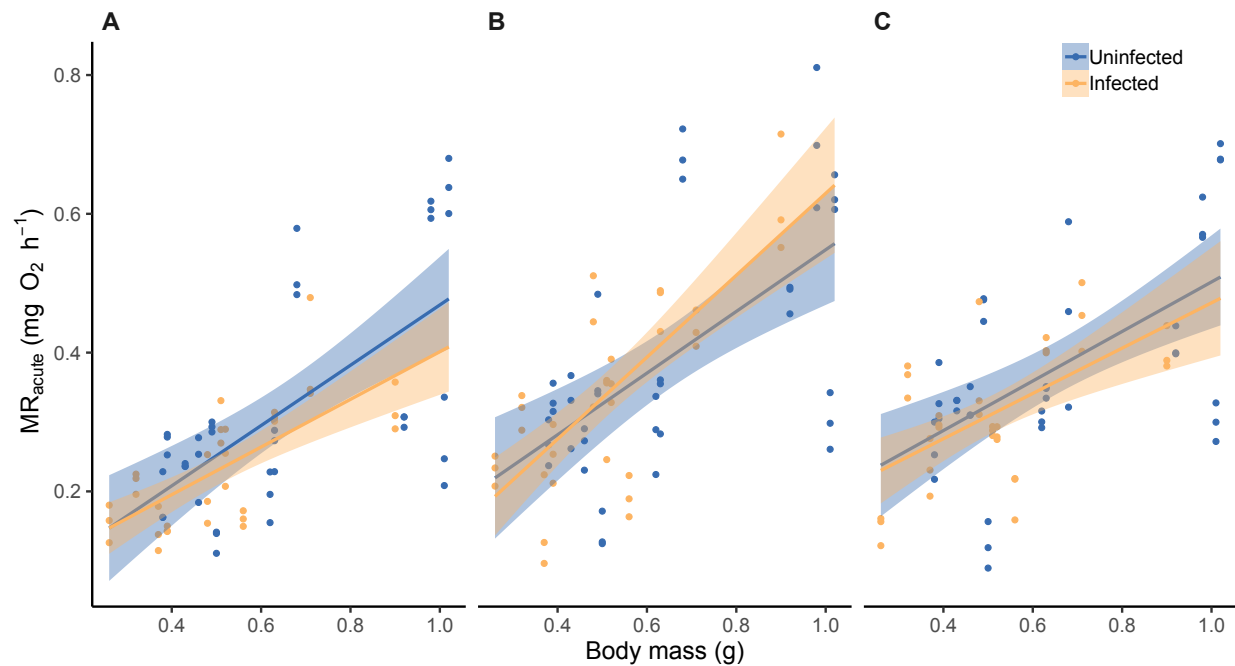


Figure S2. Effect of long-term infection treatment (uninfected or infected with the trematode parasite *Euhaplorchis californiensis*) and body size on the change in oxygen uptake (ΔMR_{acute}) during (A) exposure to cercaria (infectious parasites) and (B) one-hour post-exposure of the California killifish (*Fundulus parvipinnis*; $n = 13$ uninfected and $n = 11$ infected). ΔMR_{acute} is the difference between each individual's MR_{acute} during cercaria exposure and the average of that fish's response to sham seawater exposures. Shading represents 95% confidence intervals for the best fit lines. The data represent a repeated measure, with each individual exposed to cercaria three times and measured three times post-exposure. There was an indication of a three-way interaction among body mass, exposure treatment, and long-term infection treatment (linear mixed-effects model, $p = 0.076$).

