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IMMUNOLOGY AND HOST-PARASITE INTERACTIONS - ORIGINAL PAPER

Endocrine and immune responses of larval amphibians to trematode exposure

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Abstract

In nature, multiple waves of exposure to the same parasite are likely, making it important to understand how initial exposure or infection affects subsequent host infections, including the underlying physiological pathways involved. We tested whether experimental exposure to trematodes (*Echinostoma trivolvis* or *Ribeiroia ondatrae*) affected the stress hormone corticosterone (known to influence immunocompetence) in larvae representing five anuran species. We also examined the leukocyte profiles of seven host species after single exposure to *R. ondatrae* (including four species at multiple time points) and determined if parasite success differed between individuals given one or two challenges. We found strong interspecific variation among anuran species in their corticosterone levels and leukocyte profiles, and fewer *R. ondatrae* established in tadpoles previously challenged, consistent with defense "priming." However, exposure to either trematode had only weak effects on our measured responses. Tadpoles exposed to *E. trivolvis* had decreased corticosterone levels relative to controls, whereas those exposed to *R. ondatrae* exhibited no change. Similarly, *R. ondatrae* exposure did not lead to appreciable changes in host leukocyte profiles, even after multiple challenges. Prior exposure thus influenced host susceptibility to trematodes, but was not obviously associated with shifts in leukocyte counts or corticosterone, in contrast to work with microparasites.

Keywords Disease · Stress · Hormone · Immunity · Glucocorticoids · Amphibian · Parasite

Introduction

Infectious diseases of wildlife are of increasing concern and relevance given the recent emergence or re-emergence of many pathogens and parasites and the resulting implications for conservation and zoonotic transmission (Daszak et al. 2000; Thompson et al. 2010). There is thus substantial interest in factors that can affect host susceptibility to pathogens and parasites, including environmental influences such as pesticides and altered temperatures (e.g., Murdock et al. 2012; Pettis et al. 2013). However, a potentially important factor that may underpin variation in susceptibility to infection is the range in host physiological responses following previous parasite/pathogen exposure (Warne et al. 2011; Blaustein et al. 2012). This is particularly significant considering that most hosts simultaneously harbor several pathogen/parasite types, and macroparasite burdens often represent the outcome of multiple exposure events through time (Graham et al. 2007; Blaustein et al. 2012). Importantly, parasite exposure and infection may trigger endocrine and immunological responses that affect host susceptibility during subsequent exposures to the same or different parasites, thereby mediating positive or negative feedback effects through which secondary infections can be facilitated or hampered, respectively. The direction of these feedback effects is widely known to depend on the nature of the immune mechanisms involved, with any increase in host resistance termed "immune priming" (reviewed by Graham et al. 2007).

Infection and the resulting pathology are the product of complex interactions among host immunity, endocrine regulation, and parasite/pathogen traits (Stoltze and Buchmann 2001; Blaustein et al. 2012). Especially pertinent for parasite

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exposure is the host "stress response" to adverse conditions, i.e., a suite of adaptive physiological responses to overcome a challenge, as well as to restore internal homeostasis (see Dantzer et al. 2014 for a review), which can alter susceptibility to infection. Glucocorticoids (GCs) represent a major class of vertebrate steroid hormones widely tied to the general stress response triggered by various negative stimuli (Sapolsky et al. 2000), including parasite/pathogen exposure and infection (e.g., Stoltze and Buchmann 2001; Warne et al. 2011; Blaustein et al. 2012; Rollins-Smith 2017). This group of hormones (primarily cortisol for fish and mammals, but corticosterone for amphibians, birds, and reptiles) is critical to the short-term stress response by mobilizing energy stores and suppressing non-vital processes such as reproduction to reallocate resources (Sapolsky 1993; Romero 2004). Among other influences, GC alterations can affect cognitive and cardiovascular functioning, as well as various behavioral aspects (reviewed by Sapolsky et al. 2000; Korte et al. 2005). With respect to host GCs, various studies have reported elevations, decreases, or no change in relation to infection by parasites or pathogens. For example, helminth-infected baboons showed a decrease in GCs relative to uninfected individuals as infections progressed (Morales-Montor et al. 2001), whereas the opposite pattern was seen for helminth-infected mice (Barnard et al. 1998).

Understanding how host GCs are affected by parasite and pathogen infections is important because these hormones also play a vital role in modulating the vertebrate immune response via activation of the hypothalamus-pituitary-adrenal (HPA, for mammals), or hypothalamus-pituitary-interrenal (HPI, for non-mammalian vertebrates), axes (Sapolsky et al. 2000; Rollins-Smith and Woodhams 2012). Exposure to stressful conditions in vertebrates can activate the HPI/HPA axis (see review by Herman and Cullinan 1997), resulting in elevated levels of GCs that can alter immune responsiveness directly or through secondary means (see Cain and Cidlowski 2017 for a review), with implications for infection dynamics. For instance, long-term exposure to exogenous corticosterone has been shown to decrease both the levels of key leukocytes and resistance to helminth infection in larval amphibians (e.g., Belden and Kiesecker 2005; LaFonte and Johnson 2013), while elevated GCs in influenza-infected mice increased their susceptibility to secondary bacterial infections (Jamieson et al. 2010).

However, while chronic elevation of GCs is generally considered immunosuppressive, their well-known anti-inflammatory properties may be essential in preventing an "over-shoot" of the innate immune response to infections which can harm hosts, suggesting that short-term GC increases could actually be adaptive (reviewed by Sapolsky et al. 2000). In addition, high levels of stress hormones do not always lead to immunosuppression, and even enhance immunity under certain conditions (Martin 2009). Chronic GC reductions can also have potentially detrimental effects, including the suggested link between stress-associated neuropsychiatric disorders such as chronic fatigue syndrome and lowered activity of the HPA axis (reviewed by Yehuda and Seckl 2011).

To date, most investigations regarding parasitism and GCs in wildlife have focused on whether stress-altered levels of these hormones affect subsequent host resistance and/or tolerance to infection (e.g., Harris et al. 2000; Saeij et al. 2003). The extent to which infection itself affects host GCs has received considerably less attention (e.g., Parris and Cornelius 2004). As an additional complication, it is difficult to determine whether hosts with initially high GCs were more susceptible to parasitism, or whether GC levels were instead raised post-infection owing to the correlational nature of many studies. This distinction is critical to understand the degree to which parasites constitute a stressor in themselves rather than primarily considering infection an outcome of host exposure to other aversive stimuli that raise GCs. Experimental infections are thus an important tool to determine the effects of parasitism on host stress responses (reviewed by Graham et al. 2011), as are manipulations where GCs are blocked after the induction of stress (e.g., Gabor et al. 2018).

The negative effects of parasites/pathogens on amphibians have become of particular concern in the past three decades, especially the chytrid fungus *Batrachochytrium dendrobatidis* (a.k.a. *Bd*), ranaviruses, and trematodes (flatworms) such as *Ribeiroia ondatrae*, which collectively pose a significant conservation issue (Daszak et al. 2003; Blaustein et al. 2012). Compared to microparasites, the effects of macroparasitic infections on amphibian stress hormones and immune responses are less well understood (Rollins-Smith and Woodhams 2012) despite the ubiquity of helminths and their potentially detrimental effects (reviewed by Koprivnikar et al. 2012). Typically, macroparasites are considered to include infectious entities whose pathology is intensity-dependent (Lafferty and Kuris 2002). Exposure to macroparasites, particularly helminths, may thus provoke different host physiological responses relative to microparasites/pathogens (e.g., bacteria, fungi, and viruses). For instance, Marino et al. (2014) found that larvae of two species of ranid frog exposed to the infectious stage of a trematode parasite (*Echinostoma trivolvis*) had decreased corticosterone levels relative to controls, in contrast to studies with *Bd* and ranavirus (Warne et al. 2011; Gabor et al. 2015, 2018; Rollins-Smith 2017).

However, given the overall paucity of studies, it is not yet possible to conclude whether there is a fundamentally different response by amphibians to helminths compared to microparasites. As the encapsulation and elimination of larval trematodes by larval amphibians rely heavily on the inflammatory response (Holland et al. 2007), dampening of this pathway by GCs could affect the degree of pathology experienced in this host-parasite system, as well as the response to subsequent parasite exposure. The functioning of the

neuroendocrine-HPI axis may therefore be central to understanding variation in amphibian resistance and tolerance to infection by parasites and pathogens across host species, as well as in different environmental contexts (Rollins-Smith 2001, 2017; Blaustein et al. 2012). Considering the complexity of interactions among the HPA/HPI axis, circulating GCs, and immune function, it is important to consider both endocrine and immune responses (Fast et al. 2006; Rollins-Smith 2017).

