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ARTICLE

Experimental Infections of Bluegill with the Trematode *Ribeiroia ondatrae* (Digenea: Cathaemasiidae): Histopathology and Hematological Response

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Abstract

Infections by the digenetic trematode, *Ribeiroia ondatrae*, cause severe limb malformations in many North American amphibians. *Ribeiroia ondatrae* also infects fishes as second intermediate hosts, but less is known about the pathology and immune responses initiated in infected fish, even though reports of infected fish date back to early 1900s. To this end, we experimentally exposed juvenile Bluegills *Lepomis macrochirus* to three doses of *R. ondatrae* cercariae and monitored the pathology, parasite infection success, and humoral responses over 648 h. All exposed fish became infected with metacercariae, and the average infection load increased with exposure dose. Histologically, infection was associated with acute hemorrhages in the lateral line and local dermis at 36 h, followed by progressive granulomatous inflammation that led to the destruction of encysted metacercariae. Correspondingly, over the course of 648 h we observed an 85% decline in average infection load among hosts, reflecting the host's clearance of the parasite. Infection was not associated with changes in fish growth or survival, but did correlate with leukocytosis and neutrophilia in circulating host blood. Understanding the physiological responses of *R. ondatrae* in Bluegill will help to clarify the ecological effects of this parasite and provide a foundation for subsequent comparisons into its effects on behavior, individual health, and population dynamics of Bluegill.

Ribeiroia ondatrae (Digenea: Cathaemasiidae) is a trematode that sequentially infects freshwater snails (family Planorbidae) that act as first intermediate hosts, fish or amphibians that act as second intermediate hosts, and birds or, less commonly, mammals that are definitive hosts (Johnson et al. 2004). This parasite has gained recent notoriety for its capacity to induce severe limb malformations, including missing, malformed, or duplicated limbs, in a broad range of North American amphibian species (Blaustein and Johnson 2003; Johnson et al. 2012). Metamorphosing frogs that have limb deformities caused by *R. ondatrae* have impaired locomotion and decreased foraging success (Goodman and Johnson 2011a, 2011b) and are suspected to be more prone to predation by definitive hosts, thereby enhancing trophic transmission. Although recent attention has

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focused on interactions between *R. ondatrae* and its amphibian hosts, less is known about patterns of infection within fish or the relative importance of fish versus amphibians for supporting transmission in natural systems. To date, *Ribeiroia* spp. have been reported in 15 species and 9 genera of fishes throughout North and Central America (updated from Johnson et al. 2004) and was recently reported in South America (Pinto et al. 2014). Comparatively, *Ribeiroia* spp. infections have been detected in 21 species and 7 genera of amphibians (Johnson and McKenzie 2009). Similar to amphibians, fish become infected when free-swimming cercariae contact and penetrate fish epithelial tissue. Previous reports describe metacercariae as localizing superficially along the cornea and other epidermal surfaces, often in depressions or grooved areas such as the periocular area, nares, and especially in the lateral line canal (Beaver 1939; Basch and Sturrock 1969; Simmons 1971; Malek 1977; Nassi 1978; Molnár 1994; Hoffman 1999; Johnson et al. 2004; Pinto et al. 2014). How these infections affect fish health or their ability to detect potential predators—a key role of the lateral line canal system (Barber and Wright 2005)—remain largely unknown.

In the current study, we sought to characterize the direct physiological effects and tissue pathology associated with *R. ondatrae* infection in Bluegills *Lepomis macrochirus*, which act as intermediate hosts. Our specific goals were to (1) establish whether fish, like some amphibians, demonstrate the ability to clear *R. ondatrae* metacercariae over time, (2) characterize the pathology of lateral-line infection and how it changes with time postexposure (PE), and (3) investigate humoral responses to the parasite by comparing the leukocyte profiles of infected and uninfected fish.