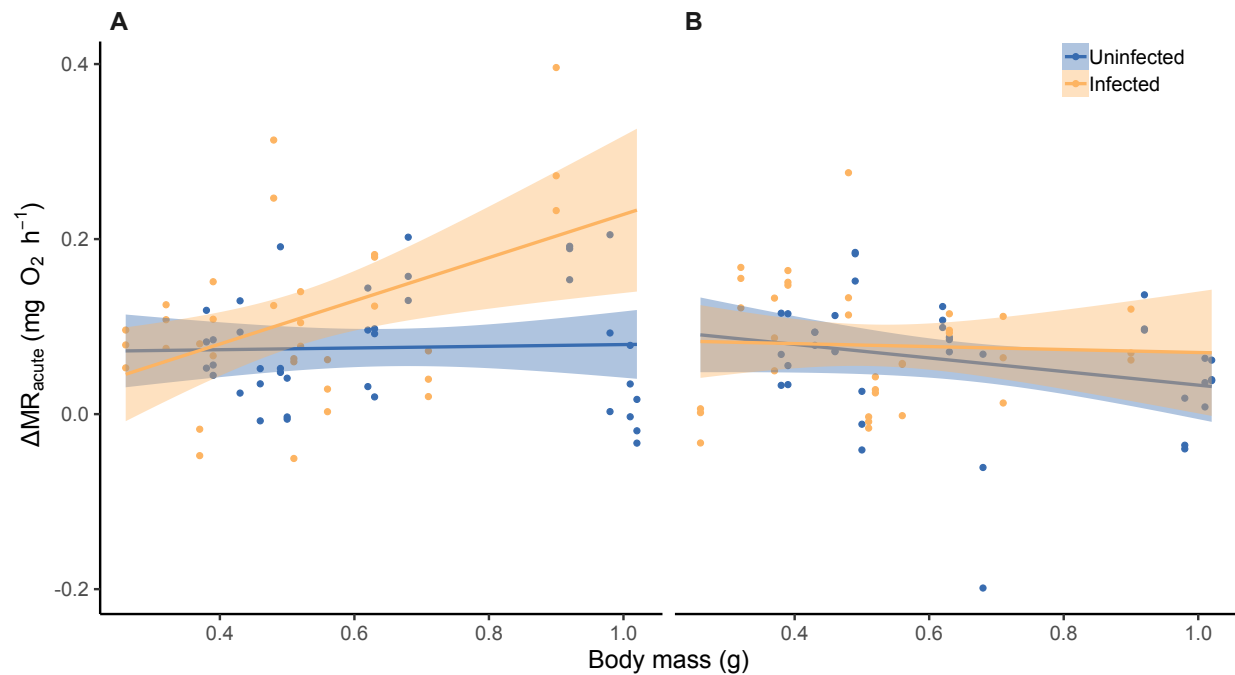


Figure S3. Effect of body mass and long-term infection treatment (uninfected or infected with the trematode parasite, *Euhaplorchis californiensis*) on the number of new *E. californiensis* parasite infections following acute exposure to infectious parasites in the California killifish (*Fundulus parvipinnis*; n = 13 uninfected and n = 11 infected), as revealed by a Poisson generalized linear model. The blue and orange shading represents 95% confidence intervals for the uninfected and infected treatments, respectively.

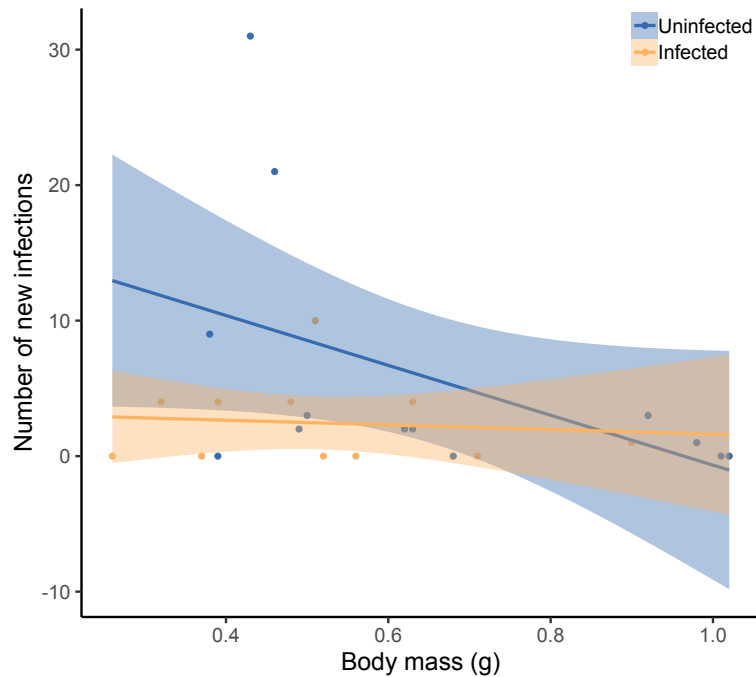


Figure S4. Effect of long-term infection treatment (uninfected or infected with the trematode parasite *Euhaplorchis californiensis*) on California killifish (*Fundulus parvipinnis*) enzyme activity in brain, gill, and white muscle tissue, for (A) lactate dehydrogenase (LDH, an indicator of anaerobic energy production), and (B) citrate synthase (CS, a key enzyme in aerobic metabolism). Due to limitations in gill tissue, only the LDH assay was conducted on the gills (n = 13 uninfected and n = 11 infected for all assays, except gill LDH, where n = 13 uninfected and n = 10 infected). Bars represent the mean $\pm$ s.e. Letters above bars represent significant differences ($p < 0.05$) in Tukey's multiple comparisons post-hoc tests following generalized linear model analysis. In panel B, "n.s." denotes non-significance.

