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Original Contribution

Role of Antimicrobial Peptides in Amphibian Defense Against Trematode Infection

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Abstract: Antimicrobial peptides (AMPs) contribute to the immune defenses of many vertebrates, including amphibians. As larvae, amphibians are often exposed to the infectious stages of trematode parasites, many of which must penetrate the host's skin, potentially interacting with host AMPs. We tested the effects of the natural AMPs repertoires on both the survival of trematode infectious stages as well as their ability to infect larval amphibians. All five trematode species exhibited decreased survival of cercariae in response to higher concentrations of adult bullfrog AMPs, but no effect when exposed to AMPs from larval bullfrogs. Similarly, the use of norepinephrine to remove AMPs from larval bullfrogs, Pacific chorus frogs, and gray treefrogs had only weak (gray treefrogs) or non-significant (other tested species) effects on infection success by *Ribeiroia ondatrae*. We nonetheless observed strong differences in parasite infection as a function of both host stage (first- versus second-year bullfrogs) and host species (Pacific chorus frogs versus gray treefrogs) that were apparently unrelated to AMPs. Taken together, our results suggest that AMPs do not play a significant role in defending larval amphibians against trematode cercariae, but that they could be one mechanism helping to prevent infection of post-metamorphic amphibians, particularly for highly aquatic species.

Keywords: disease ecology, wildlife immunology, antimicrobial peptides, trematodes

Introduction

Antimicrobial peptides (AMPs) are a critical component of the innate immune system in vertebrates. These diverse proteins engage in a variety of antimicrobial activities ranging from membrane permeabilization to actions on cytoplasmic targets; however, they lack specific antigen recognition and thus function broadly in innate defense against a range of foreign agents (Brogden et al. 2003; Yeaman and Yount 2003; Batycka-Baran et al. 2014). The highest concentrations of AMPs are often found in vertebrate tissues regularly exposed to microbes, such as skin and the gastrointestinal lining (Levy 1996; Brogden et al. 2003; Ganz 2003; Bulet et al. 2004). Some AMPs are produced continuously while others are induced in response to a threat (Ganz 2003). In general, AMPs are most effective as a form of chemical defense against bacteria and fungi (Brogden et al. 2003).

While AMPs have been relatively well studied in humans (Bahar and Ren 2013; Batycka-Baran et al. 2014) and

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other vertebrate model systems (Zasloff 2002; Brogden et al. 2003; Yeaman and Yount 2003; Li et al. 2012; Pask et al. 2012), their importance for wildlife diseases remains poorly understood. Owing to their biphasic life cycles, amphibians are exposed to a wide range of both micro- and macroparasites and research into their immune defenses has increased steadily (Densmore and Green 2007; Todd 2007; Koprivnikar et al. 2012; Rollins-Smith and Woodhams 2011). Amphibian AMPs are synthesized and stored in the granular glands of the dermal skin layer (Rollins-Smith et al. 2005); when an amphibian becomes alarmed or injured, the peptides are secreted, attach to the phospholipid bilayer of a pathogen, and can lead to a disruption of pathogen functionality (Carey et al. 1999; Rollins-Smith et al. 2005; Ramsey et al. 2010). Evidence suggests that AMPs may be an important determinant of amphibian susceptibility to *Batrachochytrium dendrobatidis* (Bd), the causative agent of chytridiomycosis. For instance, among four species of Australian frogs experimentally exposed to Bd, those with higher concentrations of AMPs as adults were more likely to survive following Bd exposure (Woodhams et al. 2007). Similarly, Conlon (2008) showed that 16 purified proteins isolated from *Rana pretiosa* inhibited the growth of Bd zoospores. Beyond Bd, amphibian AMPs have also been shown effective against some bacteria, protists, and viruses (Nicolas and Mor 1995; Rinaldi 2002; Apidianakis et al. 2005; Pinto et al. 2013; Pretzel et al. 2013). Pinto et al. (2013) found that four AMPs isolated from adult *Phyllomedusa nordestina* were effective against infections by *Leishmania infantum* and *Trypanosoma cruzi* in culture, which may contribute to future human drug design.