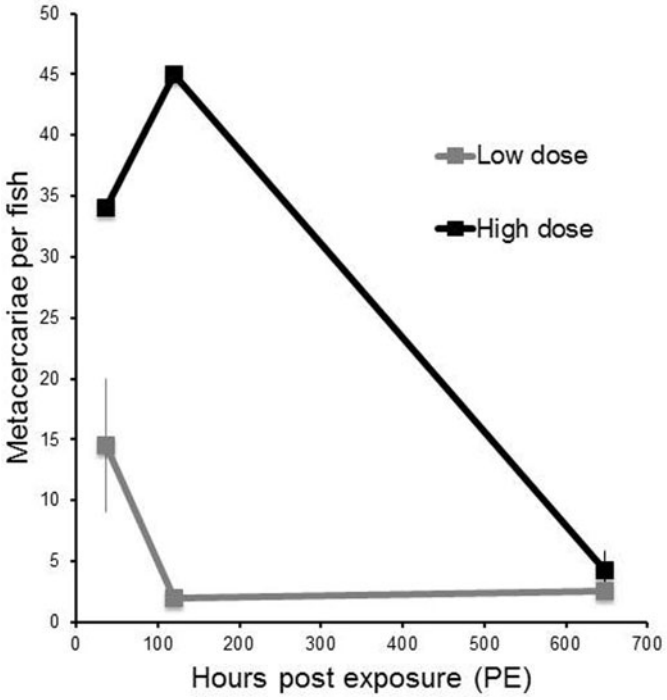

FIGURE 1. The average number of *Ribeiroia ondatrae* metacercariae recovered at each time point (36, 120, and 648 h) for low- and high-exposure treatment groups of Bluegills; vertical bars represent SE. Control treatment is not included as no parasites were ever detected.

METHODS

Fish husbandry.—Fifteen juvenile Bluegills of mixed sex and ranging in size from 76 to 127 mm TL were acquired from Aqua Sierra, Morrison, Colorado, and housed individually in 2.8-L containers in an Aquaneering Stand Alone Zebrafish Aquatic Rack (Aquaneering) (protocol approved by the Institutional Animal Care and Use Committee [IACUC] of the University of Colorado, Boulder). Bluegills were allowed to acclimate for 48 d prior to parasite exposure and were fed Omega One Freshwater flakes daily to satiation. Water was maintained at 23°C and pH 8.0 with an automated 80% water change completed every 12 h. The temperature in the experimental room was 25 ± 2°C, and a light timer was set to provide 12 h of light and 12 h of dark.

Individual specimens of marsh rams-horn snails *Helisoma trivolvis* infected with *R. ondatrae* were collected from freshwater ponds located in the San Francisco Bay Area of California within the following three California counties: Alameda, Contra Costa, Santa Clara (see Figure 1 in Richgels et al. 2013) between the months of May and July, 2014, using dip nets and/or a 2-m seine as described by Richgels et al. (2013). Snails were housed in the laboratory in 40-L Rubbermaid containers containing freshwater that was dechlorinated, sterilized with ultraviolet light, and carbon filtered (referred to as "treated water" hereafter). Water in the snail containers was replaced every other day. Snails were fed a mixture of ground Tetramin, agar, and calcium daily. Air temperature for the snails was 28 ± 2°C and the photoperiod was adjusted to a 16 h light : 8 h dark cycle to match the field environment from which they were collected.

Parasite exposures.—To obtain cercariae, *H. trivolvis* infected with *R. ondatrae* were placed individually into 50-mL conical tubes filled with 40−45 mL of treated water and housed in the dark, which is known to induce shedding (Paull et al. 2012). Approximately 7 h later, released cercariae were collected from each vial and pooled into a single water sample to avoid any snail-specific effects. The density of cercariae per milliliter was calculated using an Olympus SZX10 to count the number of cercariae in a 50-mL subsample of water. This allowed us to estimate the total number of cercariae within the entire sample (i.e., by multiplying the number of cercariae per milliliter in the subsample by the total remaining volume in the entire sample).

