PHILOSOPHICAL TRANSACTIONS B

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Research

Cite this article: Ramsay C, Rohr JR. 2023 Ontogeny of immunity and potential implications for co-infection. *Phil. Trans. R. Soc. B* **378**: 20220127.
https://doi.org/10.1098/rstb.2022.0127

Received: 18 May 2022
Accepted: 28 September 2022

One contribution of 14 to a theme issue 'Amphibian immunity: stress, disease and ecoimmunology'.

Subject Areas:
ecology, immunology

Keywords:
amphibian, *Batrachochytrium dendrobatidis*, immunity, metamorphosis, ontogeny, tradeoffs

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Electronic supplementary material is available online at https://doi.org/10.6084/m9.figshare.c.6631860.

THE ROYAL SOCIETY PUBLISHING

Ontogeny of immunity and potential implications for co-infection

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Immunity changes through ontogeny and can mediate facilitative and inhibitory interactions among co-infecting parasite species. In amphibians, most immune memory is not carried through metamorphosis, leading to variation in the complexity of immune responses across life stages. To test if the ontogeny of host immunity might drive interactions among co-infecting parasites, we simultaneously exposed Cuban treefrogs (*Osteopilus septentrionalis*) to a fungus (*Batrachochytrium dendrobaditis*, Bd) and a nematode (*Aplectana hamatospicula*) at tadpole, metamorphic and post-metamorphic life stages. We measured metrics of host immunity, host health and parasite abundance. We predicted facilitative interactions between co-infecting parasites as the different immune responses hosts mount to combat these infectious are energetically challenging to mount simultaneously. We found ontogenetic differences in IgY levels and cellular immunity but no evidence that metamorphic frogs were more immunosuppressed than tadpoles. There was also little evidence that these parasites facilitated one another and no evidence that *A. hamatospicula* infection altered host immunity or health. However, Bd, which is known to be immunosuppressive, decreased immunity in metamorphic frogs. This made metamorphic frogs both less resistant and less tolerant of Bd infection than the other life stages. These findings indicate that changes in immunity altered host responses to parasite exposures throughout ontogeny.

This article is part of the theme issue 'Amphibian immunity: stress, disease and ecoimmunology'.

1. Introduction

Immune function and composition can vary across ontogeny [1,2] and influence host susceptibility to infections [3,4]. In humans, immunity is weakest at the beginning and end of life [1,2]. For example, effective phagocytosis by neutrophils is reduced in elderly patients owing to a reduction of CD16 expression when compared with younger patients [5]. Not only can the overall strength of immunity change through life, but so too can the composition of immune responses, which can affect the kinds of infectious agents that hosts most successfully combat [6,7]. As an example, there is a convex relationship between age and parasite intensity in rabbits, indicating that acquired immunity plays an important role in limiting parasite intensity [8].

Hosts are often simultaneously infected with more than one parasite species, termed co-infection [9–11]. Co-infections can change disease progression in hosts, which can significantly alter host health relative to infections by a single parasite [12,13]. Whether interactions between co-infecting parasites are synergistic or antagonistic and how they alter host health can be highly dependent on host immune responses to parasites, especially when parasites do not compete directly for space or resources [12,14–16].

Host co-infections with micro- and macroparasites can force hosts to mount a different immune response to combat each infection. Mounting immune responses is energetically costly and this can lead to energetic tradeoffs between different immune responses [17]. Microparasite infections, such as fungi, are targeted by neutrophils and macrophages of the hosts' cellular immune response,

and Toll-like receptors (TLRs) can play a major role in pathogen identification [18–20]. Additionally, the skin barrier and mucosal surfaces provide innate immunity [19,21]. Microparasites typically activate the T helper 1 (Th1) acquired immune response [22]. By contrast, when combatting macroparasites, such as gastrointestinal nematodes, hosts predominately use basophils and eosinophil cellular immunity [23]. Additionally, vertebrate hosts can combat macroparasites through the production of cytokines such as interleukin (IL)-4, IL5, IL9 and IL13 [24]. Hosts typically activate the T helper 2 (Th2) arm of the host acquired immune response [22,25,26]. While co-infecting parasites can inhibit one another if the host can mount a single immune pathway to combat both parasites, co-infections requiring different immune responses, such as a co-infection with a macro- and a microparasite, are more likely to facilitate each other, as investment in one response often means less investment in the other response [11,25,27].

Amphibians are an ideal model system to study the ontogeny of immunity and its effects on co-infections. Infectious disease is a primary factor in worldwide declines of amphibians, the most highly threatened vertebrate taxon [28]. Additionally, the amphibian immune system changes drastically through metamorphosis [29,30]. Tadpoles have a functional, but less complex immune system than adult frogs. Amphibians remake their acquired immune cells after metamorphosis so that they do not mistake their own newly formed tissue as foreign [29]. Therefore, amphibians are believed to have little acquired immunity during metamorphosis, potentially neutralizing any Th1/Th2 tradeoff but leaving intact effects from co-infection driven by innate immune responses. Thus, amphibians that metamorphose provide us with a unique system of consistent innate immunity but varying levels of acquired immunity across their life stages. This should allow us to address whether immune ontogeny and co-infections both need to be considered when assessing how a parasite affects host health.