The importance of AMPs against other amphibian parasites is less clear, particularly for macroparasitic infections such as flukes (Pretzel et al. 2013). Because some of these parasites are waterborne as larvae and acquired through the skin, it is possible that AMPs play an important role in immunodefense. While amphibian immunodefenses are not well studied in general, some species are known to produce AMPs as larvae, and expression increases through development (Clark et al. 1994, Katzenback et al. 2014). The acquired immune system is also less developed in larval amphibians (reviewed by Rollins-Smith 1998; Rollins-Smith and Woodhams 2011), which is perhaps another reason to expect greater dependence on components of innate immunity, such as AMPs. For instance, larval trematodes such as *Ribeiroia ondatrae*, which can cause severe limb deformities in amphibians, penetrate the skin of larval amphibians and encysts subcutaneously, where they are presumably exposed to AMPs if they are present (Johnson et al. 2002, 2012). Among amphibian species tested, there is substantial variation in susceptibility to *R. ondatrae* (Johnson and Hartson 2009; Rohr et al. 2010; Johnson et al. 2012; LaFonte and Johnson 2013), some of which is associated with variation in immune function. LaFonte and Johnson (2013), for instance, showed that the exogenous addition of the immunosuppressant corticosterone led to a 191% increase in *R. ondatrae* infection, an 80% increase in *Echinostoma* sp. infection, and a 67.8% increase in *Alaria* sp. (two other species of trematodes) relative to controls. Similarly, Belden and Kiesecker (2005) found that *Hyla versicolor* tadpoles treated with exogenous corticosterone developed $\sim$2 times higher *Alaria* sp. loads compared with controls. Whether variation in larval amphibian AMPs helps explain observed variation in trematode infection susceptibility among amphibian host species remains an open question.

Here we explored the role of AMPs in defending against larval trematode infections in amphibians. Specifically, we (1) determined the effects of variable concentrations of amphibian AMPs on infectious trematode cercariae, (2) assessed how such effects varied among five different trematode species and across amphibian life stages (larval versus adult), and (3) evaluated whether AMP removal can help explain extreme differences in susceptibility between larval *H. versicolor* and *Pseudacris regilla* to infection by *R. ondatrae*. This work has implications for understanding patterns of amphibian deformities and other forms of pathology associated with macroparasite infections in amphibians (Blaustein et al. 2011; Koprivnikar et al. 2012; Paull and Johnson 2014).

Methods

Effects of AMPs on Cercariae Survival

We collected snails (*Helisoma trivolvis* and *Physa acuta*) from ponds in the San Francisco Bay Area of California between May and August of 2013 and 2014 (see Richgels et al. 2013 Fig. 1 for map). To find infected snails, we isolated snails individually into 50-ml centrifuge tubes and allowed them to release cercariae over a 24-h period following a natural light:dark cycle; snails were checked every 6–10 h, and any released cercariae were identified using morphological features (Beaver 1939; Schell 1970, 1985;

Johnson et al. 2002). We focused on the following trematode taxa: *R. ondatrae*, *Echinostoma* sp. (likely *E. trivolvis*), *Alaria* sp., and an unidentified trematode with armatae cercariae, all from the rams horn snails *H. trivolvis*, as well as *Manodistomum syntomentera* from *P. acuta*. To obtain cercariae for experimental exposures, we placed previously identified infected snails into 50-ml centrifuge tubes with 40 ml of dechlorinated, UV-sterilized, and carbon-filtered freshwater (referred to as 'treated water' hereafter) and harvested parasites within 5–6 h. Some parasites, *Echinostoma* sp., *Alaria* sp., and armatae cercariae, were induced to shed under light, whereas *R. ondatrae* and *M. syntomentera* were placed in the dark to induce shedding (Paull and Johnson 2014).

To obtain AMPs for trematode exposure experiments, we collected natural skin secretions from larval (first- and second-year tadpoles) and adult bullfrogs, *Lithobates catesbeianus*, according to previously established methods (Rollins-Smith et al. 2002, 2005). Tadpoles ($n = 25$) were individually exposed to an aqueous solution of 100 μM norepinephrine-bitartrate (Sigma), and adults ($n = 6$) were subcutaneously injected with 40 nM per g body mass. After administration of norepinephrine, skin secretions were acidified with 0.1% HCl to inhibit degradation, concentrated and partially purified over C-18 Sep-Pak cartridges (Waters Inc.), as well as quantified as previously described (Rollins-Smith et al. 2002).