Fifteen Bluegills were measured (TL, mm), weighed (wet mass, g), and randomly assigned using the random function in Microsoft Excel software into one of three experimental treatments: control, low exposure, and high exposure. There were no significant differences in initial host TL (ANOVA: $F_{1, 13} = 0.11$, $P = 0.74$) or wet mass ($F_{1, 13} = 0.24$, $P = 0.62$). Exposure doses

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of *R. ondatrae* were based on the dose of *R. marini* known to cause pathology in Goldfish *Carassius auratus* lateral lines (320 cercariae per fish: Huizinga and Nadakavukaren 1997). Six fish were exposed as a group to a "low exposure" of approximately 994 cercariae (or 199 cercariae per fish) within a volume of ~18 L of treated water. Six fish were exposed to a "'high exposure" of 2,158 cercariae (or 432 cercariae/fish) in a comparable volume (~18 L). The three remaining fish served as controls and were subjected to the same handling procedures as exposed fish, but were not exposed to cercariae. The low- and high-exposure groups were incubated with cercariae for a total of 17 h, which is the viable lifespan of free-swimming cercariae (Paull et al. 2012), after which fish were transferred back into individual 2.8-L containers. Fish were maintained for a maximum of 648 h PE.

Two fish from the low-exposure treatment were humanely euthanized following guidelines in the IACUC protocol at the following time points: 36, 120, and 648 h PE. One fish from the high-exposure treatment was humanely euthanized at 36 h and another at 120 h PE, and the remaining four fish were euthanized at 648 h when the experiment was terminated. One control fish was also euthanized at each time point. Cardiac blood was collected immediately post mortem using a capillary tube, and a fresh blood smear was prepared following blood-staining procedures described in Calhoun (2009). After blotting dry each fish was measured (mm) using digital calipers and weighed (g) on a digital scale.

Leukocyte profiles.—Two different methods were used to quantify leukocyte profiles in the Bluegills. First, following La-Fonte and Johnson (2013), the number of white bloods cells (WBCs) (neutrophils, eosinophils, lymphocytes, monocytes, and thrombocytes) was counted per 2,000 erythrocytes (RBCs), which offers a metric of the total number of each cell type relative to circulating RBCs. Second, leukocyte differentials were performed, which quantified the number of each WBC type within a sample of 100 WBCs (see Calhoun 2009). We were unable to collect blood from one Bluegill in the low-exposure group.

Gross pathology and histopathology.—We examined the entire fish, including the surface and all organs, using an Olympus SZX10 dissection scope. The number of *R. ondatrae* metacercariae was recorded and sites of infection were labeled using tissue-marking dye (Cancer Diagnostics). The right and left body walls containing the lateral lines were dissected free from each fish and preserved in Davidson's solution for histopathology. Sites on the lateral line marked with tissue dye were targeted for specific evaluation. These sites were trimmed transversely, such that the sections produced would include a transverse cross-sectional profile of the lateral line, the associated scale, dermis with epidermis, and underlying skeletal muscle. These trimmed sections were routinely processed, embedded on the transverse edge in paraffin, sectioned at 4-μm intervals. The resulting sections were mounted on microscope slides and stained with hematoxylin and eosin for histological evaluation.

Data analysis.—To assess how the number of metacercariae varied with treatment and time PE, we used a generalized linear model with a negative binomial distribution, which frequently approximates the statistical distribution of parasite abundance within hosts. Predictor variables were parasite exposure treatment (low versus high exposure, controls were not included in this analysis as they had no detected infections), hours PE (36, 120, and 648 h), and initial fish size (to account for any effects host size might have on the probability of parasite encounter). We also included an interaction effect between treatment and time; however, when this was nonsignificant we removed it and ran the analyses again. For fish size, we evaluated how infection treatment, hours PE, and their interaction affected final size (final length [mm] or mass [g] minus initial length or mass) using a general linear model (i.e., ANOVA). Finally, to analyze leukocytes cell counts among exposed and unexposed hosts, we used generalized linear regressions with the number of a particular blood cell type (scaled per 2,000 RBCs) as the response and exposure treatment as the predictor (control, low, or high). We ran the models with the response variables modeled as either a Poisson or negative binomial distribution and used the Akaike information criterion (AIC) values to choose the better-fitting model.