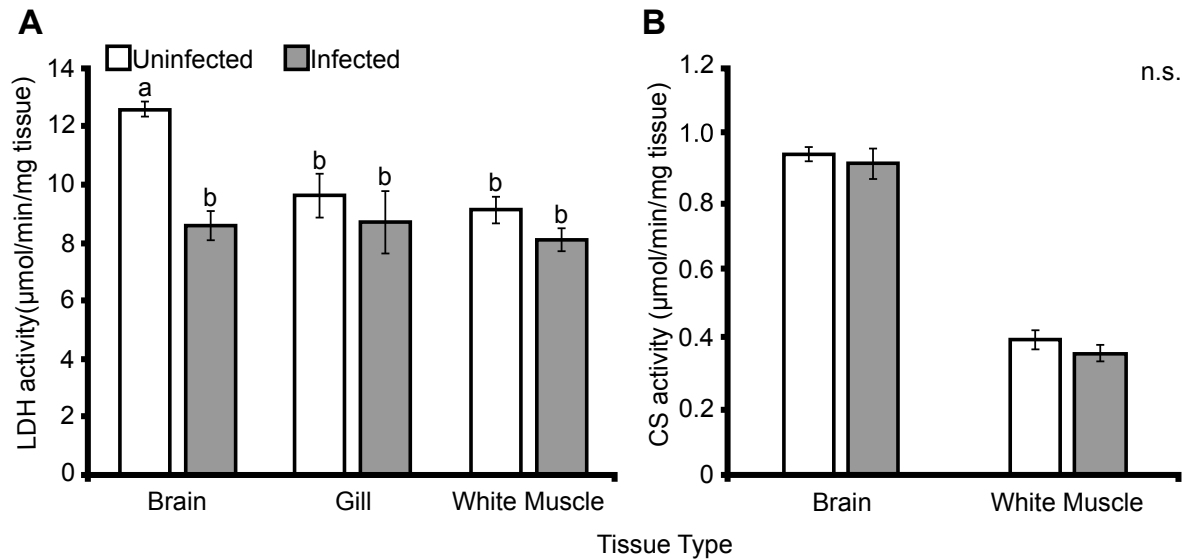
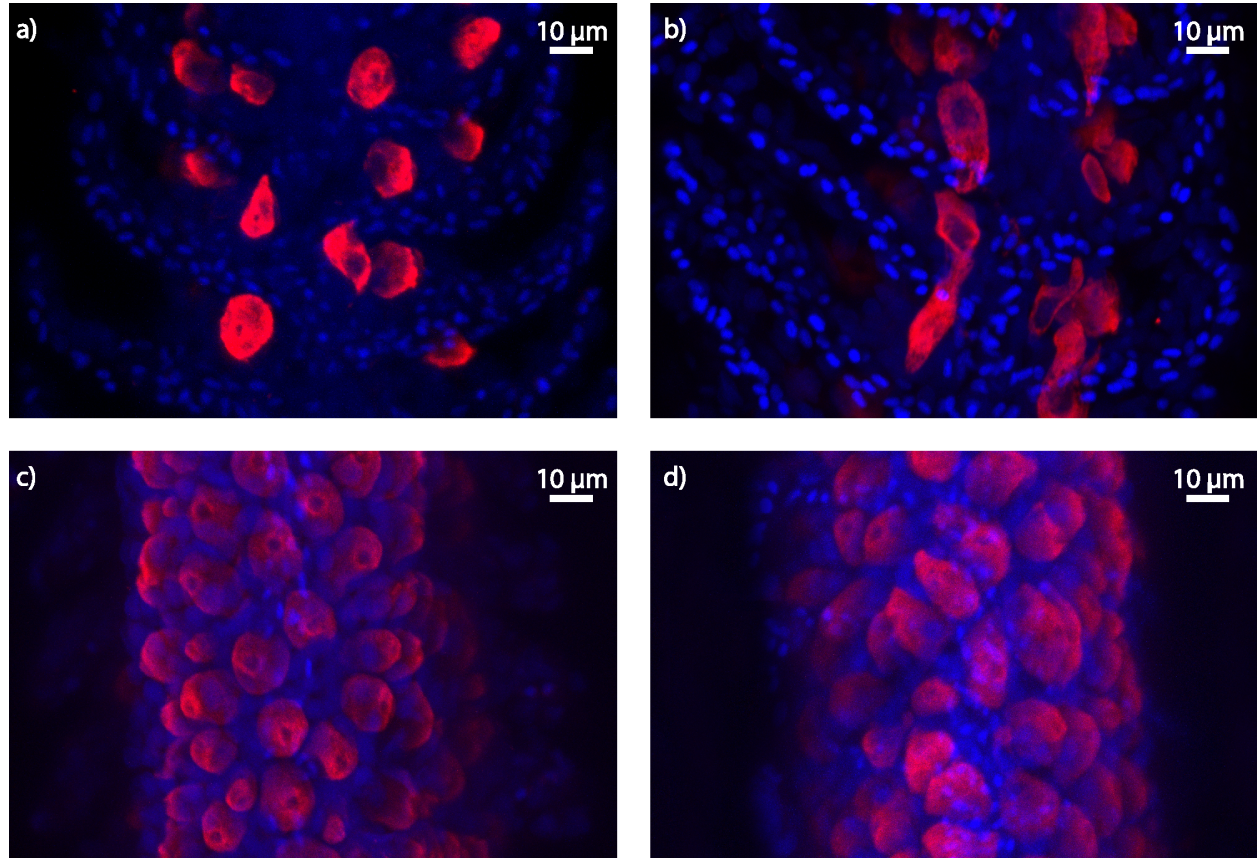