To test the sensitivity of different trematode species to amphibian AMPs, we placed individual cercaria of each parasite species into wells within a sterile 96-well plate using a sterile glass 1-ml pipette. Prior to the addition of cercariae, we added one of four dosages of AMPs (0, 12.5, 50, and 100 μg/ml) collected from different life stages of *L. catesbeianus* (early stage tadpoles [Gosner (1960) stages 25–30], late stage tadpoles [Gosner 31–42], and adults), a species known to support moderately high AMP concentrations (Conlon 2008). The four concentrations were achieved by mixing purified AMP with treated water to a total volume of 100 μl per well. Each concentration was replicated 16 times per parasite; with four concentrations and three host life stages, this yielded 192 cercariae for each parasite species, with the exception of *Echinostoma* sp., for which we only tested AMPs from adult *L. catesbeianus* at the four concentrations (64 cercariae). Replicates representing each concentration and host life stage were included in every well plate to avoid any plate-level effects; parasites were maintained in separate well plates owing to differences in the time of their emergence from snails. Every 2 h, we recorded the status of each cercaria as alive (movement) or dead (no movement) for a maximum of 24 h, which in all but a few cases concluded with the death of all cercariae.

In addition, we also tested the sensitivity of *R. ondatrae* cercariae to AMPs collected from tadpoles of two other amphibian species: *H. versicolor* and *P. regilla*, which are known to differ strongly in their susceptibilities to infection (Johnson and Hartson 2009; LaFonte and Johnson 2013). Following the protocol outlined above, we added 250 μl of AMP solutions randomly to 24 wells within a sterile 96 well plate. We then pipetted one *R. ondatrae* cercaria using calibrated pipette into each well with 20 μm of water. We monitored cercariae every 2 h for a maximum of 24 h.

Effects of AMPs on Trematode Infection Success

To assess how AMPs affected trematode infection success within larval amphibians, we conducted two experiments, both of which used previously established protocols with norepinephrine exposure to induce larval amphibians to release stored AMPs, prior to trematode exposure (Gammill et al. 2012). In 2014, we collected *L. catesbeianus* larvae (first and second year) from a pond in Boulder, Colorado, and housed them in individual containers filled with 1 L of treated water and fed ad libitum a diet consisting of equal parts TetraMin (Tetra, Melle, Germany) and ground *Spirulina*. We changed water semiweekly and maintained the light cycle at 12:12 h light:dark. Prior to parasite exposure, we randomly assigned each larva to one of three treatment groups: 0 μM norepinephrine (control), 100 μM norepinephrine, or 200 μM of norepinephrine. We maintained tadpoles in 200 ml treated water and norepinephrine (concentration depending on treatment group) for 15 min (Holden et al. 2015). Following treatment with norepinephrine, we transferred tadpoles to a new container filled with 1.5 L of treated water. Twelve hours later, we transferred all tadpoles to individual containers filled with 700 ml of treated water and exposed them to 50 cercariae of *R. ondatrae*, isolated using the techniques described above. After 48 h we quantified infection within each host.

In 2013, we collected amphibian egg masses of *H. versicolor* from University Park, Pennsylvania, and of *P. regilla* from Lake Penhollow, Oregon. Hatching larvae were raised in 40 L of treated water and fed an ad libitum diet as described above. Water was changed once per week and the light cycle was maintained at 12:12 h light:dark. Upon reaching Gosner stage 28 (Gosner 1960), larvae were ran-

domly assigned to one of four treatments, each with 20 replicates: control (no norepinephrine), AMPs removed (2 ml of 10 mM stock norepinephrine solution per 200 ml of treated water), AMPs removed with reciprocal transfer of AMPs from the other amphibian species, and a vehicle control (see details below). To reduce AMP levels in each species, we exposed 20 tadpoles housed together to 2 ml of a stock norepinephrine within a volume of 200 ml of treated water for 30 min. To add AMPs from one host species to another, we added 0.5 ml of the water (and norepinephrine) remaining after the 30 min exposure period to isolated tadpoles of the second species (i.e., AMP solution from *P. regilla* was added to individual *H. versicolor* larvae and vice versa). Tadpoles in the vehicle control treatment were exposed to an extra 0.5 ml of treated water from a 200 ml solution with 2 ml of norepinephrine but no tadpoles. Animals in the control treatment were given 2 ml of treated water as a sham exposure. Thirty minutes after norepinephrine treatment, larvae from all treatment groups were exposed to 14 *R. ondatrae* cercariae and subsequently examined to quantify metacercariae. All applicable institutional and/or national guidelines of the care and use of the animals used in all listed experiments were followed.