RESULTS

Infection Success

All Bluegills exposed to cercariae became infected with *R. ondatrae,* regardless of whether they were in the low- or high-exposure treatments or the time point at which they were examined. In both exposure treatments, an estimated 8% of administered cercariae successfully established as metacercariae by 36 h PE. At the end of the study (648 h), approximately 1% of administered cercariae persisted as metacercariae in both exposure treatments. Based on a generalized linear model with a negative binomial distribution, Bluegills exposed to the high-exposure treatment of cercariae had consistently more metacercariae (mean = 16.0, SE = 7.63) at each time point compared with fish exposed to the lower exposure treatment (mean = 6.3, SE = 2.95) (Figure 1) (low- versus high-dosage effect = 1.587, SE = 0.4199, $Z = 3.779$, $P = 0.00016$). Concurrently, time PE had a strong negative effect on the number of metacercariae detected (hours PE = 0.00324, SE = 0.000732, $Z = -4.429$, $P < 0.000001$). Initial fish size (mm) had no effect on infection ($P = 0.15$). Thus, between 36 and 648 h PE, the number of metacercariae detected dropped by 83% in the low-exposure treatment and by 88% in the high-exposure treatment (Figure 1). There was no interaction between dose and hours PE, nor did fish size (included as a covariate) explain any additional variation ($P = 0.15$).

Gross Pathology

We further evaluated whether infection by *R. ondatrae* resulted in detectable effects on Bluegill growth during the

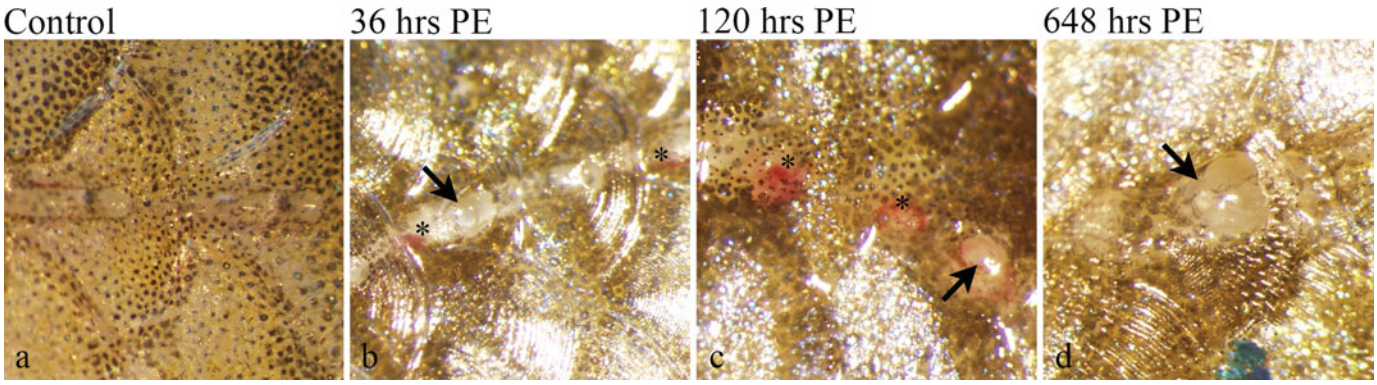

FIGURE 2. Magnification (57.5 ×) of the lateral line in Bluegills after exposure to *R. ondatrae*. (**a**) Control: The normal lateral line is a delicate structure created by the overlap of specialized scales along the body wall; no hemorrhages are observed. (**b**) 36 h PE: Multiple hemorrhages are present along the lateral line (*). There is increased mucous at the lateral line pores (arrow). (**c**) 120 h PE: Multiple hemorrhages are present along the lateral line (*). There is increased mucous at the lateral line pores (arrow). (**d**) 648 h PE: There is increased mucous at the lateral line pores (arrow). The focal green discoloration at the bottom edge of the image is tissue dye. [Figure available in color online.]