Here, we sought to evaluate whether exposure to macroparasites affected two aspects of larval amphibian physiology: (1) endocrine profiles, specifically levels of corticosterone, and (2) host immunity, as measured by leukocyte profiles. To investigate the corticosterone response, larvae from five anuran species were experimentally exposed to one of two pathogenic trematodes (*R. ondatrae* or *E. trivolvis*). Both parasites are known to have detrimental effects (see reviews by Johnson and McKenzie 2009; Koprivnikar et al. 2012), but differ in their modes of host invasion, site of infection, as well as degree and form of pathology. To assess how parasitism affected leukocyte (a.k.a. white blood cell, WBC) profiles, we evaluated changes in seven host species 36 h after exposure to *R. ondatrae*. For four of the host species, WBC profiles were examined for an additional 4 days to test for temporal changes. Lastly, we used fluorescently labeled cercariae to test how previous exposure (1 week prior) to *R. ondatrae* affected subsequent susceptibility in one host species, and whether this was reflected in their WBC profiles (i.e., "immune priming"). Taken together, these experiments thus aimed to determine how prior parasite exposure/infection affects subsequent susceptibility in a vulnerable group of hosts (amphibians), as well as potential underlying mechanistic pathways.

Material and method

Parasite life cycles and pathology

The life cycles of *E. trivolvis* and *R. ondatrae* are similar in that both are complex and often utilize larval amphibians as second intermediate hosts (see Johnson and McKenzie 2009 for a review). However, these two trematode species differ with respect to their encystment site and pathology. Cercariae of *E. trivolvis* enter the cloaca of tadpoles and encyst within the nephric system. Pathology is dependent on infection intensity and host life history such that early-stage tadpoles with many cysts often exhibit edema, reduced kidney function, and decreased survival (Fried et al. 1997; Schotthoefer et al. 2003a). In contrast, *R. ondatrae* cercariae primarily target the site of developing hindlimb buds in larval amphibians, forming subcutaneous cysts after burrowing into host tissue (Johnson and McKenzie 2009). The pathology of *R. ondatrae* is similarly influenced by infection level and host stage (Schotthoefer et al. 2003b; Johnson et al. 2011), but is more severe relative to that of *E. trivolvis* at the same dose. Exposure to as few as 10–15 *R. ondatrae* cercariae is often lethal to young tadpoles and those that survive typically develop a range of malformations (Rohr et al. 2010; Johnson et al. 2011, 2012). For *E. trivolvis*, a dose of 25 cercariae can substantially reduce survival of amphibian larvae in early developmental stages, and survivors of exposure to as few as 15 cercariae can exhibit reduced growth (Schotthoefer et al. 2003a; Koprivnikar 2010). There is also substantial variation among amphibian species with respect to *R. ondatrae*- and *E. trivolvis*-induced pathology, but the physiological reason(s) underlying these differences remain poorly understood (Holland 2010; Johnson et al. 2012).

Larval amphibian maintenance

Rana clamitans (green frog) and *Xenopus laevis* (African clawed frog) eggs were procured from a commercial supplier (Charles D. Sullivan Co. Inc.). Eggs of the following species were collected from field sites: *Rana pipiens* (Northern leopard frog) and *Bufo americanus* (American toad) from Manitoba; *B. boreas* (Western toad), *Rana cascadae* (Cascades frog), and *Pseudacris regilla* (Pacific chorus frog) from Oregon; *P. triseriata* (Western chorus frog) from Colorado; *R. sphenocephalus* (Southern leopard frog) and *Osteopilus septentrionalis* (Cuban tree frog) from Florida; and *Hyla versicolor* (Gray tree frog) from Pennsylvania. Considering that these species do not occur in the same geographic regions or breed at the same time, it was necessary to use both field collections and commercially supplied eggs; however, all eggs were reared under the same laboratory conditions during the same time period. Multiple egg masses for each species were separately maintained in dechlorinated water on a 14:10 light-dark cycle at 22 °C, and, after hatching, fed a mixture of agar, *Spirulina*, and Tetramin fish food until larvae reached the desired developmental stage (see below).

Larval amphibian parasite exposure and corticosterone response

After hatching, larvae from different egg batches were mixed for each of five species (*X. laevis*, *R. clamitans*, *R. pipiens*, *B. boreas*, and *B. americanus*) and reared in separate 40-L containers of dechlorinated water until they reached Gosner developmental stage 27–29 (Gosner 1960). Parasites were obtained from field-collected planorbid snails (*Helisoma trivolvis*) known to harbor natural infections of *E. trivolvis* or *R. ondatrae* based on previous morphological and molecular identification of trematode infectious stages (cercariae) from snails at these field sites (Huver et al. 2015), with consistent cercariae morphotypes found in the present study. Tadpoles of all host species were exposed to cercariae on the

same day to minimize potential batch effects. To do so, we haphazardly chose 30–50 individuals of each species from their group containers (availability differed) and randomly assigned them to 1 of 3 treatments in roughly equal proportions: control (sham addition of water), 20 *R. ondatrae* cercariae, or 20 *E. trivolvis* cercariae. As each group container held tadpoles hatched from multiple egg masses, this accounts for any inherent variation among masses. For the rest of the experiment, tadpoles were individually housed in 1-L containers filled with dechlorinated water. Cercariae were obtained as they emerged from snails kept in water-filled Petri dishes and were less than 4 h old when used. We added 20 cercariae to each tadpole container with a disposable plastic pipette, or the sham treatment by adding approximately the same amount of dechlorinated water (~ 2 mL). Tadpoles were then maintained in their containers for 72 h before euthanization in a solution of buffered 0.2% MS-222.

Owing to the small size of individual larval amphibians at relatively early developmental stages, sufficient plasma cannot be extracted for corticosterone analysis, thus requiring whole-body homogenates (see review by Burraco et al. 2015). Accordingly, specimens were placed into individual 1-mL microcentrifuge tubes, homogenized, and the contents were flash-frozen using an ethanol dry ice bath after they were centrifuged at 4000 rpm for 15 min at 4 °C. In accordance with established procedures (e.g., Yeh et al. 2013; Burraco and Gomez-Mestre 2016), the supernatant was extracted and kept at − 80 °C until corticosterone analysis took place. Given the possibility for rapid deterioration of corticosterone in fresh or thawed tadpoles, it is not possible to perform necropsies to determine both individual infection status and corticosterone level (LaFonte and Johnson 2013). However, previous studies (e.g., Johnson et al. 2012; Koprivnikar et al. 2014) suggest that our parasite exposure dosages and methodology were adequate to induce infection in all animals, albeit likely with varying levels of intensity. In addition, the same parasite exposure method for the leukocyte-related experiments (detailed below) resulted in successful infections.

Corticosterone analysis

The use of whole-body homogenates in tandem with enzyme immunoassay (EIA) procedures has become increasingly used over radioimmunoassay (RIA) procedures for studies of larval amphibians and other very small animals where it is difficult to extract sufficient plasma (reviewed by Burraco et al. 2015). While RIA is more sensitive to sample differences in GCs at very low concentrations, EIA is more effective for relatively high GC levels as commercial kits do not appear to have upper saturation limits (Burraco et al. 2015). EIA may therefore be better for detecting sample differences across a larger range of GC concentrations, especially when species have unknown basal GC levels (Burraco et al. 2015).

However, because many commercial EIA kits for corticosterone were developed for use with mammalian plasma, they may not be optimal for other vertebrates (Burraco et al. 2015), and these kits also appear to have lower repeatability relative to specific procedures developed in-house (Schoenemann and Bonier 2018). We thus followed established procedures (e.g., Yeh et al. 2013; Burraco et al. 2015) with a few modifications for whole-body GC quantification of small aquatic vertebrates via EIA without a commercial kit. Owing to the small volumes for some individual tadpoles (especially small-bodied species such as *B. boreas* and *B. americanus*), we reconstituted the supernatant samples with 1 mL of phosphate-buffered saline (PBS) in order to obtain sufficient material to run each sample in triplicate but did not add bovine serum albumin (BSA). The immune reaction was also blocked with SuperBlock® (Thermo Scientific) rather than BSA, and all incubations were for 1 h. Samples for the five different tadpole species were run on separate days. Two microplates were needed to perform the EIA for each species so as to accommodate samples for individual tadpoles in triplicate, as well as the controls and standards. Each microplate thus contained samples from individuals in the three experimental treatments, but the order of these was rotated among the microplate wells to minimize within- and between-plate variation.

Corticosterone standards were made by diluting analytical-grade corticosterone (Sigma-Aldrich) with ethanol. Sheep polyclonal cortisol antibody (LS-C152801) was obtained from LifeSpan BioSciences and used in a 1:1000 dilution with SuperBlock®. Based on supplier documentation, this antibody has a sensitivity of 5 pg/well when used for EIA and < 4% total cross-reactivity with other glucocorticoids. Donkey polyclonal anti-sheep IgG antibody (conjugated to horseradish peroxidase) was obtained from Novus Biologicals (NB7190) and used in a 1:1000 dilution with SuperBlock®. After the final reaction, we read the absorbance at 450 nm using a VersaMax® microplate reader (Molecular Devices). Standard curves were generated using the serial dilutions of corticosterone with SoftMax® Pro Data acquisition and analysis software. The corticosterone concentration for each well was thus determined based on the standard curves run in duplicate on each plate, and that for each individual tadpole was taken as the mean for its three triplicate samples. We validated our assay procedure for northern leopard frogs by serially diluting the supernatant extractions for another three individuals and comparing these values to the standard curves of known corticosterone concentrations.