Analysis

We used a Cox proportional hazards regression model (coxph) in the R package survival (Fox 2002) to evaluate how different treatments affected the survival of trematode cercariae. We used this approach to assess how AMP concentration, amphibian life stage, and parasite identity affected cercariae survival time. We first tested how survival varied among parasite species, independent of any AMP treatments, and then ran parasite-specific models to test the influence of dose and host life stage. Any cercaria that survived the entire observation period (i.e., 24 h) were treated as censored. Because one parasite (*M. syntomentera*) had 100% parasite survival in several treatments, we used the Firth correction to the proportional hazards model for any analyses involving this parasite (coxphf). For the subsequent trials involving AMPs from *P. regilla* and *H. versicolor*, we used this same approach to evaluate how AMP treatment affected the survival of *R. ondatrae* cercariae relative to the control and vehicle control treatments.

We also calculated the lethal concentration required to kill 50% of cercariae (LC50) for each parasite species. We focused on adult bullfrog AMPs both because it was the only stage tested for all parasites and because each of the species showed a strong, negative response in survival. To estimate the LC50, we analyzed the AMP concentrations using a generalized linear model with a binomially distributed response (alive vs. dead after 12 h, which was halfway through the observation period) and used this model to interpolate the LC50 value (using the dose.p function in the MASS package).

To test how treatments affected the success of cercariae in infecting tadpoles, we used generalized linear models with either a negative binomial or Poisson distribution based on AIC comparisons and model fit. We used this approach to evaluate how host stage (first or second year tadpole of *L. catesbeianus*) and norepinephrine dose (0, 100 μM, or 200 μM) affected the number of *R. ondatrae* metacercariae detected per tadpole. In each case, we first tested the full model including any interaction terms, and subsequently fit the reduced model if the interaction was non-significant. We also used this framework to evaluate how norepinephrine dose and the reciprocal transfer of AMP solution between *P. regilla* and *H. versicolor* influenced *R. ondatrae* load per host.

Results

Among the five trematode species tested, trematode cercariae differed in their survival times even among the control treatments (Wald test = 18.83; df = 4, $P < 0.001$; $n = 160$). *Mandistomum syntomentera* had the longest survival time, for which none of the cercariae in the control treatment died during the 24-h observation period (i.e., all observations were censored), followed by *R. ondatrae* (average survival ± 1 SE = 1044 ± 68.2 min), *Echinostoma* sp. (1005 ± 68.1 min), and *Alaria* sp. (876 ± 93.2 min), with the armatae cercaria exhibiting the shortest survival time (768 ± 70 min) (Fig. 1). Similarly, based on the LC50 analysis, all parasite species showed a significant, negative response in survival with increasing concentrations of adult bullfrog AMPs. The estimates of LC50 ranged from 17.9 μg/ml for *Alaria* sp. to 64.3 μg/ml for *M. syntomentera* (Fig. 2).

For each parasites species, we also detected significant effects of both AMP dosage and host life stage on parasite survival. For *M. syntomentera*, the AMPs from adult bullfrogs substantially reduced cercariae life span, particularly at high concentrations (Firth correction; Wald test = 24.55462, df = 3, $P < 0.00001$; $n = 176$; dose

