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RESEARCH ARTICLE

A brain-infecting parasite impacts host metabolism both during exposure and after infection is established

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Abstract

1. Metabolic costs associated with parasites should not be limited to established infections. Even during initial exposure to questing and attacking parasites, hosts can enact behavioural and physiological responses that could also incur metabolic costs. However, few studies have measured these costs directly. Hence, little is known about metabolic costs arising from parasite exposure.

2. Furthermore, no one has yet measured whether and how previous infection history modulates metabolic responses to parasite exposure.

3. Here, using the California killifish *Fundulus parvipinnis* and its brain-infecting parasite *Euhaplorchis californiensis*, we quantified how killifish metabolism, behaviour and osmoregulatory phenotype changed upon acute exposure to parasite infectious stages (i.e. cercariae), and with long-term infection.

4. Exposure to cercariae caused both naïve and long-term infected killifish to acutely increase their metabolic rate and activity, indicating detection and response to parasite infectious stages. Additionally, these metabolic and behavioural effects were moderately stronger in long-term infected hosts than naïve killifish, suggesting that hosts may develop learned behavioural responses, nociceptor sensitization and/or acute immune mechanisms to limit new infections.

5. Although established infection altered the metabolic response to parasite exposure, established infection did not alter standard metabolic rate, routine metabolic rate, maximum metabolic rate, aerobic scope or citrate synthase enzyme activity.

6. Unexpectedly, established infection reduced lactate dehydrogenase enzyme activity in killifish brains and relative Na^+/K^+-ATPase abundance in gills, suggesting novel mechanisms by which *E. californiensis* may alter its hosts' behaviour and osmoregulation.

7. Thus, we provide empirical evidence that parasites can disrupt the metabolism of their host both during parasite exposure and after infection is established. This response may be modulated by previous infection history, with probable knock-on effects for host performance, brain energy metabolism, osmoregulation and ecology.

1 | INTRODUCTION

Hosting parasites can be metabolically costly (Dallas et al., 2016). These costs arise from several sources, including direct energy consumption by parasites, repairing or replacing damaged tissue or mounting immune responses (Sadd & Schmid-Hempel, 2009; Walkey & Meakings, 1970). However, even before infection is established, parasites may metabolically impact their hosts if the hosts respond to the presence of parasite infectious stages (Luong et al., 2017). Although studies in a range of taxa have quantified how *established* infection impacts host metabolic rate (Robar et al., 2011), little work has examined the metabolic consequences of the host's response to *initial* parasite exposure (i.e. Luong et al., 2017; Voutilainen et al., 2008) or compared these effects to the metabolic impacts of *established* parasite infection. Furthermore, to the best of our knowledge, no studies have tested if the metabolic response to parasite exposure is modulated by previous infection history, which would be particularly relevant for energy metabolism in systems where hosts frequently encounter infectious parasite stages.

Hosts may experience elevated metabolic rate upon parasite exposure (i.e. encountering questing and attacking parasite stages; Lafferty et al., 2015) through several mechanisms. Similar to the 'ecology of fear' described for predator–prey relationships (reviewed by Buck et al., 2018; Raffel et al., 2008), after detection of infectious stages, hosts may minimize infection risk by modifying activity, changing social behaviour or physically dislodging attacking parasites (James et al., 2008; Stumbo et al., 2012), all of which can increase metabolic rate (Speakman & Selman, 2003). Hosts can also exhibit physiological signs of stress following exposure to infectious parasite stages, including changes in ventilation, respiration and heart rates (Laitinen et al., 1996; Luong et al., 2017; Voutilainen et al., 2008). Such behavioural and physiological responses may also be learned or developed following initial parasite exposures, which could result in amplified host responses to subsequent encounter with the same parasite. For example, following previous exposure to trematode parasites, fathead minnows reduce activity in response to parasite cues to lower their risk of new infections (James et al., 2008). In contrast, some pathogens may elicit diminished immune responses upon subsequent exposure, due to acquired immunity; for instance, humans show substantially reduced cellular immune responses to yellow fever booster vaccinations when compared to the response to primary vaccination (Kongsgaard et al., 2017). Despite such suggestive studies, how previous exposure to parasites modulates a host's metabolic response (e.g. stress and energy metabolism) to new parasite exposure events remains unquantified.

In this study, we examined the impacts of acute parasite exposure and established infection on host metabolism for a small estuarine fish and its brain-infecting parasite *Euhaplorchis californiensis*. This trematode parasite leaves its first intermediate host, the California horn snail *Cerithideopsis californica*, as a free-swimming stage (i.e. cercaria) that seeks out and infects its next host, the California killifish *Fundulus parvipinnis* (Martin, 1950). After encounter, the parasite burrows through the fish's epithelium and makes its way to the brain's meningeal surface, where it encysts as a metacercaria (Helland-Riise et al., 2020; Martin, 1950; Shaw et al., 2009). Fish with established parasites on the brain may exhibit more conspicuous behaviours than uninfected conspecifics (e.g. surfacing, spontaneous burst-swimming behaviour), potentially driven by altered neurotransmitter activity in the brain (Shaw et al., 2009; Shaw & Øverli, 2012), leading to 10–30 times greater predation by the parasite's final host, fish-eating marsh birds (Lafferty & Morris, 1996). In estuaries where *E. californiensis* is present, infection prevalence and abundance in killifish is extremely high, ranging from 94% to 100% of the population, with each fish typically hosting hundreds to thousands of parasites on the brain (Shaw et al., 2010). These high levels of *E. californiensis* infection in killifish are readily explained by the widespread and common nature of first intermediate host horn snails infected by *E. californiensis* (Hechinger et al., 2007; Kuris et al., 2008), the source of parasite infectious stages (cercariae). The *E. californiensis* cercariae are present in the water throughout the year, reaching particularly high densities in the summer and on rising tides (Fingerut et al., 2003). Given the high likelihood of repeated encounter with *E. californiensis* cercariae, and the neurophysiological and fitness impacts of established infection, we hypothesized that selection has favoured killifish behavioural and physiological responses to defend against *E. californiensis* cercariae. However, so far, evidence that killifish detect or respond to the cercariae is lacking. If they do respond, the metabolic costs of the response could be an important factor in host metabolism and total energy budget.