With concerns regarding corticosterone degradation, individual tadpoles were not weighed at the time of euthanasia, but the volume of the homogenate obtained for each was recorded. To account for intra- and interspecific differences in tadpole size, the mean corticosterone concentration for each tadpole (in ng/mL) was divided by its initial homogenate volume (i.e., before extracting the supernatant and adding PBS). This allows for standardized comparisons of corticosterone concentrations among tadpoles of different sizes by adjusting

for the initial homogenate volume. Corticosterone concentrations from here on thus refer to those adjusted for the initial homogenate volume. Given the established relationship between the volume and wet mass of animal tissues (typically 1.1 g/mL—see Peters 1983), larger individuals should ultimately yield more homogenate and supernatant. Using 11 individuals each of *R. pipiens* and *B. americanus* that were frozen from pilot trials after their masses were recorded, we prepared homogenized samples in the same manner as described above to later examine the correlation between individual initial homogenate volume and wet mass so as to ensure that our use of the former adequately accounted for tadpole size and its potential effect on corticosterone concentrations.

The coefficient of variation (CV) can be used to evaluate consistency in readings for each individual when there are replicate samples; thus, tadpoles were removed from the data set if this value exceeded 20% following Freitas et al. (2017); < 7% of all samples exceeded this CV threshold. Given the variation in initial availability among amphibian species, and removal of individuals with large CV values, the following sample sizes for each host species were included in the statistical analysis: 46 *B. americanus*, 25 *X. laevis*, 35 *R. clamitans*, 52 *B. boreas*, and 26 *R. pipiens*. For the 3 treatments, there were 64, 63, and 57 tadpoles corresponding to the sham, *E. trivolvis*, and *R. ondatrae* exposures, respectively. Corticosterone concentrations were then $\log_{10}$-transformed before using a general linear model (GLM) in SPSS 24.0 to examine the factorial combination of host species and treatment (control, *E. trivolvis*, or *R. ondatrae*), followed by Tukey post hoc tests (LSD adjusted for multiple comparisons). A generalized linear mixed model (GLMM) was used to examine the relationship between individual log-transformed homogenate volume (normal distribution and log link function) and mass for our 22 pilot procedure tadpoles, including tadpole species (*R. pipiens* or *B. americanus*) as a random categorical effect.

Larval amphibian parasite exposure and leukocyte profiles

Leukocyte profiles were measured for tadpoles given a single or a repeated parasite exposure. For the single exposure experiment, we compared the WBC profiles of tadpoles exposed to *R. ondatrae* at a single time point to comparably treated animals that were not exposed to parasites by using standardized leukocyte measures (see below) across seven different host species (*B. boreas*, *R. cascadae*, *R. sphenocephalus*, *O. septentrionalis*, *H. versicolor*, *X. laevis*, and *R. clamitans)*. Egg masses were raised in 20-L containers containing dechlorinated water (~ 50 individuals per container) until they reached Gosner stage 28–30, whereupon experimental animals were transferred into individual 1-L containers. For each species, individuals were randomly assigned to either a parasite exposure treatment (20 *R. ondatrae* cercariae isolated from field-collected *H. trivolvis*) or the control treatment (sham exposure to 2 mL of water). For all species, blood smears were obtained from 12 individuals at 36 h post-exposure (P.E.) following euthanasia. For a subset of host species (*R. clamitans*, *R. sphenocephalus*, and *H. versicolor*), an additional 12 individuals were examined at 72 and 120 h P.E. to capture any temporal variation in leukocyte profiles and identify the time scale over which these occurred. Twelve *P. triseriata* were also assessed at the 72 and 120 h P.E. time points, respectively, as well as after 24 (but not 36) h.

In the repeated parasite exposure experiment, we assessed the potential for larval amphibians to exhibit immune priming after initial exposure to *R. ondatrae*, and whether this related to host resistance during a subsequent challenge with the same parasite. This was done by assigning *P. regilla* tadpoles in Gosner stage 27 to 1 of 2 different treatments ($n = 32$ individuals per treatment; 64 total): repeated exposure to cercariae (5 cercariae on day 0 followed by 30 cercariae on day 7) or single exposure to 30 cercariae (day 7 only). To distinguish between cysts resulting from the two separate exposure events, a red-fluorescing dye was used to label the cercariae used for tadpoles exposed on day 0 (for methodology, see Hoverman et al. 2013; Lafonte and Johnson 2013), and a green-fluorescing dye for the cercariae used on day 7. In this way, we were able to quantify parasite encystment from both challenges during host necropsy using a fluorescence-equipped stereomicroscope. The leukocyte profiles from a subset ($n = 16$) of surviving tadpoles were also examined 36 h after parasite exposure on day 7 to determine whether these differed as a function of one versus two parasite challenge events.

Quantification of leukocyte types

For both the single and repeated parasite exposure experiments, we quantified the proportion of circulating WBCs. These WBC counts were used as an indicator of immune activity in anuran hosts, in keeping with previous studies examining immunosuppression and parasitic infection in larval amphibians (e.g., Belden and Kiesecker 2005), and the importance of leukocytes for vertebrate immune responses to macroparasites, including that of tadpoles to trematodes (e.g., Martin and Conn 1990; Holland et al. 2007). Leukocyte profiles are of broad utility in evaluating vertebrate physiological stress via GC alterations (Davis and Maney 2018), including for larval amphibians (Burraco et al. 2017).

In brief, our approach for using blood smears to evaluate WBC profiles entailed a sagittal incision through the heart of each tadpole after euthanization to collect blood using a heparinized glass capillary tube that was then transferred to a clean microscope slide and smeared using a cover slip. After air drying, each slide was immersed in Wright's stain (R9350, Ricca Chemical, Arlington, TX, USA) for 5 s, drained for 2 min, and then rinsed twice in deionized water. The number

of lymphocytes, neutrophils, eosinophils, basophils, and monocytes per 2000 erythrocytes (i.e., red blood cells—RBC) were quantified for each smear following standard methods (Hadji-Azimi et al. 1987; Davis 2009). After obtaining blood samples, hosts were necropsied to quantify the number of encysted *R. ondatrae* (metacercariae) using standard methods (see Johnson et al. 2011).

Leukocyte profile analysis

Because basophils and monocytes tended to be quite rare (1–2 cells per 2000 RBC), we focused on the remaining types of WBCs, as well as total number of WBC, in our analyses as they are believed to play larger roles in the amphibian immune response to larval trematodes (Kiesecker 2002; Belden and Kiesecker 2005; Maizels and Yazdanbakhsh 2003; LaFonte and Johnson 2013). All cell counts were $\log_{10}$-transformed prior to analysis to help meet assumptions of normality. To examine differences in response among the seven host species 36 h after one-time *R. ondatrae* exposure, we analyzed each of the WBC types (number of lymphocytes, neutrophils, and eosinophils per 2000 RBCs), as well as total number of WBC (all types per 2000 RBCs) and the ratio of lymphocytes to neutrophils (L/N) given that stress-induced GC secretions can cause elevations of the latter (Davis et al. 2008). For these measures, we used linear mixed models (LMM) with a normal distribution and identity link function, including the fixed effects of host species (as a factor), parasite exposure (yes or no), and their interaction, as well as Gosner developmental stage as a covariate. After running the full model, non-significant terms were removed such that final models only contained significant main effects and/or interactions. To test for the effect of parasite load on WBC profiles, rather than just exposure, this analysis was repeated for *R. ondatrae*-exposed individuals only ($n = 58$, i.e., omitting control animals); here, infection intensity (observed number of metacercariae as a continuous variable) was substituted for the fixed effect of parasite exposure. All parasite-exposed individuals became infected, with the exception of *H. versicolor*, which are known to eliminate *R. ondatrae* cysts within 72 h (LaFonte and Johnson 2013), and were thus omitted from the analyses of parasite load and WBC counts. For the four host species sampled at multiple time points, we ran additional LMM analyses to assess the effects of parasite exposure, time P.E. (24, 36, 72, or 120 h), and their interaction on the abundance of each major WBC type (lymphocytes, neutrophils, eosinophils), total WBC and L:N, also including Gosner stage as a covariate.

To test for evidence of immune priming in *P. regilla*, we used a GLMM to evaluate whether prior parasite exposure (on day 0, yes or no) affected the number of *R. ondatrae* cercariae successful in forming cysts when administered on day 7 (which were labeled with green fluorescing dye). The number of green cysts only within each individual tadpole was thus modeled using a Poisson distribution with a log link function. Lastly, we examined whether prior exposure affected WBC profiles at 36 h, for which Gosner stage was included as a covariate. All leukocyte-related analyses were performed using SPSS 24.0.