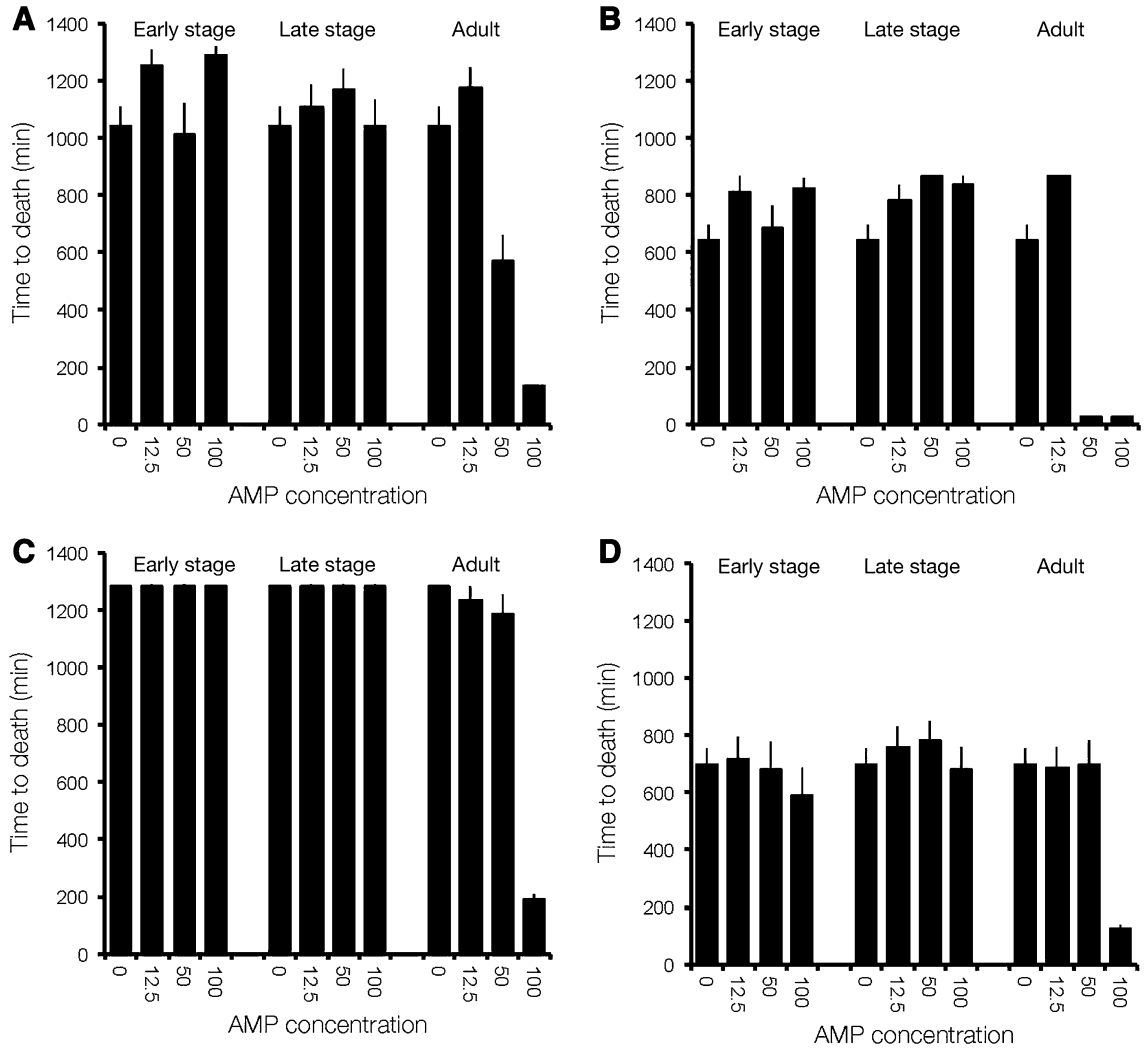

Figure 1. Average time of death (in minutes) + 1 SE of *Ribeiroia ondatrae* cercariae (**a**), *Alaria* sp. cercariae (**b**), *Manodistomum syntomentera* cercariae (**c**), and an unknown species with armatae cercariae (**d**) to three antimicrobial peptide concentrations (12.5, 50, 100 μg/ml) collected from early stage larvae, late stage larvae, and adult bullfrogs. Data for *Echinostoma* sp., which was only exposed to adult bullfrog AMPs, are not shown.