To evaluate how changes in host immunity across life stages affect co-infections in amphibians, we exposed Cuban treefrogs (*Osteopilus septentrionalis*) to a chytrid fungus and a nematode either singly or together. The chytrid fungus, *Batrachochytrium dendrobatidis* (Bd), has been widely implicated in mass mortality and extinction of amphibians [31,32]. Zoospores of this fungus infect amphibian skin and proliferate, limiting osmoregulation and leading to cardiac arrest [33]. Hosts can combat Bd infection through a variety of immune responses, anti-microbial peptides [21] and increasing their rate of skin sloughing to remove infected skin [34]. Bd is also known to occasionally reduce host lymphocyte levels, suggesting it has the potential to be immunosuppressive [35,36]. Larvae of the focal nematode, *Aplectana hamatospicula*, penetrate amphibian skin and migrate to the gastrointestinal tract [37]. There, adult nematodes mature and produce juveniles, which are released in the faeces and can reinfect frogs [37]. Cuban treefrog hosts have been shown to increase their food intake to lessen the negative effects of *A. hamatospicula* infections [37]. This parasite is common in amphibians in the southeastern USA and Latin America [38–40]. Bd and parasitic helminths frequently co-occur in wild amphibian hosts, with 80% of amphibians co-infected with Bd, ranavirus and parasitic helminths in sites across California [41].

We hypothesize that acquired immunity will vary across amphibian life stages, but innate immunity will not. We predict that acquired immunity will be strongest in post-metamorphic frogs and weakest in metamorphic frogs because amphibians have very little acquired immune response during metamorphosis and a less complex immune system as tadpoles, whereas innate immunity shows less variation with ontogeny [29,30]. We also hypothesize pathogen loads will be affected by host ontogeny. As Bd and *A. hamatospicula* activate differing arms of the immune system, which are challenging to upregulate simultaneously [22,27,42], we expect that these parasites will facilitate each other when the acquired immune system is potent (i.e. in post-metamorphic amphibians). We also expect that this facilitation will be weaker in tadpoles and non-existent in metamorphic frogs. We hypothesize that host cellular immunity will depend on parasite identity, but not on ontogeny, as innate immunity does not vary as drastically as acquired immunity across life stages [30]. We expect to see higher basophil and eosinophil counts in hosts exposed to the macroparasite, *A. hamatospicula* [23], and higher macrophage and neutrophil counts in hosts exposed to the microparasite, Bd [18,36]. Finally, we hypothesize that host health will be affected by an interaction between infection and ontogeny. As tadpole and metamorphic frogs have less well-developed immune responses than post-metamorphic frogs, we expect that parasite loads and adverse health effects in single infections will be greatest in these early ontogenetic stages [29]. By contrast, we expect adverse effects on host health from co-infection to be highest after metamorphosis owing to facilitation between these parasites.

2. Methods

(a) Animal husbandry

Cuban treefrog (*Osteopilus septentrionalis*) tadpoles were collected from 140 l pools filled with rainwater at the University of South Florida botanical gardens (Tampa, FL, USA) in the summer of 2017. Individuals collected early in the season (May), midseason (July) and later in the summer (August) were all reared to post-metamorphic, metamorphic and tadpole life stages respectively. See electronic supplementary material, methods for additional details.

(b) Experimental design

To examine how the life stage of amphibians affects host–parasite dynamics, we exposed *O. septentrionalis* to one of four treatments at each of three amphibian life stages. Treatments included no parasite exposure, and exposures to either Bd alone, *A. hamatospicula* alone, or Bd and *A. hamatospicula* together. These four treatments were applied at the tadpole (Gosner development stage 28–35; [43]), metamorphic (stage 42–45 frogs), and post-metamorphic stages. In total, there were 5, 8 and 9 control individuals for tadpole, metamorphic and post-metamorphic frogs, respectively, and 18 individuals for each of 9 treatment groups (3 life stages and 3 treatment groups), for a total of 184 individuals.

Bd (SRS-JEL 212 strain) was cultured in a 1% tryptone solution and *A. hamatospicula* larvae were harvested from adult worms and developed in Petri dishes (see electronic supplementary material for more details). Exposures were applied to post- and mid-metamorphic frogs in Petri dishes (25 × 100 mm) with 1 and 10 ml of artificial spring water (ASW, see supplementary material), respectively, for 24 h. Tadpoles were exposed in cups containing 30 ml of ASW. Hosts were exposed to 1 ml of deionized water containing either 30 J3 *A. hamatospicula* larvae or 10^5 zoospores of Bd. All frogs received sham exposures for parasites to which they were not exposed.

(c) Assessing amphibian responses to parasites

To assess Bd abundance, hosts were swabbed 8 days after treatment exposures because that is late enough for parasite

establishment, but early enough that few hosts have experienced parasite-induced mortality [44]. Frogs were either swabbed five times from hip to toe on rear legs (if frogs had rear legs) or around the mouth for tadpoles. DNA was extracted from swabs (Qiagen DNeasy Blood & Tissue Kit) and analysed using quantitative polymerase chain reaction (qPCR) to quantify Bd abundance [45]. *Aplectana hamatospicula* abundance was assessed by counting adult worms in the gastrointestinal tract of the host after either mortality or euthanasia (for individuals surviving the entire experiment).

Subsets of experimental animals were euthanized on day 11, 19 or 28 to test host immunity during early, but detectable, Bd infections and the peak immune response for *A. hamatospicula* and Bd, respectively. Blood was drawn using micro-haematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA) and used to create blood smears. Blood smears were stained using Camco Quik Stain II (Cambridge Diagnostic Products, Fort Lauderdale, FL). Three separate researchers counted 2000 cells on each slide and identified white blood cell types, and the results were averaged [46]. Enzyme-linked immunosorbent assays (ELISAs) from remaining plasma were used to detect IgY antibody presence (adapted from [37]; see electronic supplementary material, methods).