Although naturally infected killifish populations exhibit high parasite abundance (0.5%–1.7% of their body mass; Shaw et al., 2010), there is no evidence for density-dependent limitations on *E. californiensis* body volume in wild-caught killifish (Weinersmith et al., 2014), suggesting that the parasites do not take a large amount of host resources that would substantially impact host fish. Consistent with this idea, comparisons between naturally infected killifish with those from uninfected populations indicate that body condition and reproduction are maintained after infection (Shaw et al., 2009, 2010; Shaw & Øverli, 2012). However, parasites may induce tissue-specific metabolic modifications that are not evident at the whole-animal level. For example, many parasites, including trematodes, produce lactate as their primary metabolic end product, which is subsequently released into the host's surrounding tissues (Barrett, 1981; Perrot-Minnot et al., 2016). Hence, for example, *E. californiensis* may

directly release lactate in the killifish brain, which could affect brain function as lactate is a vital metabolic fuel for neurons and astrocytes (Boumezbeur et al., 2010). Additionally, the conspicuous swimming bursts induced by the parasite must be driven by white muscle activity, which is predominantly anaerobic. Thus, parasitic infection could conceivably increase the need for anaerobic glycolytic capacity in the white muscle.

Another physiological process that may be altered by parasitic infection is osmoregulation. Indeed, parasitized eel (Fazio et al., 2008; Lorin-Nebel et al., 2013) and sea bream (Moreira et al., 2018) demonstrated alterations in gill Na^+/K^+-ATPase (NKA), an enzyme that is key for maintaining ion balance in fish (Evans et al., 2005). Since killifish live in estuarine habitats where the salinity of the water is highly variable on both temporal and spatial scales (Desmond et al., 2000; Valentine & Miller, 1969), similar alterations in NKA could affect killifish habitat preference and distribution.

Here, we quantified killifish metabolism, behaviour and osmoregulatory phenotype to address the following linked questions concerning *E. californiensis*:

1. Does established infection influence aerobic metabolic rate? As *E. californiensis* parasites both consume host energetic resources and modify host behaviour, we hypothesized that long-term infection would increase metabolic demand and reduce aerobic capacity.
2. Do killifish metabolically or behaviourally respond to cercaria exposure, and do any such responses vary between uninfected versus long-term infected killifish? If killifish detect questing or attacking parasite stages, we expected that metabolic rate and activity would increase during cercaria exposure, particularly for previously exposed fish, which may have developed a 'fear' or sensitization to parasite exposure.
3. Does established infection affect killifish metabolic activity in the brain and white muscle? We measured activity of the key metabolic enzymes lactate dehydrogenase (LDH) and citrate synthase (CS), from which the LDH/CS activity ratio was calculated as a proxy for relative anaerobic/aerobic metabolic potential (Hochachka et al., 1982). We hypothesized that this ratio would change in both the brain and white muscle following established infection, due to changes in metabolic substrate availability and parasite-induced modifications in behaviour, in the brain and white muscle respectively.
4. Does established infection alter host osmoregulatory capacity in the gills? Given the previously observed effects of infection on gill NKA abundance in other host–parasite systems, we investigated whether *E. californiensis* induces similar alterations in killifish gills, as killifish are frequently subjected to salinity challenges that could alter their access to foraging grounds in the estuarine habitat.

Taken together, the above questions allowed us to compare the energetic costs of acute exposure to infectious parasite stages to those arising from established infection, and determine if the energetic response to parasite exposure is altered by established infection (i.e. established metacercariae on the brain).

2 | MATERIALS AND METHODS

This study was part of a larger project examining the influence of *E. californiensis* on killifish physiology, neurobiology and behaviour throughout development, and involved laboratory rearing of fish from wild-caught gametes (Helland-Riise et al., 2020). These fish were exposed for 13 months post-hatch under different 'long-term treatments' (repeatedly exposed or sham exposed to parasites). In this study, we measured the metabolic responses of long-term uninfected and infected killifish following acute exposure to infectious parasite stages (i.e. cercaria). Individuals were then euthanized to quantify parasite infection levels and collect tissues for assays of metabolic enzymes and NKA. Below we provide methodological details, with further materials and methods available for sections (2.1)–(2.6) in the electronic supplementary material.

2.1 | Gamete collection

Wild, adult California killifish were caught in August 2016 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 *E. californiensis*), from which gametes were collected following the procedure outlined in Strawn and Hubbs (1956). Eggs from each gravid female were fertilized using sperm from approximately 4–10 males, then returned to the laboratory at Scripps Institution of Oceanography and housed in glass bowls until hatching at ~21 days post-fertilization.