$P < 0.00001$; host stage $P < 0.00001$). Exposure to 100 μg/ml of adult bullfrog AMPs reduced survival by 86% relative to both the control treatment and those involving early or late stage tadpole AMPs. For *R. ondatrae* and the armatae cercaria, there was a significant interaction between AMP dosage and host life stage ($P < 0.00001$), such that dose strongly increased cercariae mortality but only for adult AMPs (*Ribeiroia*: Wald test = 49.25, df = 1, $P < 0.00001$, $R^2 = 0.601$; $n = 64$ armatae cercaria: Wald test = 26.63, df = 1, $P < 0.00001$, $R^2 = 0.386$; $n = 60$), with no effects of dose for AMPs from early or late stage bullfrog tadpoles ($P > 0.1$). The results for *Alaria* sp. also revealed a significant interaction between dose and AMP life stage ($P < 0.00001$); however, while the dose of adult AMPs again increased cercariae mortality (Wald test = 35.04, df = 1, $P < 0.00001$; $R^2 = 0.483$), higher dosages of early and late stage tadpole AMPs had a weakly positive effect on cercariae survival (Early stage: Wald test = 5.77, df = 1, $P = 0.016$; $R^2 = 0.12$; Late stage: Wald test = 5.52, df = 1, $P = 0.019$, $R^2 = 0.146$). This effect stemmed primarily from a lower survival among cercariae in the control treatment. *Echinostoma* sp. cercariae were exposed only to adult AMPs, for which we detected a strong, negative effect of dosage on survival (Wald test = 14.44, df = 1, $P = 0.00015$, $R^2 = 0.15$).

Treating larval bullfrogs with norepinephrine had no measurable effects on the number of detected *R. ondatrae* metacercariae (GLM with negative binomial distribution;

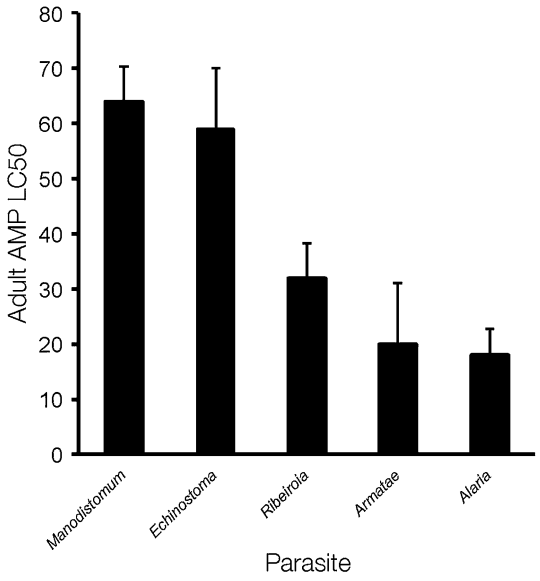

Figure 2. The estimated lethal concentrations of adult bullfrog AMPs (μg/ml) + 1 SE at which 50% of cercariae were dead by 12-h post-exposure.

norepinephrine dose = −0.00054 ± 0.0035, $z = -0.151$, $P = 0.88$). However, we did detect a strong effect of host age (tadpole year = −3.339 ± 0.556, $z = -6.00$, $P < 0.000001$; pseudo-$R^2 = 0.537$). First year tadpoles exhibited 30 times more metacercariae than second year tadpoles (average ± 1 SE = 10.72 ± 2.045 vs. 0.375 ± 0.272, respectively), regardless of norepinephrine treatment. Most (14/16; 85.7%) second year tadpoles had no parasites whatsoever, whereas 15/18 (83.3%) of the first year hosts were infected with at least one metacercariae (2–30 per host). Comparable results were generated if we used host snout-vent length as the predictor rather than the categorical estimate of first vs. second year (pseudo-$R^2 = 0.651$).

For the experiment involving the reciprocal transfer of AMPs between *P. regilla* and *H. versicolor*, we found no effect of treatment on *R. ondatrae* cercariae survival (Wald test = 0.38, df = 3, $P = 0.945$, $R^2 = 0.004$). Across treatments, cercariae survival averaged (± 1 SE) 890 ± 15.7 min in the control, 865 ± 20.0 min in the vehicle control, 870 ± 19.0 min in the *H. veriscolor* AMP treatment, and 890 ± 17.2 min in the *P. regilla* AMP treatment. As expected, *P. regilla* supported substantially higher infection loads with *R. ondatrae* relative to *H. versicolor* tadpoles exposed to identical numbers of cercariae. On average, each *P. regilla* had 8.65 ± 0.483 metacercariae compared with 0.15 ± 0.082 per *H. versicolor* (Fig. 3). Interestingly, we detected a significant host species-by-treatment interaction ($P = 0.0109$); while there was no effect of treatment on *R. ondatrae* load in *P. regilla*, the AMP deficient treatment caused an increase in infection among *H. versicolor* relative to both the control and the reciprocal AMP transfer. The magnitude of this effect was