Individuals were measured (snout–vent length (SVL)) and weighed weekly for four weeks. Amphibians were also checked twice daily for mortality. If mortality occurred, frogs were weighed, measured, swabbed and dissected as described above. Twenty-eight days after treatment exposures, all the remaining frogs were euthanized. Growth rates were calculated as the final mass or SVL minus the initial mass or SVL divided by weeks alive (g week^{-1} or mm week^{-1}).

(d) Statistical analyses

All analyses were run with R v. 3.6.1 [47] and figures were created using the *visreg* function and package [48] or the *ggplot* function and *ggplot2* package [49]. For all models host life stage, exposure to Bd, and exposure to *A. hamatospicula* were included as interacting independent variables unless stated otherwise. Tukey's *post hoc* tests to compare across life stages were run using the *multcomp* package and using the *glht* function [50].

To investigate how host immune response and parasite abundance are affected by host life stage and co-infection, we ran models with all types of white blood cells counted (i.e. lymphocytes, neutrophils, basophils, monocytes, thrombocytes and eosinophils), total white blood cell count, neutrophil to lymphocyte ratio, IgY antibody level and Bd or *A. hamatospicula* abundance as separate dependent variables. Neutrophil, monocyte, eosinophil and thrombocyte counts and Bd abundance were analysed with negative binomial error distributions. The proportion of J3 larvae that hosts were exposed to that successfully penetrated the host's skin and the proportion of J3 larvae that penetrated the host's skin that successfully established as an adult worm in the amphibian guts were run as separate analyses with binomial error distributions. Weight was also used as a predictor variable in these models to control for differences in host size. All other immune responses were analysed as generalized linear models with normal error distributions. Life stage was based on the Gosner stage of the amphibian when immune metrics or parasite abundance were quantified.

We also investigated how co-infections and host life stage alter host health. We conducted a survival analysis using the *survival* package and the *coxph* function [51]. Individuals that survived to the end of the experiment were right-censored. Total development and growth (g or mm) were divided by four weeks, or time to metamorphosis for development, to give rates (in g week^{-1} or mm week^{-1}) and used as dependent variables in separate models. To test how host life stage and identity of the co-infecting parasite affected host tolerance, all the above-described models with host health and immunity were rerun with the addition of parasite abundance (Bd and *A. hamatospicula* abundance were run separately) as a fourth, interacting independent variable. Thus, tolerance was measured as the slope of survival and growth against parasite abundance.

3. Results

(a) Host immunity

Total host white blood cell counts (WBC; $F_{2,150} = 3.67$, $p < 0.05$), lymphocyte counts ($F_{2,150} = 5.38$, $p < 0.01$), neutrophil to lymphocyte ratios ($F_{2,150} = 6.14$, $p < 0.01$), eosinophil counts ($\chi^2_2 = 64.60$, $p < 0.001$), thrombocyte counts ($\chi^2_2 = 24.35$, $p < 0.001$) and IgY antibody levels ($F_{2,121} = 9.14$, $p < 0.001$) all significantly depended on host life stage. Tadpoles ($p < 0.001$) and metamorphic frogs ($p < 0.001$) had significantly higher eosinophil counts than post-metamorphic frogs. Tadpoles also had a significantly higher neutrophil to lymphocyte ratio than post-metamorphic ($p < 0.01$), but not metamorphic frogs ($p = 0.83$). Metamorphic frogs had significantly higher thrombocyte counts than tadpoles ($p < 0.05$) or post-metamorphic frogs ($p < 0.001$). Post-metamorphic frogs had significantly higher white blood cell and lymphocyte counts than tadpoles (WBC: $p < 0.05$; lymphocyte: $p = 0.01$), but not metamorphic frogs (WBC: $p = 0.14$; lymphocyte: $p = 0.06$). Post-metamorphic frogs also had higher IgY antibody levels than tadpoles ($p < 0.001$) and metamorphic frogs ($p < 0.005$; figure 1).

Whether *A. hamatospicula* or Bd significantly altered white blood cell counts depended on the cell type. Regardless of life stage, basophil ($\chi^2_1 = 5.38$, $p < 0.05$) and monocyte counts ($\chi^2_1 = 4.63$, $p < 0.05$) were reduced by exposure to Bd (electronic supplementary material, figure S1), but not affected by exposure to *A. hamatospicula* (basophil: $\chi^2_1 = 0.47$, $p = 0.49$; monocyte: $\chi^2_1 = 1.27$, $p = 0.26$) or their interaction (basophil: $\chi^2_2 = 1.34$, $p = 0.25$; monocyte: $\chi^2_1 = 0.11$, $p = 0.74$). The abundance of Bd was also negatively associated with monocyte and neutrophil counts for metamorphic frogs (monocyte: $\chi^2_1 = 8.07$, $p < 0.01$; neutrophil: $\chi^2_1 = 11.45$, $p < 0.001$), but not for tadpoles (monocyte: $\chi^2_1 = 0.78$, $p = 0.38$; neutrophil: $\chi^2_1 = 0.77$, $p = 0.38$) or post-metamorphic frogs (monocyte: $\chi^2_1 = 0.03$, $p = 0.87$; neutrophil: $\chi^2_1 = 0.45$, $p = 0.50$; figure 2). Neutrophil and eosinophil counts were affected by the interaction between host life stage, exposure to *A. hamatospicula*, and exposure to Bd (neutrophil: $\chi^2_2 = 7.48$, $p < 0.05$; eosinophil: $\chi^2_2 = 6.32$, $p < 0.05$; electronic supplementary material, figure S2). Exposure to *A. hamatospicula*, Bd, or both decreased both neutrophil ($\chi^2_1 = 22.13$, $p < 0.001$) and eosinophil ($\chi^2_1 = 3.96$, $p < 0.05$) counts for metamorphic frogs, but not for tadpoles (eosinophil: $\chi^2_1 = 2.07$, $p = 0.15$; neutrophil: $\chi^2_1 = 0.03$, $p = 0.86$) or post-metamorphic frogs (eosinophil: $\chi^2_1 = 0.29$, $p = 0.59$; neutrophil: $\chi^2_1 = 0.09$, $p = 0.76$).