2.2 | Fish rearing

Following hatching, we held fish in groups of 20–21 fish each in 38L glass holding tanks (51 × 27 × 32 cm) that were randomly assigned to one of two treatments: (a) uninfected (*n* = 4 tanks) and (b) infected by *E. californiensis* (*n* = 5 tanks). Fish were reared in these tanks for 10 months under ambient seasonal water temperature (~18°C in winter, ~21°C in summer) and light cycle conditions (from 11:13 hr light:dark cycle in winter to 13:11 hr light:dark cycle in summer) with flow-through natural seawater. At the end of this 10-month period, some of the fish from each of these replicate tanks were removed for behavioural and neurophysiological studies that are not reported here. Twenty-four of these experimental fish were tested for this study (13 uninfected and 11 infected killifish in all tests and assays unless otherwise specified). During the final 3 months before testing, the uninfected and infected treatments were re-housed in two and one tanks respectively. Although suboptimal given potential tank effects, these fish were combined into fewer tanks to avoid stress associated with social

isolation at low densities (the group housing mimicked the group sizes and densities experienced during the initial 10-month rearing period; group size = ~12 individuals, density ~ 0.6 fish/L). Fish were not tagged prior to re-housing, so their original tank of origin could not be included in subsequent analyses. As these fish are not sexually dimorphic and were not sexually mature at the time of testing, sex of these fish could not be identified. At the time of testing (November 2017), on average, individuals were 13 months old, 0.59 g ($M \pm SE$; 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). Although infected fish were marginally smaller on average than control fish, this effect was not statistically significant ($p > 0.05$).

2.3 | Long-term infection procedure

Throughout the 13-month rearing period, groups in the infected treatment were exposed to *E. californiensis* cercariae (measuring ~ 190 μm long × 55 μm wide, Martin, 1950) twice weekly in their home tanks, while uninfected groups were concurrently exposed to a seawater sham. The cercaria used during experimental infections were shed from first intermediate host snails collected from the University of California Kendall-Frost Marsh Reserve (KF) in Mission Bay, San Diego County, CA, USA (32.80°N, 117.23°W). The cercaria exposure dose per infection event increased gradually throughout the entire rearing period, in line with the fish's growth in body size (from 2 to 100 cercariae/fish/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 1–8 days prior to testing.

The long-term infection procedure resulted in *E. californiensis* intensities ranging from 352 to 1,783 established metacercariae per killifish ($M \pm SD$: 1,190 ± 408 per fish) and densities ranging from 677 to 4,100 metacercariae g^{-1} fish (2,528 ± 1,040 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.

2.4 | Intermittent-flow respirometry and acute cercaria exposure

We measured metabolic rate following an intermittent-flow respirometry methodology tailored for social fishes (Nadler, Killen, McClure, et al., 2016), focusing on five measures of metabolic rate, including (a) standard metabolic rate (SMR, the metabolic rate of a resting, fasting and non-stressed individual), (b) routine metabolic rate (RMR, the metabolic rate of an undisturbed but spontaneously active individual), (c) maximum metabolic rate (MMR, the upper constraint on an individual's oxygen-consuming physiological activities), (d) aerobic scope (AS, the capacity to support

activities beyond basic maintenance, calculated as the difference between MMR and SMR; Chabot et al., 2016; Farrell, 2016; Killen et al., 2017) and (e) acute metabolic rate (MR$_{acute}$, oxygen uptake following parasite exposure treatments). A Fire-Sting fibre-optic oxygen meter (Pyroscience, Germany) read and logged dissolved oxygen concentration every 2 s. Fish were fasted for 24 hr prior to experimentation to ensure that they were in a post-absorptive state and left undisturbed in the respirometers for 20–23 hr. The flushing-measurement period used was 17 min followed by a 5-min flushing period. Slopes (s) were calculated from plots of oxygen concentration versus time using ordinary least squares linear regression (LabChart v6) and converted to the rate of oxygen uptake ($\dot{M}_{O_2}$). Background respiration was measured for three measurement periods before and after trials and subtracted from all fish respiration measurements, assuming a linear increase in microbial respiration (Rodgers et al., 2016). From the measurements, we analysed two measures of metabolic rate: SMR and RMR. We calculated SMR as the lowest 10th percentile of all $\dot{M}_{O_2}$ measurements on an individual fish (Chabot et al., 2016; Killen, 2014) and RMR as the mean $\dot{M}_{O_2}$ excluding the first 5 hr in the respirometer (Killen et al., 2011).

The acute response of $\dot{M}_{O_2}$ to cercaria exposure (MR$_{acute}$) was then quantified. First, to acquire a baseline, we quantified $\dot{M}_{O_2}$ of individual fish during three sequential 17-min seawater sham exposures (with each baseline measurement separated by a full 23-min flushing-measurement cycle). Following these baseline measurements, we then exposed individuals to cercaria. As with the seawater sham treatment, cercaria exposure treatments were also repeated three times in sequence (300 cercariae per exposure), with each measurement separated by one flushing-measurement cycle.

During each replicate cercaria exposure treatment, we quantified baseline oxygen consumption 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) was administered through a three-way valve in the tubing between the flushing pump and the chamber. Following the final cercaria exposure treatment, each fish's post-exposure $\dot{M}_{O_2}$ was continuously recorded for an additional three flushing-measurement cycles to determine if any cercaria-induced changes in $\dot{M}_{O_2}$ persisted after cercaria were removed through flushing.

Preliminary cercaria exposures of killifish indicate that cercariae can attach to the epithelium within minutes of exposure. The time required for cercariae to penetrate the epithelium varied depending on the point of attachment, with cercariae capable of penetrating the gill filament just within the 17-min exposure window, while penetration remained ongoing after 17 min on all outer body surfaces (details found in supplementary Materials & Methods and Results). As such, the above exposure treatments reflect the killifish's integrated response to cercaria pre-attack (i.e. cercaria in the environment), attack (i.e. attaching to and penetrating the epithelium) and early infection (i.e. just after penetrating the epithelium, but before establishing on the brain).

During the above experimental exposures, each fish's behaviour was recorded continuously using a webcam (H264 Webcam Software), and activity was quantified by measuring the number of 180° turns for the last 15 min of each measurement period (using the procedure outlined in Nadler, Killen, McClure, et al., 2016; Nadler, Killen, McCormick, et al., 2016). The first 2 min of each measurement period was not included in activity measurements to allow time for the cercariae to be injected into the chamber.