Results

Larval amphibian parasite exposure and corticosterone response

There was a strong positive correlation between individual tadpole wet mass and homogenate volume from the pilot trials ($F_{1,22} = 336.765$, $p < 0.001$, correlation coefficient = 0.534), indicating that our use of volume to account for tadpole size to standardize measured corticosterone levels for intra- and interspecific comparisons was appropriate. Measured corticosterone levels differed significantly among host species ($F_{4,169} = 12.620$, $p < 0.001$) and treatments ($F_{2,169} = 9.501$, $p < 0.001$); there was a trend for an interaction of host with treatment, but this was not significant ($F_{8,169} = 1.786$, $p = 0.083$; Fig. 1a). Tukey post hoc tests indicated that *B. americanus* had significantly lower mean corticosterone levels than *B. boreas* and *R. pipiens* ($p < 0.001$ for both), but not *X. laevis* ($p = 0.115$) or *R. clamitans* ($p = 0.259$). Similarly, *X. laevis* had significantly lower mean corticosterone than *B. boreas* and *R. pipiens* ($p < 0.001$ for both), as did *R. clamitans* ($p < 0.001$ for both). American toads (*B. americanus*) had the lowest overall mean corticosterone levels (mean 127.5 ng/mL ± 10.6 S.E.), while Northern leopard frogs (*R. pipiens*) had the highest (mean 295.2 ng/mL ± 32.2 S.E.). Post hoc tests also indicated that tadpoles exposed to *E. trivolvis* generally had lower corticosterone levels (mean 160.3 ng/mL ± 11.9 S.E.) compared to the control (mean 225 ng/mL ± 16.1 S.E.) and those in the *R. ondatrae* treatment (mean 209.7 ng/mL ± 15.5 S.E.) ($P < 0.001$ for both), regardless of the amphibian species, whereas there was no difference in corticosterone between the control and *R. ondatrae*-exposed tadpoles ($p = 0.924$; Fig. 1b). The mean intra-assay CV for each host species (after removing individuals with CV > 20%) was less than 2.5%, as was the inter-assay CV, indicating consistency in the enzyme immunoassay (EIA) procedures.

Larval amphibian parasite exposure and leukocyte profiles

One-time exposure to *R. ondatrae* cercariae had no effect on host WBC profiles 36 h P.E. (all $p > 0.05$) for seven different host species, either as a main effect or as an interaction with host species. Host species identity, in contrast, strongly influenced WBC profiles for both parasite-exposed and unexposed

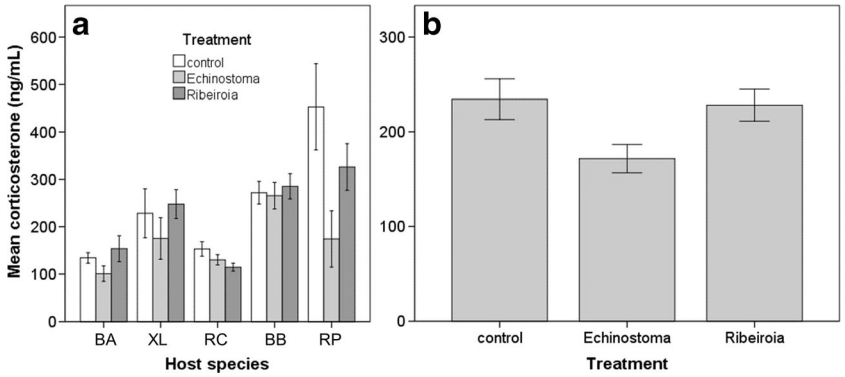

Fig. 1 Mean (±S.E.) corticosterone levels (adjusted for tadpole size) for **a** larvae of five anuran species (BA = *Bufo americanus*, XL = *Xenopus laevis*, RC = *Rana clamitans*, BB = *B. boreas*, RP = *R. pipiens*) subjected to three experimental treatments (control, exposure to 20 *Echinostoma trivolvis* cercariae, or exposure to 20 *Ribeiroia ondatrae* cercariae); **b** all five host species across the three experimental treatments

individuals (all $p < 0.001$; Table 1): while there was some variation depending on WBC type, *H. versicolor* and *R. clamitans* typically had the highest counts relative to the other host species (Fig. 2). For instance, these two species had 3–5× the number of neutrophils (per 2000 RBC) than that of *R. sphenocephalus*. Interestingly, only *H. versicolor* showed a negative L/N, and this ratio differed significantly from that of every other host species (all $p < 0.004$). Among hosts exposed to *R. ondatrae* (i.e., omitting control animals), there was no effect of infection intensity on any of the WBC measures, whereas host species remained a significant predictor (all $p < 0.001$; Fig. 3). There were also no significant interactions between species and infection intensity.

For the four host species in which WBC profiles were examined at multiple time points P.E. (24, 36, 72, and 120 h), host species identity was the only significant predictor of total WBC count, lymphocytes, neutrophils, and L/N (all $p < 0.001$; Table 2), although there was a marginal trend ($p = 0.085$) for total WBC count to increase with time. For eosinophil count, however, the final model included host species ($p < 0.001$), *R. ondatrae* exposure ($p = 0.010$), and an interaction between species and time of sample collection ($p = 0.008$). Thus, individuals exposed to *R. ondatrae* had fewer circulating eosinophils (mean 52 eosinophils/2000 RBC ± 9.5 S.E.) compared to controls (mean 67.4 eosinophils/2000 RBC ± 13.3 S.E.). Eosinophil counts also increased through time for *H. versicolor* and *R. sphenocephalus*, but decreased for *R. clamitans* (Fig. 4).

Table 1 Results of linear mixed models (LMM) for leukocyte (WBC) profiles (based on 2000 erythrocytes) of seven species of larval anuran with fixed effects of host species identity and one-time exposure to the trematode *Ribeiroia ondatrae*, or with infection intensity substituted for parasite exposure (i.e., omitting control treatment individuals). Only significant predictors are shown

WBC type	Predictor	Results
All individuals		
Total leukocytes	Host species	$F_{6,73} = 19.167$, $P < 0.001$
Lymphocytes	Host species	$F_{6,73} = 12.402$, $P < 0.001$
Neutrophils	Host species	$F_{6,73} = 16.079$, $P < 0.001$
Eosinophils	Host species	$F_{6,73} = 41.632$, $P < 0.001$
Lymphocytes:neutrophils	Host species	$F_{6,73} = 11.146$, $P < 0.001$
Infected individuals		
Total leukocytes	Host species	$F_{6,33} = 10.836$, $P < 0.001$
Lymphocytes	Host species	$F_{6,33} = 6.242$, $P < 0.001$
Neutrophils	Host species	$F_{6,33} = 8.518$, $P < 0.001$
Eosinophils	Host species	$F_{6,33} = 14.449$, $P < 0.001$
Lymphocytes/neutrophils	Host species	$F_{6,33} = 5.015$, $P = 0.01$

Exposure of *P. regilla* to *R. ondatrae* cercariae in either a single challenge (day 7 only) or multiple challenges (both days 0 and 7) was a significant predictor of how many parasites which were administered on day 7 subsequently encysted ($F_{1,55} = 4.531$, $p = 0.038$). Cercariae in the second round of exposure (on day 7, i.e., green-dyed cercariae) had lower success by virtue of forming fewer cysts if their host had been previously exposed on day 0 compared to those with hosts for whom day 7 represented the only parasite challenge (mean cysts = 6.1 ± 3.2 S.D. vs. 7.7 ± 4.9 S.D., respectively; Fig. 5). This reduced success as a function of prior exposure suggests heightened resistance on the part of tadpoles challenged on day 0. However, previous *R. ondatrae* exposure had no effect on leukocyte profiles at 36 h, as reflected by total WBC numbers ($F_{1,13} = 0.168$, $p = 0.689$), eosinophils ($F_{1,13} = 2.850$, $p = 0.115$), lymphocytes ($F_{1,13} = 0.313$, $p = 0.585$), neutrophils ($F_{1,13} = 0.244$, $p = 0.629$), and L/N ($F_{1,13} = 1.570$, $p = 0.232$).