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experiment. Based on ANOVA, parasite exposure had no significant effect on the change in fish mass (final minus initial), whereas hours PE had a positive effect (overall ANOVA: $F_{2, 12} = 4.12$, $P = 0.04$; parasite exposure t-ratio $= 0.31$, $P = 0.76$; hours PE t-ratio $= 2.65$, $P = 0.021$). Neither variable significantly influenced the change in fish length (overall ANOVA: $F_{2, 12} = 0.586$, $P = 0.57$; parasite exposure t-ratio $= -0.07$, $P = 0.95$; hours PE t-ratio $= 1.06$, $P = 0.312$).

Petechial hemorrhages were evident along the lateral lines of the fish at 36 h and 120 h PE (Figure 2b, c). Hemorrhages were not evident at 648 h PE (Figure 2d). Increased amounts of mucus were present at lateral line pores at all time points, except in the control fish (Figure 2). No hemorrhages or mucus were present in the lateral line pores of fish in the control treatment (Figure 2a).

Histopathology

A total of 291 sections, each 4 μm thick, of lateral line tissue from the control (47 sections) and infected fish (244 sections: 97 sections at 36 h PE, 50 sections at 120 h PE, and 97 sections from 648 h PE) were prepared and evaluated microscopically. Lateral line tissues from control fish were histologically normal (Figure 3a). In these sections, the lateral line lumen was empty. The lateral line was lined by a single layer of epithelial cells, supported by sparse dermis and bone of the lateral line canal.

By 36 h PE, infected fish exhibited acute and multifocal hemorrhages within the lateral line canal and in the dermal interstitium supporting the lateral line epithelium (Figure 3b). Although metacercariae were rarely observed in histologic sections at this time point, in some cases they formed cyst-like spaces immediately subjacent to the lateral line epithelium (Figure 3c). These cyst-like spaces were not associated with inflammation. In other sections, the lumen of the lateral line was almost fully occluded by the presence of a single metacercaria surrounded by infiltrates of macrophages and sparse amounts of necrotic cellular debris with variable disruption and loss of the canal epithelium (Figure 3d, g). Observed metacercariae measured ~200 μm in diameter, had an eosinophilic tegument 10 μm thick, and loose parenchyma. Ventral suckers were prominent in some sections where metacercaria were observed.

Lateral line architecture was completely disrupted after 120 h PE; there was a loss of the lining epithelium and full occlusion of the canal lumen at sites of infection (Figure 3e, h). This was caused by a heavy influx of epithelioid macrophages and multinucleated giant cells. The inflammatory cells often formed granulomas centered on small amounts of necrotic cellular debris. Small to moderate numbers of lymphocytes and plasma cells were lightly distributed in the dermis of the adjacent scale pockets. No metacercariae were observed directly within the lateral line canals at 120 h PE, likely due to the extensive inflammatory response.

By 648 h PE, some sections exhibited moderate to large numbers of lymphocytes and plasma cells in the dermis adjacent to scale pockets (Figures 3f, i). Metacercariae were not evident in any of the examined sections and granulomatous infiltrates were absent.

Leukocyte Profiles

Neutrophils, lymphocytes, thrombocytes, and RBCs were identified in all leukocyte profiles. Infected Bluegills had an elevated WBC count, averaging 21.8 WBCs (SE = 5.68) per 2,000 RBCs compared with that in the control fish, which averaged 5.6 WBCs (SE = 0.66) per 2,000 RBCs. Based on generalized regressions with a negative binomial distribution, both the number of neutrophils and the number of lymphocytes per 2,000 RBCs increased as a function of parasite exposure dosage (lymphocytes, $P = 0.05$; neutrophils, $P = 0.001$; total WBCs, $P = 0.005$). The negative binomial distribution model offered the lowest AIC value (indicating better model fit; all delta AICs > 10) in all cases.