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 cercaria exposure, this was more directly examined by calculating each individual's change in $\dot{M}_{O_2}$ during cercaria exposure compared to the mean of its previous three 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 after the above procedures were completed to ensure that individuals reached SMR prior to subsequent testing, and allowed us to look at relative differences in MMR with long-term treatment. MMR was measured using the chase protocol, in which individuals are exercised to exhaustion through manual chasing to elicit burst swimming behaviour (Killen et al., 2017; Roche et al., 2013; Rummer et al., 2016), followed by air exposure 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 then recorded for 10 min (this time frame was used to ensure 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), as the difference between these two measures.

Sampling of fish tissues commenced 1 hr following the chase procedure. Fish were euthanized using an overdose of MS-222 (250 mg/L). They were first weighed (±0.01 g) and measured (standard length, SL, ±0.5 mm), then quickly sampled. Each fish's brain, gills (second and third gill arches) and white muscle were quickly flash frozen on liquid N_2 (within ~2–3 min of euthanasia). From infected fish, metacercariae were first rapidly removed from the brain meningeal surface (which they are found on, but not in) using a combination of manual removal with forceps and rinsing the brain tissue with fish saline (0.6% saline) prior to freezing

(this process took <60 s). Last, all metacercariae were counted to quantify infection intensity. The tissues were stored at −80°C until enzyme activity analyses. 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. Given the time period between cercaria exposure and tissue sampling (<3 hr), these new infections did not have had time to reach the brain, and were found in locations (specifically, 20% in the caudal fin and 80% in muscle tissue) between the skin entry point and the brain (McNeff, 1978, pers. obs.).

2.5 | Energy metabolism enzyme assays

To assess relative differences in enzyme activity between long-term treatments, we analysed frozen brain (lacking parasites), white muscle and left second gill arch tissue from all sampled fish. Summary information on the weight of tissues collected for enzyme assays can be found in Table S1. Tissue samples were homogenized and analysed for activity of the key metabolic enzymes LDH and CS according to published protocols and equations (McClelland et al., 2005; Seebacher et al., 2003; Thibault et al., 1997). Relative anaerobic potential was calculated by dividing LDH activity over CS activity for each sample (Hochachka et al., 1982). Gills did not provide enough material for conducting both assays, so we measured only LDH activity for that tissue. Gill tissue from one individual did not provide enough material for the LDH assay either, so was not included in these analyses (i.e. data on gill LDH activity include 13 uninfected and 10 infected killifish). Although all fish were exposed to cercaria prior to tissue sampling, plasticity in enzyme activity occurs over the course of days or weeks (e.g. Rogers et al., 2004). As sampling occurred within ~3 hr of first exposure, changes in enzyme activity due to cercaria exposure would be highly unlikely (e.g. Rogers et al., 2004), and thus, these data are indicative of relative differences due to long-term treatment.

2.6 | Na$^+$/K$^+$-ATPase abundance and immunolocalization

Na$^+$/K$^+$-ATPase in killifish gills was immunodetected using the α5 monoclonal antibody (Developmental Studies Hybridoma Bank) against the α-subunit of chicken NKA (Lebovitz et al., 1989) following the protocols described in Kwan et al. (2019), with some minor modifications. 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 this assay, so were not included in final analyses, which included 11 uninfected and 10 infected killifish gills (all from the right, second gill arch). Potential differences in overall protein loading were accounted for by quantifying α-tubulin abundance in each sample. Data are presented as 'relative NKA

abundance', which was calculated as the ratio of NKA/α-tubulin abundance.

NKA-rich cells (ionocytes) were visualized by immunofluorescence in gills from three uninfected and three infected killifish (third gill arch, right side). Images were generated from the leading and trailing edges of gill filaments by confocal microscopy.

2.7 | Statistical analysis

We conducted all statistical analysis in the R Statistical Environment (v3.2.4; R Development Core Team, 2016), using the packages 'LME4', 'NLME', 'EMMEANS', 'GGPLOT2', 'MULTCOMP', 'MuMIN', 'MASS', 'GLM-MTMB' and 'CAR'. For all models, we checked that model assumptions of homoscedasticity and normality were met by visually inspecting residual and quantile–quantile plots respectively (Bolker et al., 2009). We transformed data when it readily mitigated deviations from model assumptions, and otherwise used models incorporating non-normal error distributions (detailed below). Each model's R^2 (including the marginal and conditional R^2 for mixed-effects models, indicated as R^2m and R^2c, respectively, Harrison et al., 2018) are included either below or in the supplementary material.

Aerobic metabolic rate (SMR, RMR, MMR and AS) was analysed using generalized linear models (GLMs) with a normal error distribution and an identity link with long-term treatment (uninfected, infected), body mass (in g) and tank of origin (here and below, this refers to tank following the re-housing described above) as fixed effects. SMR and RMR were log-transformed to meet the normality and homoscedasticity assumptions.

Activity was analysed using a generalized linear mixed-effects model (GLMM) with a Poisson distribution, with long-term treatment and exposure treatment as fixed effects, and individual nested within tank of origin as a random effect. Both measures of acute metabolic rate (MR_{acute} and ΔMR_{acute}) were analysed using linear mixed-effects models (LMMs) with long-term treatment, acute exposure treatment (seawater, cercaria, post-exposure) and body mass as fixed effect predictors, and individual nested within tank of origin as a random effect. Activity was also included as a fixed effect for the MR_{acute} model.