Discussion

We found profound differences in whole-body corticosterone among larvae of five amphibian species, such that *B. americanus* (American toad) tadpoles had an average of one-third the corticosterone level measured in *R. pipiens* (Northern leopard frogs). This cannot be attributed to

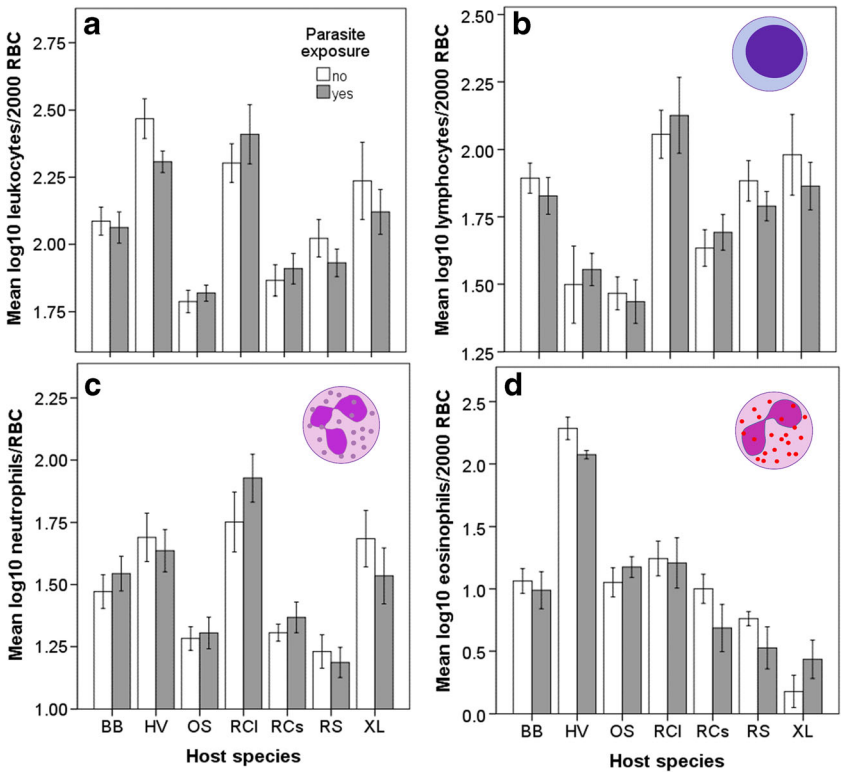

Fig. 2 No effect of exposure to 20 *Ribeiroia ondatrae* cercariae on mean (±S.E.) $\log_{10}$ leukocyte counts (all per 2000 red blood cells, RBC) 36 h later for larvae of seven amphibian host species (BB = *Bufo boreas*, HV = *Hyla versicolor*, OS = *Osteopilus septentrionalis*, RCl = *Rana clamitans*, RCs = *R. cascadae*, RS = *R. sphenocephalus*, XL = *Xenopus laevis*). Panels represent **a** total leukocytes, **b** lymphocytes, **c** neutrophils, and **d** eosinophils

differences in species mass as *B. boreas* (western toad) larvae were of similar size to *B. americanus* and yet the latter had a significantly lower overall corticosterone level, with the largest tadpoles (*R. clamitans*) having the second-lowest levels. The pattern of corticosterone levels also did not correspond to amphibian phylogeny, as the two toad species (*B. americanus* and *B. boreas*) and two ranid species (*R. clamitans* and *R. pipiens*) differed significantly from one another. Similarly, there was strong interspecific variation in the leukocyte (WBC) profiles of seven host species that were examined, sometimes spanning five orders of magnitude depending on the particular WBC type. While tadpoles for each experiment were simultaneously raised under the same laboratory conditions from the embryonic stage, these were obtained from different sources owing to differences in their life history and natural distributions; this possible influence therefore warrants investigation where feasible (e.g., comparison of commercially supplied and field-collected eggs of a given species).

In contrast to host species identity, exposure to two relatively pathogenic macroparasites (the trematodes *R. ondatrae* and *E. trivolvis*) had weak effects on these host physiological responses (corticosterone level and WBC profile) across a range of different amphibian host species. We also found that *P. regilla* tadpoles previously challenged by *R. ondatrae* had fewer parasites established during a second challenge with the

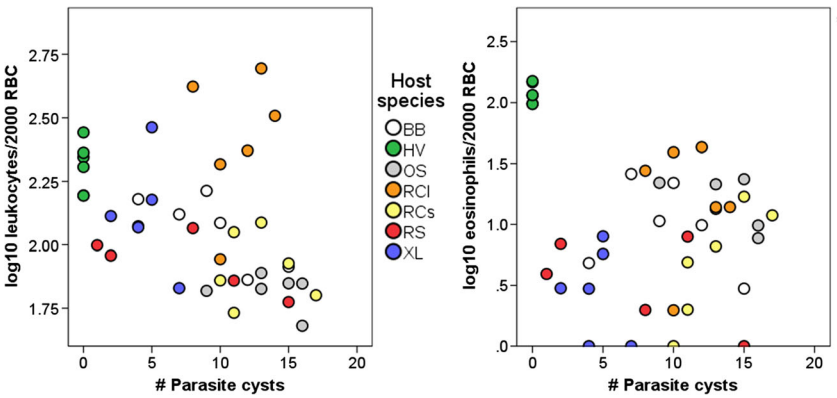

Fig. 3 No relationship between *Ribeiroia ondatrae* infection intensity (# of cysts) and **a** mean $\log_{10}$ (±S.E.) leukocyte or **b** eosinophil counts (all per 2000 red blood cells, RBC) 36 h after parasite exposure for larvae of seven amphibian host species (BB = *Bufo boreas*, HV = *Hyla versicolor*, OS = *Osteopilus septentrionalis*, RCl = *Rana clamitans*, RCs = *R. cascadae*, RS = *R. sphenocephalus*, XL = *Xenopus laevis*)

Table 2 Results of linear mixed models (LMM) for leukocyte (WBC) profiles (based on 2000 erythrocytes) of four species of larval anuran with fixed effects of host species identity, one-time exposure to the trematode *Ribeiroia ondatrae*, and sampling time post-exposure (24, 36, 72, or 120 h). Only significant predictors are shown

WBC type	Predictor	Results
Total leukocytes	Host species	$F_{3,128} = 48.704$, $P < 0.001$
Lymphocytes	Host species	$F_{3,131} = 45.387$, $P < 0.001$
Neutrophils	Host species	$F_{3,131} = 57.49$, $P < 0.001$
Eosinophils	Host species	$F_{3,122} = 232.386$, $P < 0.001$
“ ”	Parasite exposure	$F_{3,122} = 6.794$, $P = 0.01$
“ ”	Host species*sampling time	$F_{8,122} = 2.738$, $P = 0.008$
Lymphocytes/neutrophils	Host species	$F_{3,131} = 55.256$, $P < 0.001$

same species 7 days later, but the mechanism by which this occurred is not evident given that there was not a marked or consistent shift in corticosterone levels or WBC profiles from multiple host species. That previous exposure to *R. ondatrae* reduced the susceptibility of *P. regilla* tadpoles during a later challenge with the same parasite is consistent with earlier research using the same host and parasite species (Hoverman et al. 2013). Increased resistance to concomitant or secondary infection by the same pathogen/parasite has been reported for a suite of invertebrate and vertebrate hosts (e.g., Roth et al. 2009; Harvie et al. 2010) and can involve various components of the adaptive and innate immune system. For example, the recruitment of lymphocytes to the lungs of mice after vaccination by irradiated infectious stages of the trematode *Schistosoma mansoni* plays a key role in promoting host resistance to secondary challenge with live parasites (Coulson and Wilson 1997).

Despite the known pathology of *R. ondatrae* or *E. trivolvis* to larval amphibians at the dosages administered here (Schotthoefer et al. 2003a, b; Johnson et al. 2012), and the ability of many other aversive stimuli (e.g., warm temperatures, predators, and microparasite infection) to elevate corticosterone in larval amphibians (Warne et al. 2011; Middlemis Maher et al. 2013; Gabor et al. 2015; Freitas et al. 2017), exposure to either macroparasite did not induce a significant increase in corticosterone levels. Although our findings do not correspond to the general expectation for parasitism to elevate GCs (Maier and Watkins 1999), they agree with those of Marino et al. (2014) who found corticosterone reductions in *R. sylvatica* (but not *R. clamitans*) larvae following exposure to *E. trivolvis*. However, we were not able to evaluate the degree to which differences in endogenous corticosterone levels among species explained variation in infection success given the limitation in conducting necropsies on the same individuals. It was also not possible to relate individual corticosterone levels and WBC profiles given our experimental design, but associations have been established in other studies with larval amphibians, such as altered granulocyte (neutrophil) to lymphocyte ratios by individuals with elevated GCs (Burraco et al. 2017).

Although exposure to neither trematode caused corticosterone elevations given the consistent findings for the five different host species examined here, their effects differed (no influence of *R. ondatrae* vs. a GC decrease with *E. trivolvis*). Decreased GC levels in larval amphibians in response to other stressors have also been observed. For instance, Middlemis

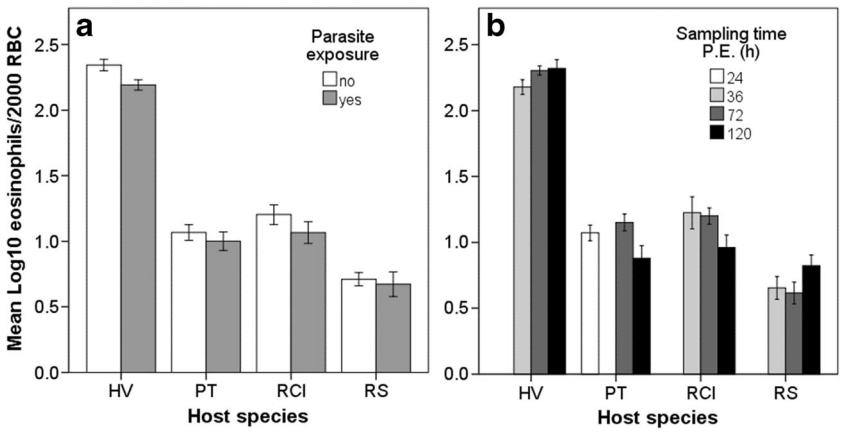

Fig. 4 **a** Negative effect of exposure to 20 *Ribeiroia ondatrae* cercariae on mean (±S.E.) $\log_{10}$ eosinophil counts (per 2000 red blood cells, RBC) for four amphibian host species (HV = *Hyla versicolor*, PT = *Pseudacris triseriata*, RCl = *Rana clamitans*, RS = *R. sphenocephalus*); and **b** variation in eosinophil counts among host species and post-exposure (P.E.) sampling times