(b) Parasite abundance

Bd abundance was significantly impacted by host life stage ($\chi^2_2 = 103.3$, $p = 0.001$; figure 3*a*) and the interaction between host life stage and exposure to *A. hamatospicula* ($\chi^2_2 = 8.09$, $p < 0.02$). Metamorphic frogs had the highest Bd abundance compared with both tadpoles ($p < 0.001$) and post-metamorphic frogs ($p < 0.001$). Post-metamorphic frogs had the next highest Bd abundance and tadpoles had the lowest

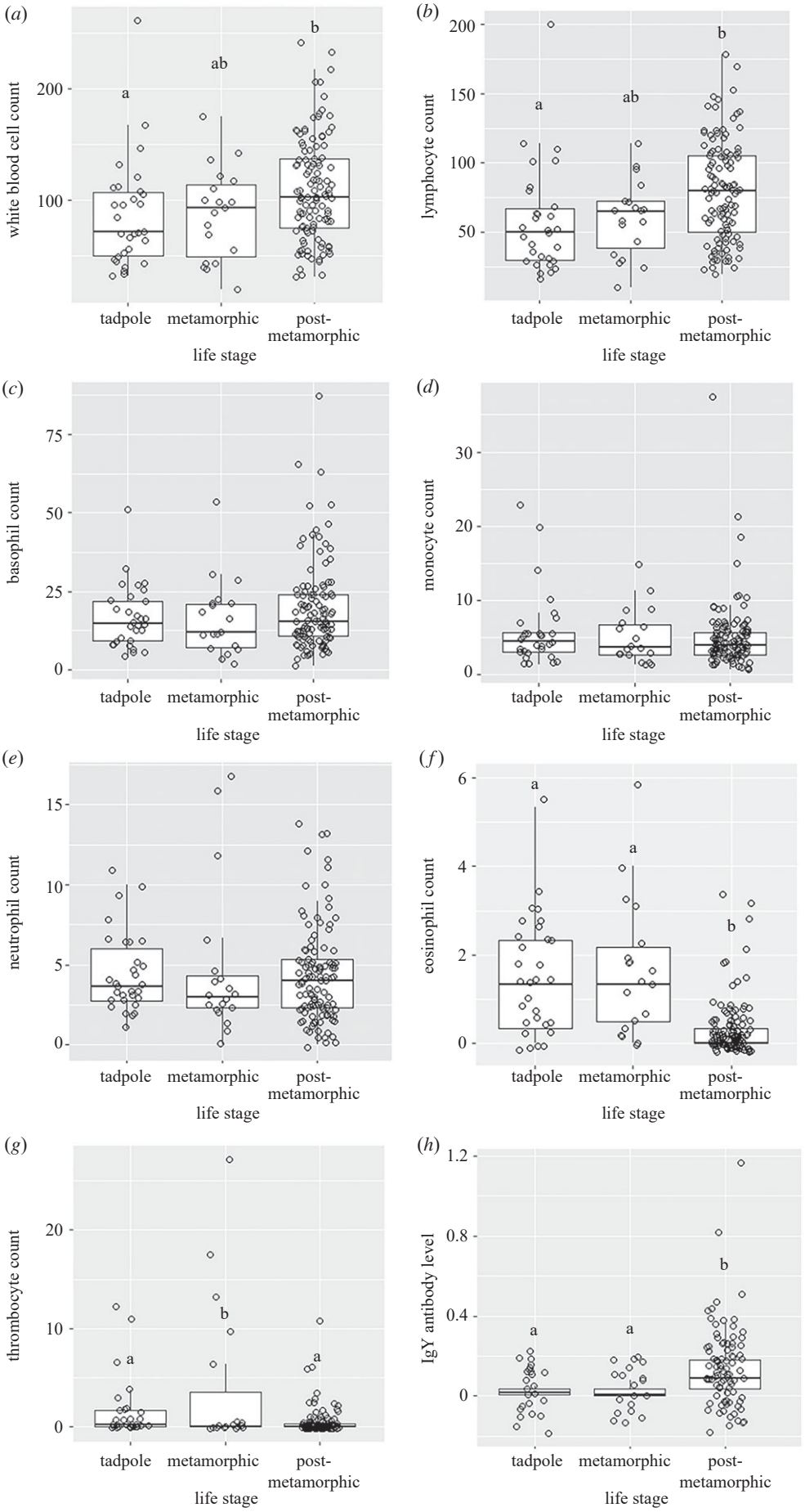

Figure 1. (*Caption overleaf.*)

($p < 0.001$). Tadpoles had higher Bd abundance when co-infected with *A. hamatospicula* than when infected with Bd only, but this was marginally non-significant ($p = 0.066$). There was no effect of *A. hamatospicula* on Bd abundance for metamorphic ($p = 0.09$) or post-metamorphic frogs ($p = 0.38$).