Each measure of enzyme activity (LDH, CS and LDH/CS) was analysed using an LMM, with long-term treatment and tissue type (brain, gill, white muscle), as fixed effects and individual nested within tank of origin as a random effect. The ratio of LDH/CS was log-transformed to meet the homoscedasticity assumption. Following square-root transformation to meet assumptions of normality and homoscedasticity, the role of long-term treatment and tank of origin in relative NKA abundance was assessed using a GLM. The number of new parasite infections following acute cercaria exposure was analysed using a GLM with a negative binomial distribution to account for overdispersion in the data, with long-term treatment, activity, body mass and tank of origin as fixed effects. Significant differences discovered for the LMM and GLMM tests were further investigated

within and among treatments using Tukey's multiple comparisons post hoc tests.

3 | RESULTS

3.1 | Does established infection influence aerobic metabolic rate?

Long-term treatment did not influence any of the measured aerobic metabolic traits. That is, SMR, RMR, MMR and AS did not significantly differ between long-term uninfected and infected killifish (Table S2; Figure 1). Tank of origin had a significant impact on MMR and AS, but not SMR or RMR (Table S2).

3.2 | Do killifish metabolically or behaviourally respond to cercariae in the environment, and do any such responses vary between uninfected versus long-term infected killifish?

Acute exposure to cercariae influenced the activity of fish from both long-term uninfected and infected treatment groups (Table S3A; Figure 2A). Activity increased 34%–36% upon cercaria exposure (compared to preceding sham exposures), and then decreased 46%–85% during the subsequent post-exposure period (relative to cercaria exposure; Tukey's multiple comparisons post hoc test: uninfected: $p_{\text{seawater-cercariae}} < 0.01$, $p_{\text{cercariae-postexposure}} < 0.01$; infected: $p_{\text{seawater-cercariae}} < 0.01$, $p_{\text{cercariae-postexposure}} < 0.01$, $p_{\text{seawater-postexposure}} < 0.01$). A significant long-term × exposure treatment interaction appears to reflect the higher activity of previously infected compared to uninfected fish during sham and, particularly, cercaria
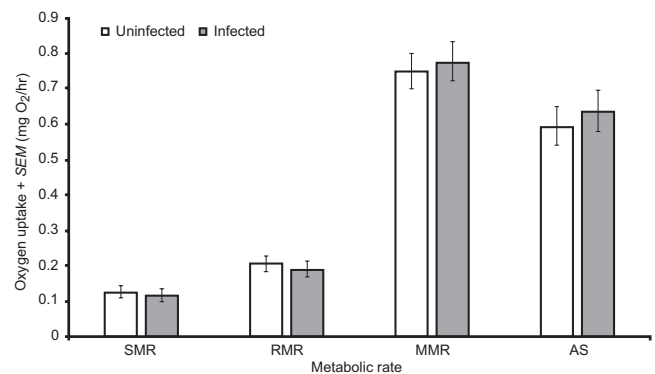

FIGURE 1 There was no effect of long-term treatment (uninfected or infected with the trematode parasite *Euhaplorchis californiensis*) on standard metabolic rate (SMR), routine metabolic rate (RMR), maximum metabolic rate (MMR) or aerobic scope (AS) of the California killifish (*Fundulus parvipinnis*; $n = 13$ uninfected and $n = 11$ infected). Bars represent the estimated marginal $M \pm SE$ (derived from the generalized linear model, $p > 0.05$ for all models), controlling for body mass, long-term treatment, their interaction and tank of origin
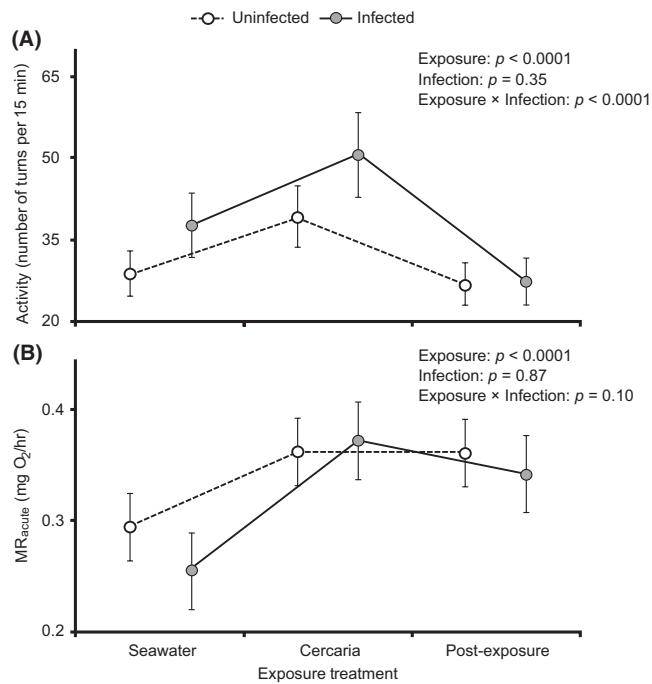
FIGURE 2 Effect of acute exposure treatments (administered sequentially: seawater sham, exposure to infectious cercaria parasites and 1-hr post-exposure period) on (A) fish activity (the number of 180° turns per 15-min exposure treatment) and (B) acute metabolic rate (MR_{acute}) of the California killifish (*Fundulus parvipinnis*; $n = 13$ uninfected and $n = 11$ infected) from different long-term treatments (previously uninfected or infected with the trematode parasite *Euhaplorchis californiensis*). Dots represent the estimated marginal $M \pm SE$ from linear mixed-effects model analysis, from which p-values were determined. For both activity and MR_{acute}, marginal means control for acute exposure treatment, long-term treatment and individual nested within tank of origin, with body mass and activity also included for the MR_{acute} model

exposure, followed by a greater relative drop in activity post-exposure (Table S3A; Figure 2A).