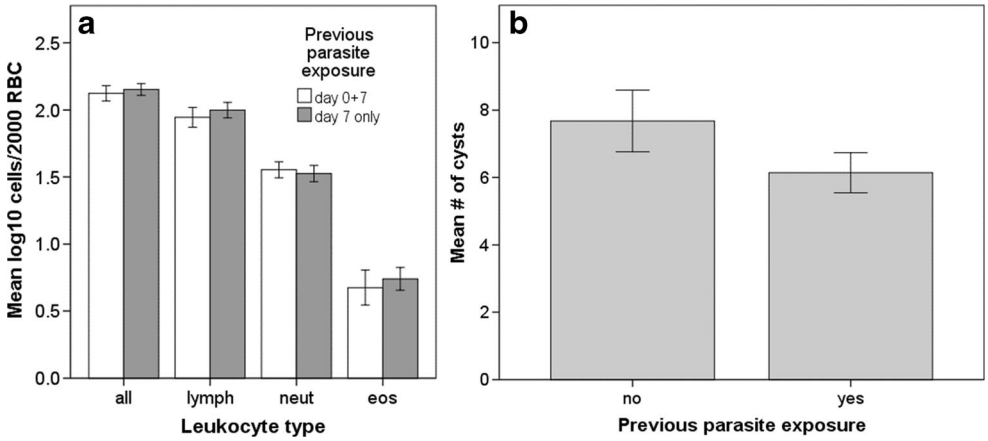

Fig. 5 Effect of exposure to *Ribeiroia ondatrae* cercariae on **a** mean (±S.E.) $\log_{10}$ leukocyte counts (all per 2000 red blood cells, RBC; lymph = lymphocytes, neut = neutrophils, eos = eosinophils) in larval *Pseudacris regilla* 36 h after parasite challenge on day 7 following either single (on day 7) or repeated (on both day 0 and day 7) exposure; and **b** host infection intensity (# cysts) from parasites given on day 7 challenge following no previous exposure (day 7 only) or previous exposure (on both day 0 and day 7)

Maher et al. (2013) reported both GC and activity level reductions in tadpoles that were exposed to visually oriented predators and suggested this promoted survival in this context. While we did not measure tadpole activity, there may be a similar association with corticosterone after parasite exposure, although it is not evident why this would only occur for *E. trivolvis*. Studies with other hosts and parasites have reported that infected individuals have higher, lower, or similar GC levels relative to uninfected or unexposed conspecifics (e.g., Laidley et al. 1988; Morales-Montor et al. 2001; Mougeot et al. 2010; Marino et al. 2014). Where GC decreases occurred, this was suggested to promote certain innate immune responses, such as granuloma formation by macrophages and lymphocytes, given the known anti-inflammatory properties of GCs (Morales-Montor et al. 2001). As such, a sustained increase in corticosterone levels could adversely affect defenses against some parasites based on the nature of the host immune response (Laidley et al. 1988). Our results thus support previous suggestions that the influence of infection on GC levels is dependent on host species and parasite identity (Fleming 1997; Sures et al. 2001; Belden et al. 2010; Fuxjager et al. 2011; Marino et al. 2014; Sutherland et al. 2014).

Rapid increases in GCs in response to aversive stimuli can have several short-term benefits; however, chronically elevated levels of GCs are immunosuppressive in many vertebrates, including larval amphibians, particularly dampening the inflammatory response (Sapolsky et al. 2000; Belden and Kiesecker 2005; LaFonte and Johnson 2013). For this reason, no change in corticosterone after parasite infection, or even a reduction, could be beneficial to hosts in certain circumstances (Morales-Montor et al. 2001; Marino et al. 2014). Importantly, the amphibian response to infection by echinostomatid trematodes involves inflammation, and some host species can actually clear cysts (Holland 2009). Similar to echinostome cysts (Holland 2009), some larval amphibians can effectively clear those of *R. ondatrae*, and this ability is hampered by elevated levels of corticosterone (LaFonte and Johnson 2013). Previous experimental immunosuppression of larval amphibians via exogenous corticosterone increased *E. trivolvis* intensity by 80% in treated tadpoles, whereas *R. ondatrae* increased by 191% (LaFonte and Johnson 2013), indicating a strong influence of this hormone on host defenses. Consequently, elevations of corticosterone over a prolonged period could be detrimental if this interferes with the host's immune response, especially inflammation, and the lack of such a response to trematode infection by larval amphibians may be adaptive.

Given that other studies have found elevated corticosterone levels in amphibians exposed to *Bd* and ranaviruses (e.g., Warne et al. 2011; Gabor et al. 2015), this discrepancy in response suggests that infectious agents which exploit different host structures, or have different life histories within their hosts, may not affect stress hormones in the same way. Such differences could occur for two main reasons. First, individual macroparasite burdens typically represent the cumulative effect of exposure events through time rather than a single infection followed by within-host replication as for microparasites/pathogens (Blaustein et al. 2012). Microparasites and pathogens (e.g., bacteria and viruses) therefore constitute active infections that often rapidly replicate within hosts, likely representing a considerable stress within a brief time period. Short-term increases in GCs may thus mobilize host resources to quickly respond to infection and limit pathogen replication, but this should not be applicable to macroparasites.

Hosts are also less likely to completely clear macroparasites, and encapsulation/encystation of certain life history stages (e.g., larval trematodes) is common (Secombes and Chappell 1996). Notably, trematode cysts (metacercariae) characterize relatively long-term infections, do not replicate or reproduce, and appear to have low energetic demands

(Koprivnikar et al. 2012). For these reasons, elevated GCs in response to macroparasite infection may not be common (in contrast to that with microparasites/pathogens), but experimental infections with additional parasite species and varied dosages will be needed to determine whether our results with *E. trivolvis* and *R. ondatrae* are representative of amphibian stress hormone and immunological responses to helminths, and macroparasites more generally. Because the pathology associated with these two parasites is mediated by dose and host life history stage (Schotthoefer et al. 2003a, b; Holland et al. 2007), tadpoles may also show different responses at higher infection intensities or during later stages of development. GC alterations may also depend on time since parasite exposure and should ideally be measured at multiple points so as to capture any periods of high stress.

Although previous investigations have demonstrated a negative correlation between eosinophil counts and larval amphibian susceptibility to helminth infection (Kiesecker 2002; Maizels and Yazdanbakhsh 2003; LaFonte and Johnson 2013), these cells did not change in response to "priming" by *R. ondatrae* in the present study (nor did any of the other leukocyte measures), suggesting that increased eosinophil circulation is not an adaptive reaction to the possibility of subsequent infections. However, it should be noted that the role(s) of eosinophils in combatting helminth infections are complex and may be limited to certain situations and life history stages (Meeusen and Balic 2000; Allen and Maizels 2011). Alternative aspects of host defenses against parasitism should thus be explored. Calhoun et al. (2016) found that antimicrobial peptides (AMPs) do not play a significant role in larval amphibian resistance to trematode cercariae, but antibody-mediated responses have not been explored in this host-parasite system, and the possibility for priming of host anti-parasite behaviors also warrants consideration given their demonstrated efficacy against cercariae by tadpoles (e.g., Koprivnikar et al. 2014).

In addition to our priming experiment with *P. regilla*, seven other amphibian host species also showed little effect of *R. ondatrae* exposure with respect to WBC profiles, even across multiple time points. This differs from an earlier study which found that hosts exposed to *R. ondatrae*, *E. trivolvis*, and *Alaria* sp. showed a 17% increase in eosinophils (LaFonte and Johnson 2013), although it is not possible to determine how this may have differed among trematode species. Timing may also play a role as we examined WBC profiles 36 h P.E. up to a maximum of 5 days for most of our host species, whereas this occurred 4 and 14 days P.E. in the previous study by LaFonte and Johnson (2013). Our lack of an overall WBC response to parasite exposure was a robust finding, occurring in seven phylogenetically disparate species. In contrast, three species of amphibians are capable of acquiring immunological resistance to chytrid fungus (*Bd*) (McMahon et al. 2014), and individuals exposed to *Bd* display heterogeneity in terms of the level and type of resulting immune response (Gervasi et al. 2014).

Future investigations will be necessary to elucidate the potential for host exposure to a given macroparasite to affect immunocompetence during future challenge with the same or different parasite species, particularly given certain inherent limitations in evaluating WBC profiles via blood smears. For instance, because blood smears represent circulating WBCs, localized changes in their concentration may not be detected, nor do they reflect WBC presence in other non-blood compartments at any given time, or the release potential of these into the blood. This being said, studies involving immunosuppression as measured by WBC profiles are generally considered rigorous, with changes in eosinophil, neutrophil, and lymphocyte counts corresponding to different infection outcomes in larval amphibians (e.g., Belden and Kiesecker 2005; Davis et al. 2010; LaFonte and Johnson 2013). While we focused on the involvement of leukocytes, it is important to note that the vertebrate immune response to helminth infections is complex and includes both the adaptive and innate arms (reviewed by Allen and Maizels 2011); thus, it will be necessary to evaluate additional measures beyond those here.