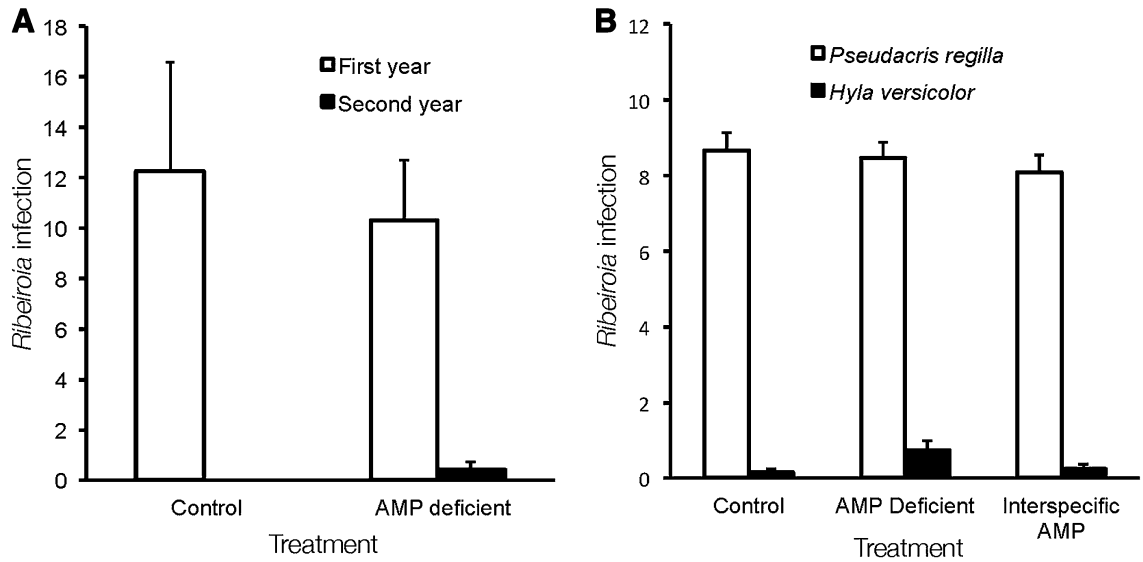

Figure 3. Effects of AMP removal on *Ribeiroia ondatrae* infection in amphibians. **a** The average number of detected *R. ondatrae* metacercariae + 1 SE in first and second year bullfrogs after exposure to norepinephrine (AMP-deficient) or to no manipulation (control). **b** The average number of *R. ondatrae* metacercariae + 1 SE in *Pseudacris regilla* and *Hyla versicolor* tadpoles following treatment with norepinephrine (AMP deficient), norepinephrine followed by addition of the interspecific AMPs (interspecific AMP), or no manipulation (control).

small, however: tadpoles in the AMP deficient treatment supported 0.75 ± 0.22 parasites relative to 0.25 ± 0.123 in the AMP transfer treatment and 0.15 ± 0.082 in the control treatment (see Fig. 3).

Discussion

Our results indicated that AMPs isolated from adult bullfrogs had strong, negative effects on the survival of larval trematode cercariae. While the five trematodes species differed in their sensitivity to AMP exposure, they all showed significantly increased mortality in the presence of adult bullfrog AMPs. This finding agrees with one of the only other studies to examine the effects of adult amphibian AMPs on a human trematode. Pretzel et al. (2013) demonstrated that an AMP isolated from the treefrog *Phyllomedusa oreades* killed adult schistosome worms at concentrations similar to those used here (e.g., 50–200 μg/mL), leading to the suggestion that drug development using amphibian AMPs could help decrease burden of schistosomiasis in humans. Similarly, in an experimental study of fish, production of alarm cells containing AMPs increased upon exposure to trematodes, although whether this affected infection risk was not determined (Chivers et al. 2007).