Cercaria exposure also increased MR_{acute}, which was 23% and 45% higher (for previously uninfected and infected individuals respectively) on average when individuals were exposed to cercariae than to seawater (Table S3B; Figure 2B). This elevation in metabolic rate was sustained for at least 1-hr post-exposure ($p < 0.01$ for all Tukey post hoc comparisons of cercaria and post-exposure with seawater sham). During cercaria exposure, killifish used 33% and 38% of their AS on average with their response (± 4% and ± 4% SE for uninfected and infected individuals respectively). Post-exposure, MR_{acute} accounted for 33% of each individual's AS (± 4% SE, both uninfected and infected killifish). In the post-exposure period, oxygen uptake remained consistent across the three measurements in both long-term treatments (ranging from 0.34 to 0.37 mg O_2/hr). Although activity had a significant effect on MR_{acute} (Table S3B), its partial R^2 was only 0.009 (when comparing the R^2m for the models with and without activity). A three-way interaction also occurred among body mass, long-term treatment and acute exposure treatment (Table S3B). This interaction was largely explained by the

medium- and large-sized previously infected killifish exhibiting the strongest MR_{acute} increases during cercaria exposure (Figure S1).

When examining ΔMR_{acute}, which allowed us to more directly examine how long-term treatment influenced the response to cercaria, there was a statistically marginal trend for a three-way interaction among body mass, long-term treatment and acute exposure treatment (LMM: $F_{1,116} = 3.2$, $p = 0.08$; R^2m $= 0.13$, R^2c $= 0.49$). The magnitude of ΔMR_{acute} during cercaria exposure was higher in infected fish, particularly in larger individuals (Figure S2A). On average, the magnitude of ΔMR_{acute} was 68% higher in previously infected than uninfected fish (uninfected: 0.076 ± 0.017 mg O_2/hr; infected: 0.127 ± 0.020 mg O_2/hr; $M \pm SE$). However, this effect was minimal post-exposure (Figure S2B).

The number of new infections declined significantly with body mass (negative binomial GLM: $F_{1,22} = 8.0$, $p = 0.005$; Figure S3). We also found weak, though non-statistically significant, relationships between the number of new infections and long-term treatment (negative binomial GLM: $F_{1,21} = 3.3$, $p = 0.07$; $R^2 = 0.49$; Figure S3) and tank of origin (negative binomial GLM: $F_{1,19} = 3.3$, $p = 0.07$). The number of new infections was more than two times higher on average in uninfected fish than in previously infected fish ($M \pm SE$; Control: 5.7 ± 2.7; Infected: 2.5 ± 1.0). For tank of origin, one of the control tanks had 7× higher numbers of new infections on average than the other (8.5 ± 4.0 parasites ($n = 8$) versus 1.2 ± 1.1 parasites ($n = 5$)). Neither activity (negative binomial GLM: $F_{1,20} = 0.0$, $p = 0.99$) nor the body mass × long-term treatment interaction (negative binomial GLM: $F_{1,18} = 1.2$, $p = 0.27$) exerted any significant effect on the number of new infections.

3.3 | Does established infection affect killifish metabolic activity in the brain, white muscle and gills?

There was a significant interaction between long-term treatment and tissue type for both LDH activity (LMM: $F_{2,43} = 5.3$, $p = 0.009$; R^2m $= 0.35$, R^2c $= 0.49$; Figure S4A) and anaerobic potential (LMM: $F_{1,22} = 26.1$, $p < 0.0001$; R^2m $= 0.83$, R^2c $= 0.92$; Figure 3A), as both traits were approximately 30% lower in the brain tissue of infected killifish than uninfected killifish (Tukey's multiple comparisons post hoc test: brain LDH activity: $p_{uninfected-infected} < 0.001$; brain anaerobic potential: $p_{uninfected-infected} = 0.001$). In contrast, LDH activity and anaerobic potential were consistent across long-term treatments in white muscle tissue and LDH activity was consistent across treatments in the gill tissue (all $p > 0.05$; Figure 3A; Figure S4A). There was no significant interaction for CS activity in either the brain or white muscle (LMM: $F_{1,22} = 0.04$, $p = 0.85$; R^2m $= 0.88$, R^2c $= 0.89$; Figure S4B).

3.4 | Does established infection alter host osmoregulatory capacity in the gills?

Relative NKA abundance in the gills of infected fish was approximately half the observed level in uninfected fish (GLM: $F_{1,19} = 8.3$,
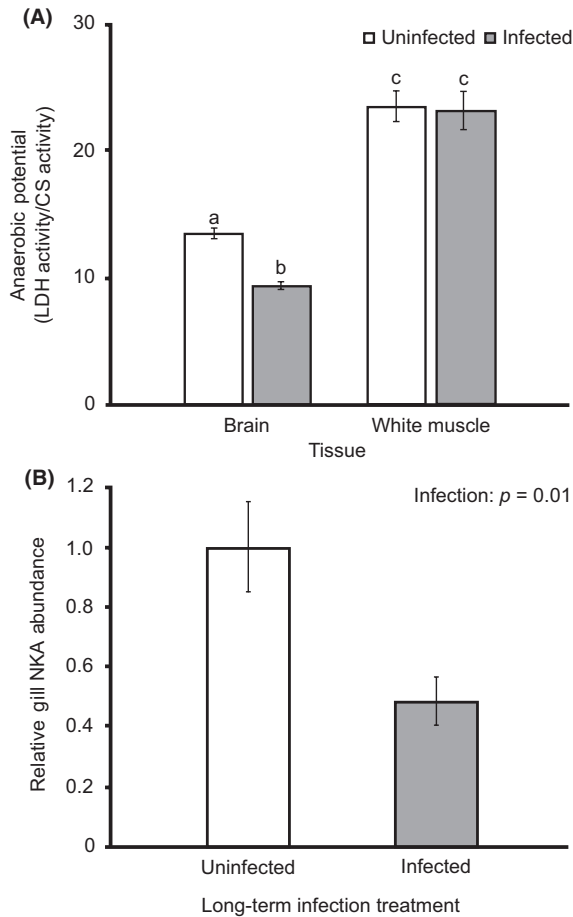
FIGURE 3 Effect of long-term treatment (uninfected or infected with the trematode parasite *Euhaplorchis californiensis*) on California killifish *Fundulus parvipinnis* enzyme activity. (A) Anaerobic potential in the brain and white muscle tissue ($n = 13$ uninfected and $n = 11$ infected), as calculated as the ratio of lactate dehydrogenase (LDH, an indicator of anaerobic energy production) to citrate synthase (CS, a key enzyme in aerobic metabolism) activity. Letters above bars represent significant differences ($p < 0.05$) in Tukey's multiple comparisons post hoc tests following generalized linear model (GLM) analysis. (B) Normalized gill relative Na$^+$/K$^+$-ATPase (NKA; $n = 11$ uninfected and $n = 10$ infected) abundance under different long-term treatments in California killifish, with *p*-value determined using GLM analysis. Bars represent the $M \pm SE$