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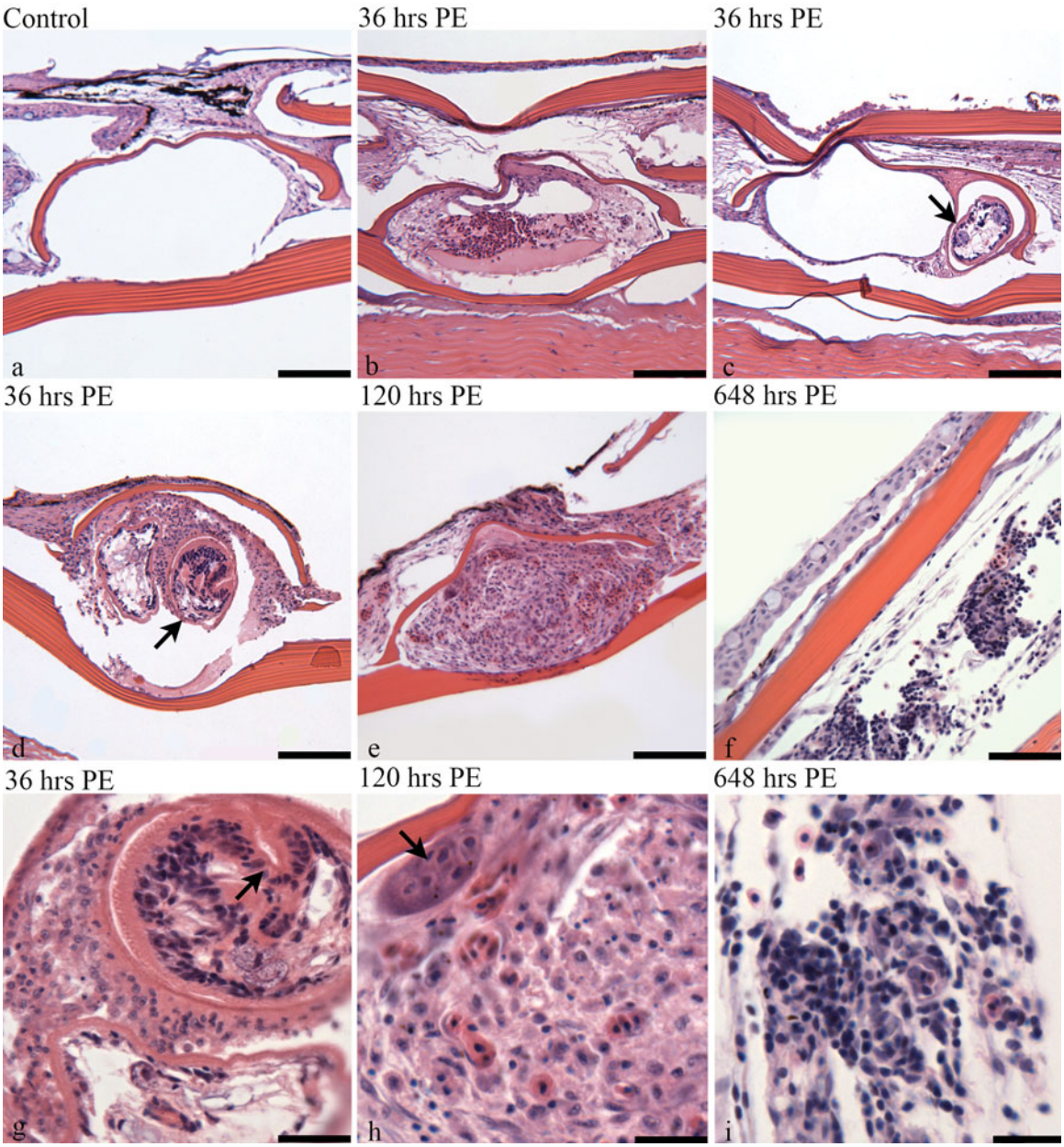