While we observed deleterious effects of adult bullfrog AMPs on larval trematode survival, no such effects were observed for AMPs from tadpoles in either their first or second year of development, even at relatively high concentrations. It is therefore less likely that AMPs—which are also more likely to be produced among post-metamorphic rather than larval amphibians (Bovbjerg 1963; Groner et al. 2013)—play a strong role in preventing initial establishment of trematodes during amphibians' aquatic development. Nonetheless, even if unlikely to account for strong variation in infection success either among amphibian species or between aquatic stages (e.g., first vs. second year tadpoles) the patterns we observed could help explain how adult amphibians either clear encysted trematodes post-metamorphosis or fail to become infected by larval trematodes when they spend time in water, particularly for highly aquatic species. Several studies indicate that metamorphic and post-metamorphic amphibians are significantly less susceptible to trematode infection (Johnson et al. 2011; Sears et al. 2012).

Our results also indicated that tadpole age and host species identity had strong effects on trematode infection success, consistent with previous studies. Specifically, we found that second year bullfrogs were 80% less likely to get infected by *R. ondatrae* cercariae compared to first year tadpoles. This pattern has been noted with other trematode species, such as *Echinostoma* sp. Raffel et al. (2011) and Schotthoefer et al. (2003), for instance, both documented strong evidence for stage-dependent susceptibility to *Echinostoma* sp. in several species of amphibians, whereas, Holland et al. (2007) found that first-year green frog tadpoles supported significantly more metacercariae of *E. revolutum* relative to second year hosts following standardized exposures. Results of the current study are notable in their magnitude: first year tadpoles supported 30 times more metacercariae than second year tadpoles, and most hosts in the latter group failed to become infected at all. However, based on our results, this pattern of age-dependent susceptibility did not appear to be explained by AMPs, given the lack of an effect of norepinephrine exposure. This suggests that other factors, including additional immune- or behavior-mediated defenses, likely accounted for observed differences in infection.

Finally, our results showed that *H. versicolor* acquired fewer *R. ondatrae* metacercariae following standardized exposures than did *P. regilla*, despite the physical and ecological similarities of these two host species. These findings are consistent with previously published results from laboratory experiments and field surveys (Johnson and Hartson 2009: LaFonte and Johnson 2013). Interestingly, treatment with norepinephrine did increase *R. ondatrae* infection in *H. versicolor*; however, the magnitude of this effect was small compared with the overall difference in infection between the two host species. On average, exposure to norepinephrine increased the number of metacercariae per *H. versicolor* by fivefold, from 0.15 to 0.75 parasites per host; in contrast, *P. regilla* supported an average of 8.65 ± 0.483 parasites per host, or roughly 60-fold higher than *H. versicolor*, regardless of norepinephrine exposure. Given previous results showing that exposure to corticosterone causes strong increases in trematode infection in *H. versicolor* (Belden and Kiesecker 2005; LaFonte and Johnson 2013), future studies should investigate the role of alternative components of amphibian immunity in explaining such patterns, including acquired and innate immunity specifically, phagocytic cells such as macrophages and neutrophils that directly phagocytize a pathogen, natural cytotoxicity provided by natural killer cells, and splenocytes with natural killer cell activity (Carey et al. 1999).

Conclusion

These findings indicate that the effects of AMPs on trematode parasites depend strongly on the stage of the host from which they are produced. AMPs isolated from adult bullfrogs dramatically reduced the survival of infectious cercariae representing a broad range of trematode species, which could be one mechanism through which amphibians are defended against larval trematode infection post-metamorphosis (see also Johnson et al. 2011), particularly for species that are highly aquatic. For larval amphibians, however, we found little evidence that AMPs played an important role in defending against cercariae. Administration of purified AMPs from early and late stage bullfrog tadpoles had no detectable effects on the cercariae of five different trematodes in vitro, nor did norepinephrine treatment of larval bullfrogs, treefrogs, or chorus frogs to reduce their AMP defenses have any biologically meaningful effects on infection success by *R. ondatrae*. These findings suggest that other physiological and/or behavioral mechanisms are needed to explain the marked differences in trematode infection observed both among larvae of different amphibian species and between first and second year tadpoles. Additional work is also needed to explore how AMPs affect the persistence of parasites once established, including for other stages in the trematode life cycle (e.g., adults, see Pretzel et al. 2013), as well as how prior host exposure affects the composition of AMPs produced (e.g., see Tennessen et al. 2009).

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