Clarifying the degree to which macroparasites generally affect amphibian endocrine parameters is important considering the various effects of GCs alone on this vulnerable group of vertebrates, including altered growth and metamorphosis (Rollins-Smith 1998, 2017). Given the decline of amphibians worldwide, a better understanding of the physiological mechanisms behind the likely drivers, including infectious diseases and environmental changes (Daszak et al. 2003; Blaustein et al. 2011, 2012; Rollins-Smith 2017), will aid in identifying species or populations of concern. Critically, many of these contributing factors may involve amphibian immunocompetence (Carey et al. 1999; Rollins-Smith 2017). Because these stressors are not unique to amphibians, more studies considering the physiological impacts of parasites/pathogens on wildlife will be needed in order to formulate the most appropriate conservation and management strategies (Blaustein et al. 2012; Dantzer et al. 2014; Hing et al. 2016), as well as to understand the ways in which host susceptibility and tolerance to parasitic infection may be affected.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed, and all procedures performed were in accordance with the ethical standards of the institution at which the studies were conducted. This article does not contain any studies with human participants performed by any of the authors.

References

Allen JE, Maizels RM (2011) Diversity and dialogue in immunity to helminths. Nat Rev Immunol 11:375–388

Barnard CJ, Behnke JM, Gage AR, Brown H, Smithurst PR (1998) The role of parasite–induced immunodepression, rank and social environment in the modulation of behaviour and hormone concentration in male laboratory mice (*Mus musculus*). Proc R Soc Lond B Biol Sci 265:693–701

Belden LK, Kiesecker JM (2005) Glucocorticosteroid hormone treatment of larval treefrogs increases infection by *Alaria* sp. trematode cercariae. J Parasitol 91:686–688

Belden LK, Wingfield JC, Kiesecker JM (2010) Variation in the hormonal stress response among larvae of three amphibian species. J Exp Zool 313:524–531

Blaustein AR, Han BA, Relyea RA, Johnson PTJ, Buck JC, Gervasi SS, Kats LB (2011) The complexity of amphibian population declines: understanding the role of cofactors in driving amphibian losses. Ann N Y Acad Sci 1223:108–119

Blaustein AR, Gervasi SS, Johnson PTJ, Hoverman JT, Belden LK, Bradley PW, Xie GY (2012) Ecophysiology meets conservation: understanding the role of disease in amphibian population declines. Philos Trans R Soc Lond Ser B Biol Sci 367:1688–1707

Burraco P, Gomez-Mestre I (2016) Physiological stress responses in amphibian larvae to multiple stressors reveal marked anthropogenic effects even below lethal levels. Physiol Biochem Zool 89:462–472

Burraco P, Arribas R, Kulkarni SS, Buchholz DR, Gomez-Mestre I (2015) Comparing techniques for measuring corticosterone in tadpoles. Curr Zool 61:835–845

Burraco P, Miranda F, Bertó A, Vazquez LA, Gomez-Mestre I (2017) Validated flow cytometry allows rapid quantitative assessment of immune responses in amphibians. Amphibia-Reptilla 38:232–237

Cain DW, Cidlowski JA (2017) Immune regulation by glucocorticoids. Nat Rev Immunol 17:233–247

Calhoun DM, Woodhams D, Howard C, LaFonte BE, Gregory JR, Johnson PTJ (2016) Role of antimicrobial peptides in amphibian defense against trematode infection. EcoHealth 13:383–391

Carey C, Cohen N, Rollins-Smith L (1999) Amphibian declines: an immunological perspective. Dev Comp Immunol 23:459–472

Coulson PS, Wilson RA (1997) Recruitment of lymphocytes to the lung through vaccination enhances the immunity of mice exposed to irradiated schistosomes. Infect Immun 65:42–48

Dantzer B, Fletcher QE, Boonstra R, Sheriff MJ (2014) Measures of physiological stress: a transparent or opaque window into the status, management and conservation of species? Conserv Physiol 2:cou023

Daszak P, Cunningham AA, Hyatt AD (2000) Emerging infectious diseases of wildlife-threats to biodiversity and human health. Science 287:443–449

Daszak P, Cunningham AA, Hyatt AD (2003) Infectious disease and amphibian population declines. Divers Distrib 9:141–150

Davis AK (2009) Metamorphosis-related changes in leukocyte profiles of larval bullfrogs (*Rana catesbeiana*). Comp Clin Pathol 18:181–186

Davis AK, Maney DL (2018) The use of glucocorticoid hormones or leucocyte profiles to measure stress in vertebrates: What's the difference? Methods Ecol Evol 9:1556–1568

Davis AK, Maney DL, Maerz JC (2008) The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. Funct Ecol 22: 760–772

Davis AK, Keel MK, Ferreira A, Maerz JC (2010) Effects of chytridiomycosis on circulating white blood cell distributions of bullfrog larvae (*Rana catesbeiana*). Comp Clin Pathol 19:49–55

Fast MD, Muise DM, Easy RE, Ross NW, Johnson SC (2006) The effects of *Lepeophtheirus salmonis* infections on the stress response and immunological status of Atlantic salmon (*Salmo salar*). Fish Shellfish Immunol 21:228–241

Fleming MW (1997) Cortisol as an indicator of severity of parasitic infections of *Haemonchus contortus* in lambs. Comp Biochem Physiol B 116:41–44

Freitas MB, Brown CT, Karasov WH (2017) Warmer temperature modifies effects of polybrominated diphenyl ethers on hormone profiles in leopard frog tadpoles (*Lithobates pipiens*). Environ Toxicol Chem 36:120–127

Fried B, Pane PL, Reddy A (1997) Experimental infection of *Rana pipiens* tadpoles with *Echinostoma trivolvis* cercariae. Parasitol Res 83:666–669

Fuxjager MJ, Foufopoulos J, Diaz-Uriarte R, Marler CA (2011) Functionally opposing effects of testosterone on two different types of parasite: implications for the immunocompetence handicap hypothesis. Funct Ecol 25:132–138

Gabor CR, Fisher MC, Bosch J (2015) Elevated corticosterone levels and changes in amphibian behavior are associated with *Batrachochytrium dendrobatidis* (*Bd*) infection and *Bd* lineage. PLoS One 10:e0122685

Gabor CR, Knutie SA, Roznik EA, Rohr JR (2018) Are the adverse effects of stressors on amphibians mediated by their effects on stress hormones? Oecologia 186:393–404

Gervasi SS, Lowry M, Hunt E, Blaustein AR (2014) Temporal patterns in immunity, infection load, and disease susceptibility: understanding the drivers of host responses in the amphibian chytrid-fungus system. Funct Ecol 28:569–578

Gosner KL (1960) A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16:183–190

Graham AL, Cattadori IM, Lloyd-Smith JO, Ferrari MJ, Bjørnstad ON (2007) Transmission consequences of coinfection: cytokines writ large? Trends Parasitol 23:284–291

Graham AL, Shuker DM, Pollitt LC, Auld SKJR, Wilson AJ, Little TJ (2011) Fitness consequences of immune responses: strengthening the empirical framework for ecoimmunology. Funct Ecol 25:5–17

Hadji-Azimi I, Coosemans V, Canicatti C (1987) Atlas of adult *Xenopus laevis laevis* hematology. Dev Comp Immunol 11:807–874

Harris PD, Soleng A, Bakke TA (2000) Increased susceptibility of salmonids to the monogenean *Gyrodactylus salaris* following administration of hydrocortisone acetate. Parasitology 120:57–64

Harvie M, Camberis M, Tang SC, Delahunt B, Paul W, Le Gros G (2010) The lung is an important site for priming CD4 T-cell-mediated protective immunity against gastrointestinal helminth parasites. Infect Immun 78:3753–3762

Herman JP, Cullinan WE (1997) Neurocircuitry of stress: central control of the hypothalamo–pituitary–adrenocortical axis. Trends Neurosci 20:78–84

Hing S, Narayan EJ, Thompson RA, Godfrey SS (2016) The relationship between physiological stress and wildlife disease: consequences for health and conservation. Wildl Res 43:51–60

Holland MP (2009) Echinostome metacercariae cyst elimination in *Rana clamitans* (green frog) tadpoles is age-dependent. J Parasitol 95: 281–285

Holland MP (2010) Echinostome-induced mortality varies across amphibian species in the field. J Parasitol 96:851–855

Holland MP, Skelly DK, Kashgarian M, Bolden SR, Harrison LM, Cappello M (2007) Echinostome infection in green frogs (*Rana clamitans*) is stage and age dependent. J Zool 271:455–462

Hoverman JT, Hoye BJ, Johnson PTJ (2013) Does timing matter? How priority effects influence the outcome of parasite interactions within hosts. Oecologia 173:1471–1480