FIGURE 3. Histological sections of the lateral line of Bluegill after exposure to *R. ondatrae*, stained with hematoxylin and eosin. (**a**) 36 h PE: The control lateral line is a hollow structure lined by a thin layer of epithelial cells (not easily distinguished at this magnification) that are supported by sparse dermis and bone of the scale. The bony canal is artifactually fractured from the plane of the scale. (**b**) 36 h PE: Free red blood cells (hemorrhage) and protein-rich fluid are consistent with plasma found in the lateral line. (**c**) 36 h PE: A metacercaria encysted in the dermis immediately adjacent to the lateral line canal with no observed inflammatory response. (**d**) and (**g**) 36 h PE: Large numbers of inflammatory cells, predominantly macrophages, destroy normal lateral line architecture and surround a metacercarial parasite (arrow). A portion of a sucker is evident (arrow) in panel (g). (**e**) and (**h**) 120 h PE: The lateral line canal is completely occluded by granulomatous infiltrates. Occasional multinucleated giant cells are present at higher power (arrow, panel h). (**f**) and (**i**) 648 h PE: The dermis of the scale pocket is expanded by moderate numbers of plasma cells and lymphocytes. (a–f, scale bar = 150 μm; g–i, scale bar = 75 μm). [Figure available in color online.]

Monocytes, neutrophils, lymphocytes, eosinophils, and thrombocytes were identified in each differential profile. Fish exposed to the high-exposure treatment developed a neutrophilia in their circulating blood compared with that in the control fish (64% [SE = 4.22%] to 54% [SE = 1.00%]). Fish exposed to low- and high-exposure treatments also developed a lymphopenia compared with that in the control fish (32.6% [SE = 1.00%] and 27.8% [SE = 3.68%], respectively, to 43% [SE = 1.54%]; $P = 0.01$). Average monocytes were slightly elevated in the high dose treatment compared with that in the uninfected fish (7.8%

[SE = 2.48%] to 3.3% [SE = 0.88%]; $P = 0.10$). Bluegills infected with either exposure treatment had eosinophils in circulating blood, whereas none were found in the control fish, but the numbers of eosinophils were too low to include in statistical analyses. Time since exposure had no significant effects on leukocyte counts, either on its own or as a covariate with infection treatment.

DISCUSSION

Our research indicated that Bluegills exposed to cercariae of *R. ondatrae* consistently became infected with metacercariae in the lateral line (range in infection intensity: 1–45) and exhibited evidence of dermal pathology associated with infection. Exposure dosage was monotonically related to observed infection, such that a ~50% increase in cercariae dose translated into a ~40% higher average incidence of infection per fish at 36 h PE. Over time, however, the number of metacercariae per host decreased sharply, averaging an 85% decline over the 648-h observation period. Although infection was not associated with changes in fish growth, it was correlated with circulating leukocytosis and neutrophilia, which is consistent with an inflammatory response (Simmons 1971; Mayer and Donnelly 2013). The lymphopenia observed in infected fish may be indicative of a stress response.