Figure S5. Ionocyte localization in gills from uninfected and infected California killifish (*Fundulus parvipinnis*; n = 6). Ionocytes were visualized using whole-mount immunofluorescence for Na⁺/K⁺-ATPase (NKA, red). Nuclei are stained with DAPI (blue). The vast majority of ionocytes were present on the gill filament, and sporadically on gill lamellae. There were no obvious differences in ionocyte abundance or localization in the gill leading (a, b) or trailing (c, d) edges between uninfected (a, c) killifish and killifish with an established infection by *Euhaplorchis californiensis* (b, d). Images are maximum intensity projections of confocal Z-stacks.



4. Supplementary Tables

Table S1. Summary information on the weight of tissues (in g, for brain, gill, white muscle) used for enzyme assays (n = 13 uninfected and n = 11 infected for tissues).

Tissue	Mean	Standard Error	Minimum	Maximum
Brain	0.010	0.0005	0.006	0.016
Gill	0.003	0.0004	0.002	0.006
White muscle	0.074	0.005	0.025	0.123

Table S2. Effect tests from generalized linear models of California killifish (*Fundulus parvipinnis*) metabolic traits in response to body mass (BM), long-term infection treatment (uninfected or infected with the trematode parasite *Euhaplorchis californiensis*; IT), and the interaction between BM and IT. Traits measured include standard metabolic rate (SMR), routine metabolic rate (RMR), maximum metabolic rate (MMR) and aerobic scope (AS) (n = 13 uninfected and n = 11 infected). The * indicates that traits were log-transformed for statistical analysis. The final column indicates the models' R² values.

Trait	Factor	d.f.	F-value	p-value	R ²
SMR*	Body mass	1,22	35.7	< 0.0001	0.62
	Infection treatment	1,21	0.5	0.47	
	Tank	1,20	1.9	0.19	
	BM x IT	1,19	0.01	0.90	
RMR*	Body mass	1,22	41.8	< 0.0001	0.65
	Infection treatment	1,21	0.6	0.44	
	Tank	1,20	0.8	0.38	
	BM x IT	1,19	0.1	0.76	
MMR	Body mass	1,22	44.3	< 0.0001	0.69
	Infection treatment	1,21	0.1	0.71	
	Tank	1,20	9.1	0.007	
	BM x IT	1,19	0.0	0.97	
AS	Body mass	1,22	10.7	0.004	0.43
	Infection treatment	1,21	0.0	0.92	
	Tank	1,20	6.3	0.02	
	BM x IT	1,19	0.2	0.63	

Table S3. Effect tests assessing variation in (A) activity (numbers of turns in respirometer per 15 minutes; analysed using generalized linear-mixed effects model with Poisson distribution) and (B) acute metabolic rate (MR_{acute} ; analysed using linear-mixed effects model analysis) with long-term infection treatment (uninfected or infected with the trematode parasite *Euhaplorchis californiensis*) and exposure treatment (sequentially: seawater sham, exposure to infectious cercaria parasites, and hour-long post-exposure period), for the California killifish (*Fundulus parvipinnis*), with individual nested within tank of origin included as a random effect (n = 13 uninfected and n = 11 infected). For MR_{acute} , body mass and activity were also included as fixed effects. The final column indicates the models' marginal (R^2_m) and conditional (R^2_c) R^2 values.

(A)

Factor	d.f.	χ^2	p-value	R^2_m , R^2_c
Infection treatment (IT)	1	0.9	0.35	0.15, 0.92
Exposure treatment (ET)	2	363.2	< 0.0001	
IT x ET	2	23.6	< 0.0001	

(B)

Factor	d.f.	F-value	p-value	R^2_m , R^2_c
Body mass (BM)	1,19	19.8	0.0003	0.45, 0.88
Infection treatment (IT)	1,1	0.04	0.87	
Exposure treatment (ET)	2,183	55.0	< 0.0001	
Activity	1,183	13.1	0.0004	
BM x IT	1,19	0.02	0.88	
BM x ET	2,183	5.0	0.008	
IT x ET	2,183	2.3	0.10	
BM x IT x ET	2,183	3.1	0.05	

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