Figure 1. (*Overleaf.*) Immune responses for *Osteopilus septentrionalis* across host life stages. Immune response data from all hosts are shown, regardless of treatment group. Host life stage significantly predicted total host white blood cell counts (WBC; $F_{2,150} = 3.67$, $p > 0.05$; (*a*)), lymphocyte counts ($F_{2,150} = 5.38$, $p < 0.01$; (*b*)), neutrophil to lymphocyte ratios ($F_{2,150} = 6.14$, $p > 0.01$), eosinophil counts ($\chi^2_2 = 64.60$, $p < 0.001$; (*f*)), thrombocyte counts ($\chi^2_2 = 24.35$, $p < 0.001$; (*g*)), and IgY antibody levels ($F_{2,121} = 9.14$, $p < 0.001$; (*h*)). Tadpoles ($p < 0.001$) and metamorphic frogs ($p < 0.001$) had significantly higher eosinophil counts than post-metamorphic frogs (*f*). Metamorphic frogs had significantly higher thrombocyte counts than tadpoles ($p < 0.05$) or post-metamorphic frogs ($p < 0.001$; (*g*)). Post-metamorphic had significantly higher white blood cell (*a*) and lymphocyte counts (*b*) than tadpoles (WBC: $p < 0.05$; lymphocyte: $p = 0.01$), but not metamorphic frogs (WBC: $p = 0.14$; lymphocyte: $p = 0.06$). Post-metamorphic frogs also had higher IgY antibody counts than tadpoles ($p < 0.001$) or metamorphic frogs ($p < 0.005$; (*h*)). Basophil ($\chi^2_2 = 3.13$, $p = 0.21$; (*c*)), monocyte ($\chi^2_2 = 1.24$, $p = 0.54$; (*d*)) and neutrophil ($\chi^2_2 = 0.48$, $p = 0.79$; (*e*)) counts were not significantly predicted by host life stage. Data are shown as boxplots, with the median and upper and lower interquartile ranges shown. Whiskers extend no further than 1.5 times the interquartile range. Points represent the immune response of individual frogs. Different letters on graphs indicate significant differences between treatments.

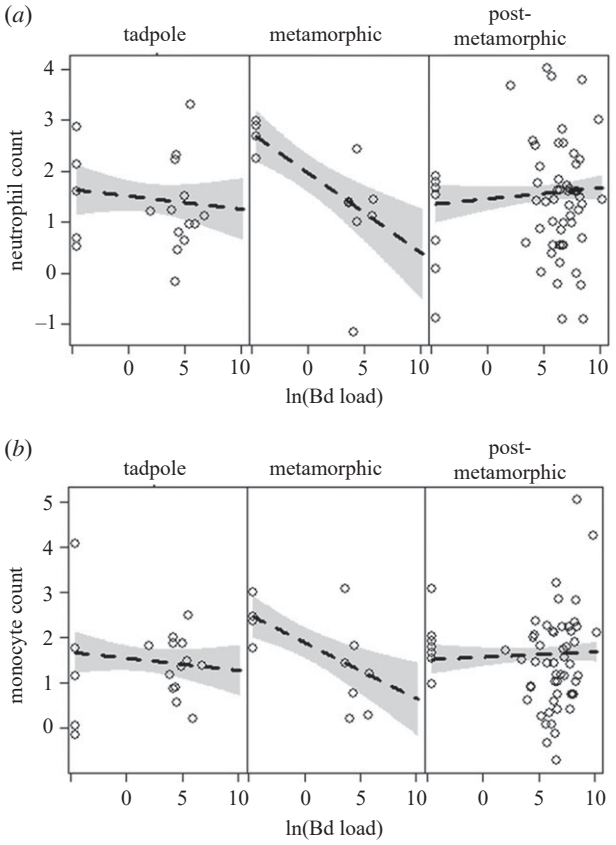

Figure 2. Immune cell counts of *Osteopilus septentrionalis* as a function of the natural log of *Batrachochytrium dendrobatidis* abundance in tadpoles, metamorphic, and post-metamorphic frogs (from left to right). The abundance of Bd was negatively correlated with neutrophil ($\chi^2_1 = 11.45$, $p < 0.001$; (*a*)) and monocyte counts ($\chi^2_1 = 8.07$, $p < 0.01$; (*b*)) in metamorphic frogs. Bd abundance was not correlated with cell counts for tadpoles (neutrophil: $\chi^2_1 = 0.77$, $p = 0.38$; monocyte: $\chi^2_1 = 0.78$, $p = 0.38$) or post-metamorphic individuals (neutrophil: $\chi^2_1 = 0.45$, $p = 0.50$; monocyte: $\chi^2_1 = 0.03$, $p = 0.87$). Data shown as a conditional plot, with the expected value (black dashed line), a 95% confidence band for the expected value (grey band), and partial residuals (points) displayed.