Based on histopathology, the infection by *R. ondatrae* metacercariae was associated with tissue inflammation and hemorrhaging. At 36 h PE, the lateral line and local dermis of infected fish exhibited acute hemorrhages observable both grossly and histologically, and identified metacercariae were surrounded by early granulomatous inflammation. By 120 h PE, metacercariae were no longer evident, likely due to the inflammatory response, and the lateral line canal had become occluded by more robust granulomatous inflammation. By the final observation at 648 h PE, neither metacercariae nor granulomatous infiltrates were identified, suggesting the infection response had subsided. The presence of moderate numbers of lymphocytes and plasma cells in the dermis is consistent with a chronic response to antigenic stimulation. Hemorrhages and inflammation were not observed in the lateral lines of any fish in the control treatment (not exposed to cercariae). These results are broadly similar to those reported previously for infected Goldfish (Simmons 1971; Huizinga and Nadakavukaren 1997). Huizinga and Nadakavukaren (1997) reported that infection in Goldfish resulted in the influx of granulocytes adjacent to the cyst wall of *R. marini*, eventually creating a necrotic zone that was further flanked by lymphocytes. Those authors concluded that the inflammation ultimately resulted in destruction or expulsion of the metacercariae from the lateral line canal (i.e., clearance of the infection); this explanation parallels our own results.

Our results also demonstrated the progressive loss of parasites over time, which is consistent with clearance by the host. Time (h) PE had a strong, negative effect on the number of metacercariae observed, such that the infection load decreased by >80% between 36 and 648 h PE. Host clearance of *Ribeiroia* metacercariae has also been documented in Goldfish (Simmons 1971; Huizinga and Nadakavukaren 1997) and in amphibians (LaFonte and Johnson 2013). Huizinga and Nadakavukaren (1997) found that metacercariae in Goldfish either failed to encyst successfully or were destroyed within 4−5 d after the initial infection, suggesting that some fish species may even be "dead end" hosts. Simmons (1971) found similar results, with 43% of the metaceracariae of *R. marini* unaccounted for after 7 d of exposure in Goldfish. In these studies as well as ours, evidence of immune activity on the part of the host suggests that parasites are being actively cleared, rather than parasite mortality occurring independent of host factors.

In contrast with the current study of Bluegills and previous work with fish as hosts for parasites in the genus *Ribeiroia*, most amphibians challenged experimentally with cercariae of *R. ondatrae* were more competent as hosts. For instance, in a study of 13 amphibian species, including larval frogs, toads, and salamanders, Johnson et al. (2012) found that exposed species maintained between 7% and 41% (mean of 25%) of administered cercariae to metamorphosis. Tree frogs in the genus *Hyla* represent an important exception, in which the estimated survival time of metacercariae was only 45 h (see Johnson and Hartson 2009 and LaFonte and Johnson 2013). These observed differences between fish and amphibians suggest that fish tend to be less competent hosts for *Ribeiroia* spp., possibly owing to the more well-developed immune system of fish at the time of exposure (relative to larval amphibians), and raises important questions about the relative importance of each host group to parasite transmission in natural systems. However, differences in the methodologies used in these experiments (individual versus group exposures, water volume) and the parasite species used (*R. ondatrae* versus *R. marini*) underscore the need for more comparative studies investigating the relative responses of fish and amphibians to metacercariae infection within a natural aquatic system.

An important future question concerns the ecological implications of these results for fish–parasite interactions in nature. The lateral line system of fish detects mechanical vibrations in water and thereby influences balance, posture, and visual stabilization, as well as detection of both prey and predator (Wubbels and Schellart 1998; Barber and Wright 2005; Mirjany et al. 2011). Infection by *R. ondatrae* metacercariae resulted in the occlusion of the lateral line canal and loss of the epithelial lining, which could therefore interfere with the fish's ability to detect and respond to environmental stimuli and swim efficiently. For instance, experimental destruction of neuromasts has reportedly caused a reduction in swimming efficiency in body–caudal-fin swimmers (Yanase et al. 2014). Parasite-induced alterations of behavior are well documented (Barber et al. 2000; Adamo 2003; McElroy and de Buron 2014) and, at least in some cases, have been linked to increased predation by suitable definitive hosts (Lafferty and Morris 1996). Additional behavioral studies are needed to assess the effects of *R. ondatrae* infection in the lat-

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eral lines of fish to determine how this affects sensory input, swimming ability, and predator avoidance.

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