$p = 0.01$; $R^2 = 0.29$; Figure 3B). There was no significant effect of tank of origin on relative NKA abundance (GLM: $F_{1,18} = 0.0$, $p = 0.99$). Immunofluorescence showed that gill NKA-rich ionocytes were predominantly localized on gill filaments and rarely on the lamellae (Figure S5). No obvious differences were observed between uninfected and infected fish.

4 | DISCUSSION

These findings demonstrate that the brain-infecting parasite *E. californiensis* impacts its killifish host during parasite exposure, even before infection is established. During initial exposure to questing and attacking cercariae, hosts increased both activity

and metabolic rate. While activity returned to baseline levels 1-hr post-exposure, metabolic rate remained elevated, suggesting ongoing physiological changes separate from behavioural effects. In contrast, established *E. californiensis* infection had no substantial effects on killifish aerobic metabolism. However, it unexpectedly reduced both brain anaerobic potential and gill relative NKA abundance. To our knowledge, this is the first empirical evidence for metabolic costs of parasite exposure in a vertebrate host species and the first time in any taxa that infection history has been shown to modulate the physiological response to infectious parasite stages.

Exposure to infective parasite stages caused an ~35% increase in both host activity and metabolic rate. The observed increase in activity was likely an attempt to either prevent cercaria attachment or dislodge those that had attached to but not yet penetrated the skin. Studies in amphibian and fish hosts illustrate similar surges of activity as an anti-parasite response to cercariae in a number of contexts (Karvonen et al., 2004; Koprivnikar & Urichuk, 2017; Taylor et al., 2004). Such anti-parasite activity can have ecological repercussions (Buck et al., 2018; Raffel et al., 2008). For instance, parasite-induced changes in host activity may increase predation rates (Marino & Werner, 2013). The concurrent metabolic response also likely has substantial consequences. For instance, it may impact the host's overall energetic budget, given the frequency with which killifish naturally encounter cercariae in the wild (see below). In another system, *Drosophila* flies exposed to ectoparasitic mites also exhibit an increased metabolic rate (Luong et al., 2017), providing evidence that the observed changes in metabolic rate with acute parasite exposure are likely relevant for a wide range of host and parasite types.

As both previously uninfected and infected killifish exhibited behavioural and physiological responses to cercariae, our findings suggest that killifish hosts have an innate recognition of parasite infectious stages. However, the relative strength of both behavioural (activity) and metabolic responses (ΔMR_{acute}) was moderately stronger in previously infected hosts than naive fish (though only marginally significant for ΔMR_{acute}). Hosts may learn to fear parasite exposure, may become sensitized to the tactile stimulation of attacking cercaria and/or may develop an acute immune response (James et al., 2008; Kalbe & Kurtz, 2006; Sitja-Bobadilla, 2008; Walker & Zunt, 2005). To our knowledge, these results provide the first evidence that prior exposure may amplify the metabolic impacts of parasite exposure.

While killifish activity returned to baseline levels, metabolic rate remained ~20% higher 1-hr post-exposure. A study by Voutilainen et al. (2008) picks up where our post-exposure period finished, recording elevated metabolic rate from 1 to 7 hr after parasite exposure, indicating that the prolonged metabolic response that we measured may continue for several hours. These prolonged physiological responses could be driven by several, non-mutually exclusive mechanisms. While excess post-exercise oxygen consumption (EPOC; Brennan et al., 2016) could contribute (to repay an oxygen debt arising from the elevated activity during cercaria exposure), the

observed activity increase did not amount to strenuous exercise (MR increases were only ~35% of the animals' total AS), so is unlikely to fully explain the prolonged metabolic response. More likely, exposed hosts increased their production of stress hormones (e.g. cortisol or epinephrine), which could have resulted in the prolonged increase in oxygen uptake (Brown et al., 1982; Morgan & Iwama, 1996). Another possibility is that the higher post-exposure metabolic rates result from a rapid immune response to acute infection, potentially as a pro-inflammatory reaction to tissue damage during epithelial penetration (Bourke et al., 2015; Ratanarat-Brockelman, 1974; Soares et al., 2014). As there was no upward or downward trend in oxygen uptake with time following exposure, it is likely that some combination of the above mechanisms contributed to the observed increase in oxygen uptake. Regardless of what factors drive the prolonged spike in metabolic rate post-exposure, the response to acute exposure may functionally impact killifish in the wild, particularly during summer when cercariae are present in the water every day at their highest densities (Fingerut et al., 2003). In flies, the substantial rise in metabolic rate in response to parasite exposure that was shown in Luong et al. (2017) resulted in both reduced life span and fecundity (Horn & Luong, 2018), suggesting that the metabolic impacts shown in this study could impact killifish long-term fitness.