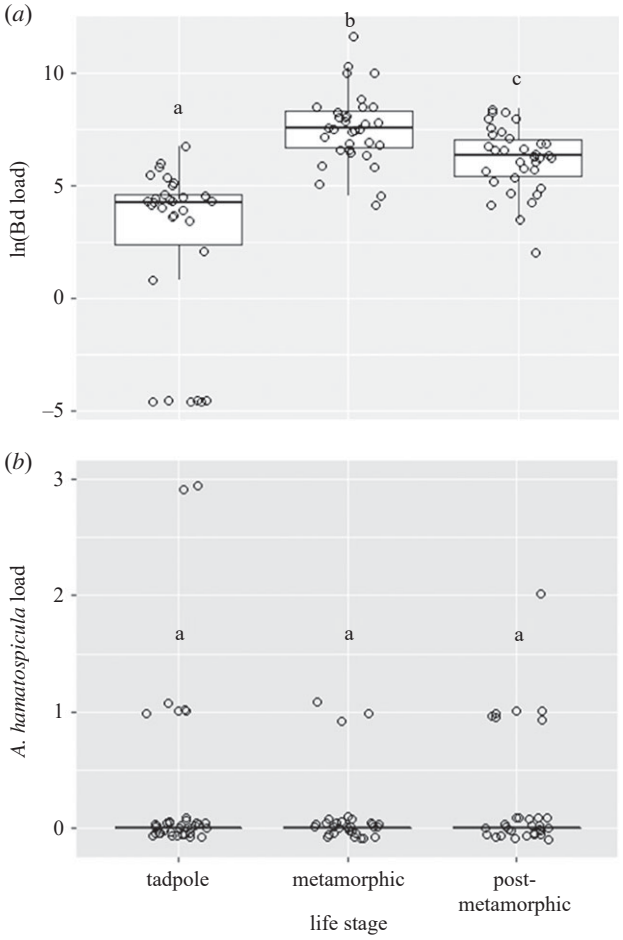

Figure 3. Parasite abundance in *Osteopilus septentrionalis* individuals exposed to *Batrachochytrium dendrobatidis* (*a*) or *Aplectana hamatospicula* (*b*). Amphibian life stage significantly predicted Bd abundance ($\chi^2_2 = 8.09$, $p < 0.02$). Metamorphic frogs had higher Bd abundance than tadpoles ($p < 0.001$) and post-metamorphic frogs ($p < 0.001$). There was no effect of amphibian life stage on the abundance of *A. hamatospicula* that successfully established in the host gut ($\chi^2_2 = 1.69$, $p = 0.43$). Data are shown as boxplots, with medians and upper and lower interquartile ranges displayed. Whiskers extend no further than values 1.5 times the interquartile range. Points are jittered and represent parasite abundance of individual frogs. Different letters on graphs indicate significant differences between treatments.

The proportion of *A. hamatospicula* successfully penetrating host skin was significantly affected by host life stage ($\chi^2_2 = 242.17$, $p < 0.001$), with fewer J3 larvae penetrating tadpoles than metamorphic ($p < 0.001$) or post-metamorphic frogs ($p < 0.001$). Additionally, post-metamorphic frogs had significantly more worms penetrate the skin than metamorphic frogs ($p < 0.02$). The proportion of *A. hamatospicula* successfully penetrating host skin was not significantly affected by exposure to Bd ($\chi^2_1 = 0.58$, $p = 0.45$), but was significantly affected by the interaction between host life stage and exposure to Bd ($\chi^2_2 = 6.79$, $p < 0.05$). Tadpoles exposed to Bd had significantly more worms penetrate the host skin ($p < 0.01$), but exposure to Bd did not affect J3 skin penetration success of metamorphic ($p = 0.13$) or post-metamorphic ($p = 0.26$) hosts (electronic supplementary material, figure S6). The proportion of J3 larvae that penetrated the host skin that successfully established in the host gut was not significantly affected by host life stage

($\chi^2_2 = 1.69$, $p = 0.43$; figure 3*b*), exposure to Bd ($\chi^2_1 = 0.03$, $p = 0.87$), or their interaction ($\chi^2_2 = 0.46$, $p = 0.8$).

(c) Host health

Host survival was significantly increased by exposure to *A. hamatospicula* ($\chi^2_1 = 5.06, p < 0.05$) and significantly decreased by exposure to Bd ($\chi^2_1 = 6.66$, $p < 0.01$) compared with controls (electronic supplementary material, figure S3). Additionally, host survival after exposure to parasites was significantly affected by host life stage ($\chi^2_2 = 24.92$, $p < 0.001$), as post-metamorphic frogs survived significantly longer than tadpoles ($p < 0.001$). Host weight and length were significantly affected by host life stage and the interaction between host life stage and exposure to Bd (see electronic supplementary material).

Hosts significantly differed across life stages in their tolerance (e.g. a host's ability to maintain its health despite high infection intensity) to Bd. When testing tolerance with host growth as the response variable, there were no effects of *A. hamatospicula* abundance on host weight ($F_{2,95} = 0.32$, $p = 0.73$) or length ($F_{2,90} = 0.54$, $p = 0.59$) across life stages. However, there were effects of Bd abundance on host weight ($F_{2,95} = 3.86$, $p < 0.05$) and length ($F_{2,90} = 5.15$ $p < 0.01$) across life stages. Higher abundance of Bd increased weight gain ($p < 0.05$) and marginally increased length gain ($p = 0.06$) for tadpoles. Conversely, higher abundance of Bd decreased length gain ($p < 0.05$) but did not affect weight gain ($p = 0.17$) for post-metamorphic frogs. Frogs undergoing metamorphosis experienced no changes in weight ($p = 0.94$) or length ($p = 0.63$) across Bd abundance (figure 4*a*). When testing host tolerance using host survival as the response variable there were no effects of *A. hamatospicula* on host survival across life stages ($\chi^2_2 = 5.57$, $p = 0.06$), but there were effects of Bd abundance on host survival across life stages ($\chi^2_2 = 7.08$, $p < 0.05$). Mortality was positively associated with Bd abundance for metamorphic frogs ($p < 0.05$), but not for tadpoles ($p = 0.29$) or post-metamorphic frogs ($p = 1$; figure 4*b*).