Huver JR, Koprivnikar J, Johnson PTJ, Whyard S (2015) Development and application of an eDNA method to detect and quantify a pathogenic parasite in aquatic ecosystems. Ecol Appl 25:991–1002

Jamieson AM, Yu S, Annicelli CH, Medzhitov R (2010) Influenza virus-induced glucocorticoids compromise innate host defense against a secondary bacterial infection. Cell Host Microbe 7:103–114

Johnson PTJ, McKenzie VJ (2009) Effects of environmental change on helminth infections in amphibians: exploring the emergence of *Ribeiroia* and *Echinostoma* infections in North America. In: Fried B, Toledo R (eds) The biology of Echinostomes. Springer, New York, pp 249–280

Johnson PTJ, Kellermanns E, Bowerman J (2011) Critical windows of disease risk: amphibian pathology driven by developmental changes in host resistance and tolerance. Funct Ecol 25:726–734

Johnson PTJ, Rohr JR, Hoverman JT, Kellermanns E, Bowerman J, Lunde KB (2012) Living fast and dying of infection: host life history drives interspecific variation in infection and disease risk. Ecol Lett 15:235–242

Kiesecker JM (2002) Synergism between trematode infection and pesticide exposure: a link to amphibian limb deformities in nature? Proc Natl Acad Sci U S A 99:9900–9904

Koprivnikar J (2010) Interactions of environmental stressors impact survival and development of parasitized larval amphibians. Ecol Appl 20:2263–2272

Koprivnikar J, Marcogliese DJ, Rohr JR, Orlofske SA, Raffel TR, Johnson PTJ (2012) Macroparasite infections of amphibians: what can they tell us? EcoHealth 9:342–360

Koprivnikar J, Redfern JC, Mazier HL (2014) Variation in anti-parasite behaviour and infection among larval amphibian species. Oecologia 174:1179–1185

Korte SM, Koolhaas JM, Wingfield JC, McEwen BS (2005) The Darwinian concept of stress: benefits of allostasis and costs of allostatic load and the trade-offs in health and disease. Neurosci Biobehav Rev 29:3–38

Lafferty KD, Kuris AM (2002) Trophic strategies, animal diversity and body size. Trends Ecol Evol 17:507–513

LaFonte BE, Johnson PTJ (2013) Experimental infection dynamics: using immunosuppression and in vivo parasite tracking to understand host resistance in an amphibian–trematode system. J Exp Biol 216:3700–3708

Laidley CW, Woo PTK, Leatherland IF (1988) The stress-response of rainbow trout to experimental infection with the blood parasite *Cryptobia salmositica* Katz, 1951. J Fish Biol 32:253–261

Maier SF, Watkins LR (1999) Bidirectional communication between the brain and the immune system: implications for behaviour. Anim Behav 57:741–751

Maizels RM, Yazdanbakhsh M (2003) Immune regulation by helminth parasites: cellular and molecular mechanisms. Nat Rev Immunol 3: 733–744

Marino JA, Holland MP, Middlemis Maher J (2014) Predators and trematode parasites jointly affect larval anuran functional traits and corticosterone levels. Oikos 123:451–460

Martin LB (2009) Stress and immunity in wild vertebrates: timing is everything. Gen Comp Endocrinol 163:70–76

Martin TR, Conn DB (1990) The pathogenicity, localization, and cyst structure of echinostomatid metacercariae (Trematoda) infecting the kidneys of the frogs *Rana clamitans* and *Rana pipiens*. J Parasitol 76:414–419

McMahon TA, Sears BF, Venesky MD et al (2014) Amphibians acquire resistance to live and dead fungus overcoming fungal immunosuppression. Nature 511:224–227

Meeusen ENT, Balic A (2000) Do eosinophils have a role in the killing of helminth parasites? Parasitol Today 16:95–101

Middlemis Maher J, Werner EE, Denver RJ (2013) Stress hormones mediate predator-induced phenotypic plasticity in amphibian tadpoles. Proc R Soc Lond B Biol Sci 280:20123075

Morales-Montor J, Newhouse E, Mohamed F, Baghdadi A, Damian RT (2001) Altered levels of hypothalamic-pituitary-adrenocortical axis hormones in baboons and mice during the course of infection with *Schistosoma mansoni*. J Infect Dis 183:313–320

Mougeot F, Martinez-Padilla J, Bortolotti GR, Webster MI, Piertney SB (2010) Physiological stress links parasites to carotenoid-based colour signals. J Evol Biol 23:643–650

Murdock CC, Paaijmans KP, Cox-Foster D, Read AF, Thomas MB (2012) Rethinking vector immunology: the role of environmental temperature in shaping resistance. Nat Rev Microbiol 10:869–876

Parris MJ, Cornelius TO (2004) Fungal pathogen causes competitive and developmental stress in larval amphibian communities. Ecology 85: 3385–3395

Peters RH (1983) The ecological implications of body size. Cambridge University Press, Cambridge

Pettis JS, Lichtenberg EM, Andree M, Stitzinger J, Rose R (2013) Crop pollination exposes honey bees to pesticides which alters their susceptibility to the gut pathogen *Nosema ceranae*. PLoS One 8:e70182

Rohr JR, Raffel TR, Hall CA (2010) Developmental variation in resistance and tolerance in a multi-host–parasite system. Funct Ecol 24: 1110–1121

Rollins-Smith LA (1998) Metamorphosis and the amphibian immune system. Immunol Rev 166:221–230

Rollins-Smith LA (2001) Neuroendocrine-immune system interactions in amphibians. Immunol Res 23:273–280

Rollins-Smith LA (2017) Amphibian immunity–stress, disease, and climate change. Dev Comp Immunol 66:111–119

Rollins-Smith LA, Woodhams DC (2012) Amphibian immunity: staying in tune with the environment. In: Demas GE, Nelson R (eds) Ecoimmunology. Oxford University Press, New York, pp 92–143

Romero LM (2004) Physiological stress in ecology: lessons from biomedical research. Trends Ecol Evol 19:249–255

Roth O, Sadd BM, Schmid-Hempel P, Kurtz J (2009) Strain-specific priming of resistance in the red flour beetle, *Tribolium castaneum*. Proc R Soc Lond B Biol Sci 276:145–151

Saeij JP, Verburg-van Kemenade L, van Muiswinkel WB, Wiegertjes GF (2003) Daily handling stress reduces resistance of carp to *Trypanoplasma borreli*: in vitro modulatory effects of cortisol on leukocyte function and apoptosis. Dev Comp Immunol 27:233–245

Sapolsky RM (1993) Neuroendocrinology of the stress-response. In: Becker JB, Breedlove SM, Crews D (eds) Behavioral endocrinology. MIT Press, Cambridge, pp 287–324

Sapolsky RM, Romero LM, Munck AU (2000) How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr Rev 21:55–89

Secombes CJ, Chappell LH (1996) Fish immune responses to experimental and natural infection with helminth parasites. Ann Rev Fish Dis 6:167–177

Schoenemann KL, Bonier F (2018) Repeatability of glucocorticoid hormones in vertebrates: a meta-analysis. PeerJ 6:e4398

Schotthoefer AM, Cole RA, Beasley VR (2003a) Relationship of tadpole stage to location of echinostome cercariae encystment

and the consequences for tadpole survival. J Parasitol 89: 475–482

Schotthoefer AM, Koehler AV, Meteyer CU, Cole RA (2003b) Influence of *Ribeiroia ondatrae* (Trematoda: Digenea) infection on limb development and survival of northern leopard frogs (*Rana pipiens*): effects of host stage and parasite-exposure level. Can J Zool 81:1144–1153

Stoltze K, Buchmann K (2001) Effect of *Gyrodactylus derjavini* infections on cortisol production in rainbow trout fry. J Helminthol 75: 291–294

Sures B, Knopf K, Kloas W (2001) Induction of stress by the swimbladder nematode *Anguillicola crassus* in European eels, *Anguilla anguilla*, after repeated experimental infection. Parasitology 123:179–184

Sutherland BJG, Koczka KW, Yasuike M, Jantzen SG, Yazawa R, Koop BF, Jones SRM (2014) Comparative transcriptomics of Atlantic *Salmo salar*, chum *Oncorhynchus keta* and pink salmon *O. gorbuscha* during infections with salmon lice *Lepeophtheirus salmonis*. BMC Genomics 15:200

Thompson RCA, Lymbery AJ, Smith A (2010) Parasites, emerging disease and wildlife conservation. Int J Parasitol 40:1163–1170

Warne RW, Crespi EJ, Brunner JL (2011) Escape from the pond: stress and developmental responses to ranavirus infection in wood frog tadpoles. Funct Ecol 25:139–146

Yeh C-M, Glöck M, Ryu S (2013) An optimized whole-body cortisol quantification method for assessing stress levels in larval zebrafish. PLoS One 8:e79406

Yehuda R, Seckl J (2011) Mini-review: stress-related psychiatric disorders with low cortisol levels: a metabolic hypothesis. Endocrinology 152:4496–4503