Although correlations with body size were observed for both ΔMR_{acute} (which increased with host body mass) and the number of new infections (which declined in larger individuals), the distribution of body sizes was imbalanced, with only a few very large individuals. While the role of body size in host response to parasite exposure and established infection is not the primary focus of this study, differences in body size can have important impacts on host resistance and parasite virulence (Cable & van Oosterhout, 2007), so should be accounted for in the analysis. As such, those results should be further investigated to determine if they are artefacts of the imbalanced body size distributions or true effects found in natural systems.

If the clear metabolic response to acute infection leads to energetic impacts, those costs were not detectable when measuring aerobic metabolic rates and capacity in hosts with established infections. That is, when infectious cercariae were not present, uninfected and infected fish did not vary in SMR, RMR, MMR, AS or CS activity. This result is consistent with previous findings that gonadal somatic index and body condition indices do not vary between killifish from naturally infected and uninfected populations (Shaw et al., 2009, 2010; Shaw & Øverli, 2012). Luong et al. (2017) also showed similar findings in a fly-ectoparasite system, in which hosts exhibited substantial changes in acute metabolic rate following parasite exposure but negligible effects on metabolic rate in response to established infection, highlighting that in diverse host–parasite systems, we may find that the costs of parasite exposure (in terms of metabolic rate) exceed those arising from established infection.

Established *E. californiensis* infection significantly decreased LDH activity and relative anaerobic metabolic capacity in the brain, but did not alter either parameter in white muscle. The reduction in LDH activity in the brain of parasitized fish could have important

implications for fish neurobiology because lactate can account for up to 60% of the brain's metabolic fuel (Boumezbeur et al., 2010). Brain LDH synthesis and activity are regulated by several factors, including lactate availability (reviewed in Valvona et al., 2016). Interestingly, many trematode parasites produce lactate in large amounts (Barrett, 1981), and several studies have reported increased lactate levels in the blood of marine animals infected with parasites (Findley et al., 1981; Mansell et al., 2005; Moreira et al., 2018). Furthermore, lactate plays major roles in neuronal signalling (Barros, 2013; Tang et al., 2014), long-term memory formation and maintenance (Suzuki et al., 2011) and consolidation of conditioned fear responses (Stehberg et al., 2012). Thus, the observed changes in brain LDH enzyme activity may be a previously unidentified avenue by which *E. californiensis* influences host behaviour. Indeed, direct manipulation of lactate levels in an amphipod induced behavioural changes associated with infection by a behaviour-modifying acanthocephalan parasite (Perrot-Minnot et al., 2016), indicating that manipulating lactate metabolism may be a general mechanism for behaviour-manipulating parasites.

Infection with *E. californiensis* also induced an ~60% decrease in NKA abundance in killifish gills. Interestingly, nematode (Fazio et al., 2008; Lorin-Nebel et al., 2013) and dinoflagellate (Moreira et al., 2018) parasites also altered NKA activity or mRNA in gills of their fish hosts, despite invading different organs (swim bladder and gills respectively). This hints at a widespread effect of parasitic infection on gill NKA of fish, perhaps acting through common hormonal pathways. The eco-physiological implications of the observed decrease in gill NKA in parasitized killifish remain unknown. However, killifish inhabit coastal and estuarine environments with heterogeneous and variable salinity (Desmond et al., 2000), and this likely requires constant osmoregulatory adjustments. Hence, it is possible that parasitic infection affects killifish salinity tolerance or preference, which could, for instance, limit killifish distribution to large creeks with more physiologically favourable salinity.

We examined how the parasite *E. californiensis* alters the energetics and osmoregulatory phenotype of its intermediate host, the California killifish. Both previously uninfected and infected fish increased activity and metabolic rates when exposed to parasite infectious stages, clarifying that the response is at least partly innate. However, previously infected fish exhibited the strongest behavioural and physiological responses to exposure, indicating that responses are strengthened with experience. In contrast, established infection had little effect on measures of host metabolic rate (i.e. SMR, RMR, MMR, AS), suggesting that the hosts reallocate energy from other functions to maintain aerobic metabolic performance. Established infection unexpectedly decreased LDH activity in killifish brains, indicating that the previously documented modifications in killifish host behaviour arising from parasitism could be due in part to changes in brain lactate metabolism. Also, established infection lowered NKA activity in the gills, suggesting that infection may influence the distribution of the fish in their estuarine habitats. Hence, our findings, in conjunction with Luong et al. (2017) and Voutilainen et al. (2008), illustrate that parasites may metabolically

disrupt their hosts, both during initial parasite exposure and after infection is established, with knock-on effects for host performance and ecology.

AUTHORS' CONTRIBUTIONS

L.E.N. and R.F.H. conceived and designed this study; L.E.N., E.B., S.H.H.-R., A.V.T., K.L.W., Ø.Ø. and R.F.H. designed and/or implemented the fish rearing and long-term experimental infection experiment; L.E.N. carried out the respirometry experiments; A.V.T. quantified new infections and completed the preliminary experiment on timing of cercaria attack; L.E.N. and I.B.J. completed the metabolic enzyme assays; E.J.E. provided equipment for respirometry and contributed to the design of the respirometry and enzyme assay analyses; C.H. and M.T. provided equipment and contributed to the design of the metabolic enzyme assay analyses; G.T.K. and M.T. completed all gill NKA and immunofluorescence assays; L.E.N. performed all statistical analyses and created the figures, with input from R.F.H.; L.E.N. and R.F.H. drafted the manuscript. All authors contributed to editing the final manuscript and gave final approval for publication.

DATA AVAILABILITY STATEMENT

All data and code are available through the NSUWorks Data Repository https://nsuworks.nova.edu/occ_facdatasets/11/.

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