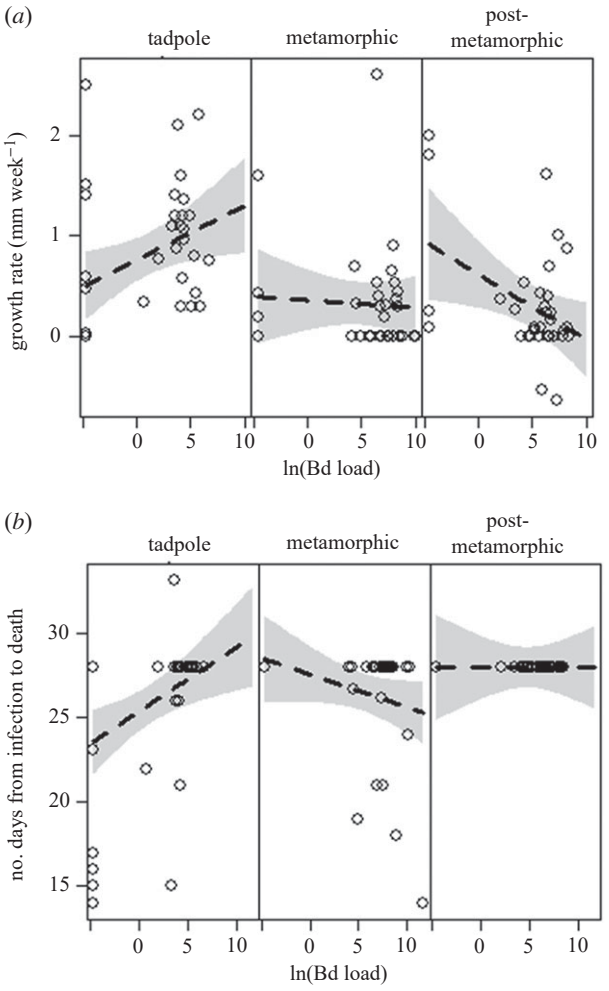

Figure 4. Host tolerance, measured as growth rate (mm week^{-1}; (*a*)) and survival (*b*) of *Osteopilus septentrionalis* as a function of the natural log of *Batrachochytrium dendrobatidis* abundance in tadpoles, metamorphic, and post-metamorphic frogs (from left to right). (*a*) There were significant effects of Bd abundance on host growth rate across life stages ($F_{2,90} = 5.15$ $p < 0.01$). Higher abundance of Bd marginally increased growth for tadpoles ($p = 0.06$) and decreased growth for post-metamorphic frogs ($p < 0.05$). Metamorphic frogs experienced no significant change in length across Bd abundance ($p = 0.63$). (*b*) Host survival was affected by abundance of Bd across host life stage ($\chi^2_2 = 7.08$, $p < 0.05$). Days alive decreased with higher Bd abundance for metamorphic frogs ($p < 0.05$) but was not significantly dependent on Bd abundance for tadpoles ($p = 0.29$) and post-metamorphic frogs ($p = 1$). Data shown as a conditional plot, with the expected value (black dashed line), a 95% confidence band for the expected value (grey band), and partial residuals (points) displayed.

4. Discussion

While co-infection outcomes can be determined by host immunity, immunity is not static and can change across a host's lifetime [3,4,13,15]. Therefore, it is important to understand how immune ontogeny can change outcomes from co-infections. We found that acquired immunity was lower in tadpoles and metamorphic frogs than in post-metamorphic frogs. However, we found no evidence that co-infection affected host health or immunity at any of the tested life stages. We did find that neutrophil and monocyte counts were negatively correlated with Bd abundance in only metamorphic frogs. Finally, we found that this life stage had higher Bd abundance and lower survival at higher Bd abundance than the other life stages, suggesting that metamorphic frogs were both less resistant and less tolerant to Bd infections.

Immunity changed across life stages in the Cuban treefrog in ways slightly different from what we predicted. We found that acquired immune responses, measured by IgY and lymphocyte counts, were higher in post-metamorphic frogs than the other life stages (figure 1). However, we did not observe lower acquired immunity in metamorphic frogs than tadpoles. Studies conducted on *Xenopus* found that lymphocyte counts were high after metamorphosis and low during metamorphosis [29,30]. These differences could simply represent species-level differences in immune ontogeny. Additionally, *Xenopus* has a somewhat unusual life history including an entirely aquatic adult life stage, which could lead to post-metamorphic immunity that is distinct from more common amphibious frog species. Although susceptibility to parasites throughout amphibian ontogeny has been tested [52,53], to our knowledge the ontogeny of immunity across all life stages in anurans outside *Xenopus* has not been studied (but see [54] for immune differences through time in post-metamorphic frogs). Additionally, rapid amphibian development, as was facilitated by Bd in tadpoles in this study (figure 4*a*), has been shown to be negatively associated with immune function, including cellular immunity [55]. This could potentially account for the lower-than-expected acquired immunity

in tadpoles. Eosinophil counts were higher in tadpoles than in post-metamorphic frogs and thrombocyte counts were the highest in metamorphic frogs (figure 1). Higher eosinophil counts in tadpoles, which have also been found in other amphibian species [56], could be an adaptive response to abundant macroparasites that tadpoles have to combat in their aquatic habitat, such as digenean trematodes released from snails [41,57].

Amphibian ontogeny changed how Bd exposure affected amphibian cellular innate immunity and Bd abundance. Basophil and monocyte (immature macrophage) counts were reduced in amphibians exposed to Bd regardless of host life stage (electronic supplementary material, figure S1). However, for metamorphic frogs only, neutrophil and monocyte counts were negatively correlated with abundance of Bd (figure 2), and exposure to either or both tested pathogens reduced eosinophil and neutrophil counts compared with control animals (electronic supplementary material, figure S2). Finally, abundance of Bd changed across life stages, with metamorphic frogs having the highest Bd abundance (figure 3*a*). Bd infects keratinized skin cells, which are present throughout the skin of post-metamorphic frogs, but only present in the mouthparts of tadpoles [58]. Therefore, evidence of immunosuppression from Bd infection and high Bd abundance in metamorphic frogs was potentially due to the combined effects of lower immunity compared with post-metamorphic individuals ([29,30], figure 1) and increased Bd abundance compared with tadpoles owing to more keratin-containing tissue ([58]; figures 2 and 3 and electronic supplementary material, figure S2). Studies testing whether Bd is immunosuppressive to leucocytes other than lymphocytes have generated mixed results [18,35,36]. Macrophages and neutrophils are known to combat Bd [35], and the downregulation of other immune responses at metamorphosis could make amphibians particularly susceptible to infection.

The proportion of *A. hamatospicula* that successfully penetrated host skin increased with frog age, but this pattern did not hold for the proportion of larvae that successfully established in the gut (figure 3*b*). This result suggests that either *A. hamatospicula* struggles to find and penetrate host skin in the three-dimensional environment (i.e. 30 ml of water) in which tadpoles were exposed relative to the more two-dimensional terrestrial environment that nematodes experienced during exposure of metamorphic and post-metamorphic frogs, or that localized immune responses in the skin of tadpoles were better at combatting *A. hamatospicula* penetration than those of metamorphic and post-metamorphic frogs. Additionally, exposure to Bd increased worm penetration in tadpoles, but not in the other host life stages. However, we found no evidence that tadpoles have subsequently better host health when exposed to this parasite or that this leads to lower adult parasite load in hosts. Additionally, co-infection did not alter *A. hamatospicula* establishment in any life stage.

We hypothesized that Bd and *A. hamatospicula* would facilitate one another owing to tradeoffs in the host acquired immune system [22,27]. However, we observed no effects of co-infections on parasite abundance at any life stage (figure 3) and consequently no negative effects on host health from co-infection. Although immune suppression was predominately found in metamorphic frogs, immune suppression from Bd could have limited the expected acquired immune facilitation (electronic supplementary material, figures S1 and S2). Additionally, fungal immunity has been shown to also upregulate the Th17 arm of the host immune system [42,59]. If this is true for Bd, it could limit the expected tradeoff between the Th1/Th2 arms of the acquired immune system and allow the host to upregulate immunity to combat both parasites.

We found that tolerance of infections depended on host life stage. Bd abundance was positively and negatively associated with growth rates of tadpoles and post-metamorphic frogs, respectively (figure 4*a*). This positive association could be adaptive for tadpoles if it allows them to more quickly exit a dangerous environment, as has been seen in amphibians in rapidly drying ponds [60,61]. Conversely, frogs post-metamorphosis may have to invest more in immunity because they cannot easily escape their environment and have a higher surface area that can be infected [33]. As has been seen in other studies, Cuban treefrogs infected with Bd had significantly lower survival than frogs infected with *A. hamatospicula* ([44,62]; electronic supplementary material, figure S3). Bd abundance was correlated with mortality for metamorphic frogs, but not for the other two life stages (figure 4*b*). As Bd abundance was also higher for metamorphic frogs (figure 3), this suggests that these frogs were both less resistant and less tolerant of Bd infections than the other life stages.

Overall, we found that host immunity varies across host life stage, which affected host responses to single parasites and host health, but not co-infections. Frogs undergoing metamorphosis have limited immunity to combat parasites compared with those post-metamorphosis. They also have a higher surface area that immunosuppressive Bd can infect compared with tadpoles. These factors may have combined to create a scenario where Bd immunosuppression is greatest in metamorphic frogs. This immunosuppression potentially caused metamorphic frogs to be both less resistant and less tolerant to Bd infections, with reductions in host survival. Our results support that changes in immunity and parasite infectivity across life stages can alter how hosts respond to parasite exposure. However, other parasite pairings and host species should be tested to determine the importance of considering ontogeny when evaluating amphibian responses to co-infections.

Ethics. All animal husbandry throughout the experiment was carried out according to IUCAC protocol no. W IS00002203.

Data accessibility. Data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.44j0zpck7 [63].

Data are also provided as electronic supplementary material [64].

Authors' contributions. C.R.: conceptualization, data curation, formal analysis, investigation, methodology, project administration, validation, visualization, writing—original draft; J.R.R.: conceptualization, funding acquisition, methodology, project administration, resources, software, supervision, validation, writing—review and editing.

Both authors gave final approval for publication and agreed to be held accountable for the work performed herein.

Conflict of interest declaration. We declare we have no competing interests.

Funding. This research was supported by grants from the National Science Foundation (grant nos DEB-2017785, DEB-1518681, IOS-1754868), National Institutes of Health (grant no. R01TW010286), and US Department of Agriculture (2021-38420-34065) to J.R.R.

Acknowledgements. We would like to thank S. Knutie and P. Snyder for guidance with immune assays, all the undergraduates who made this experiment possible, and the reviewers for their constructive feedback.

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