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Resistance and tolerance to trematode parasites in larval anurans

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Resistance and Tolerance to Trematode Parasites in Larval Anurans

by

Brittany F. Sears

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy with a concentration in Ecology and Evolutionary Biology
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October 11, 2013

Keywords: amphibian, immunity, susceptibility, Lechriorchis tygarti, Renifer aniarum

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DEDICATION

I dedicate this dissertation to my family: a maternal pedigree of academics and unwavering paternal support.
ACKNOWLEDGMENTS

Gratitude is owed to my advisor, Dr. Jason Rohr, who has been an unequivocal source of support. I also thank my committee members, Drs. My Lien Dao, Marc Lajeunesse, and Christina Richards for their guidance and expertise. Past and present members of the Rohr Lab were also sources of endless assistance and commiseration, as were Courtney Coon and Andrea Liebl.

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ABSTRACT

Nearly every species on the planet has at least one parasite, which, by definition, incurs a cost in the host. Therefore, organisms must resist parasites – preventing or reducing infections – or tolerate parasites – reducing the costs of infection – in order to maintain their fitness despite the presence of parasites. Here, I investigated: 1) whether parasitic, larval trematodes (cercariae) can detect the least resistant tadpole host species, 2) a hypothetical framework for how host life history impacts the utilization of inflammation and thus, resistance and tolerance, 3) whether a common anesthesia technique used in experimental infections immunocompromises tadpoles, 4) the relationship between tadpole host life history, tolerance, and behavioral resistance to cercarial infection, 5) how tadpole behavior affects trematode infection location, and 6) how host life history impacts trematode infection location and the implications of this for host tolerance.

In the first chapter, I investigated whether trematode cercariae could discriminate among several tadpole host species to identify the most susceptible hosts. Cercariae were consistently more attracted to *Bufo terrestris* tadpoles, which was the most susceptible host species. Other tadpole species varied in attractiveness in an order similar to their susceptibility to infection. Furthermore, there was consistent and significant variation among individual attractiveness and susceptibility within host species. If susceptibility to infection is heritable, chemical cues used by cercariae to identify susceptible hosts could represent a substrate on which natural selection acts, setting up a “Red Queen” arms race between host cues and parasite detection of those cues.

In the second chapter, I proposed a framework which outlined the cost-benefit relationship between host life history and immune responses. Because inflammatory immune responses are known to cause self-damage to hosts, anti-inflammatory immune responses should
be used for long-lived, slowly-developing ("slow-paced") hosts, those infected with relatively less virulent parasites, and/or ongoing but ineffective inflammatory responses. Conversely, the cost of inflammation might be less expensive than the cost of infection among short-lived, rapidly-developing ("fast-paced") hosts, those infected with virulent parasites, and/or those undergoing protracted but ineffective anti-inflammatory immune responses.

In the third research chapter, I investigated whether two common anesthesia agents, benzocaine and tricaine mesylate (MS-222), immunocompromise tadpoles. These chemicals are used extensively to study the behavioral resistance of tadpoles to cercariae; if treatment increases infections not only by removing these behaviors but also by suppressing the immune system, behavior might appear artificially effective at preventing trematode infection. I found that neither benzocaine nor MS-222 affected the abundance of circulating white blood cells relative to water-exposed control tadpoles. Furthermore, there was no difference in trematode infection success when tadpoles were anesthetized, allowed to recover from anesthesia, and subsequently experimentally infected. The results of this experiment indicate that benzocaine and MS-222 are both practical, non-immunosuppressive anesthesia agents to use when studying trematode infections in amphibians.

In the fourth research chapter, I quantified tadpole hosts’ use of behavioral resistance (parasite-induced behaviors) and tolerance of exposure to cercariae. Across seven host species, parasite-induced behaviors were negatively correlated with pace-of-life, with rapidly-developing ("fast-paced") tadpoles exhibiting significantly more behavior than slowly-developing ("slow-paced") tadpoles. The opposite pattern was true of tolerance, where fast-paced species had poorer tolerance of cercarial exposure than slow-paced species. Given that slow-paced species are more likely to be exposed to cercariae because they 1) occur in water more likely to harbor cercariae and 2) have longer developmental times, tolerance to trematode exposure might be an
evolutionary adaptation that circumvents the costs of behavioral – and, possibly, immunological – resistance to infection.

In the fifth research chapter, I investigated whether parasite-induced behaviors were capable of affecting encystment location of trematode cercariae in *Hyla femoralis* tadpoles and whether the resulting encystment location affected tolerance of infection. Benzocaine-anesthetized and control tadpoles had similar infection intensities. However, among benzocaine-anesthetized tadpoles, the majority of cercarial infections occurred in tadpoles’ heads, but unanesthetized control tadpoles were predominantly tail-infected. Furthermore, the number of head infections were negatively associated with mass change (poor tolerance), whereas the number of tail infections was positively associated with mass change (good tolerance). These results suggest that parasite-induced behavior is not only an important mechanism of resistance to trematodes, as other researchers have described, but also a mechanism of tolerance, whereby tadpoles can prevent the deleterious effects of trematode infection by controlling infection location.

The body of work that I have produced demonstrates that variation in resistance and tolerance to trematode parasites is ubiquitous among tadpole hosts. Furthermore, this variation is predictable based on host life history. Because tadpole life history can dramatically impact the likelihood of exposure to cercariae and encounters are necessary for host-parasite selective pressure to occur, life history can predict the adaptations of hosts and parasites. Given amphibians’ status as the most rapidly declining taxon on the planet and the ubiquity of emerging infectious diseases for amphibians and other organisms, these findings should inform future research on host- and parasite-mediated mechanisms of disease.
INTRODUCTION

Organisms face frequent life history conflicts. No host has infinite time or energy to devote to all of its life history traits (e.g., growth, reproduction, and immune responses) (Sheldon and Verhulst 1996, Zuk and Stoehr 2002), so some traits will inevitably be prioritized over others. When the risk of exposure to a parasite is predictable, investment in parasite responses should be prioritized accordingly, with high-risk hosts allocating resources differently than low-risk hosts. However, it is not clear which of the myriad classes of parasite responses (e.g., physiological vs. behavioral) should receive the greatest investment. Nor has previous research made clear how likelihood of parasite exposure affects the functional outcome of these responses, which fall broadly into two categories: 1) resistance, which reduces the intensity of an infection, and 2) tolerance, which ameliorates the effects of infection (e.g., intestinal damage) without necessarily affecting parasite numbers (Raberg et al. 2009). Thus, hosts must balance not only the magnitude of investment in overall parasite responses versus other life history traits, but within “parasite responses,” they much also balance the class of response used, the function of each class, and then balance each combination of these against one another and life history traits (e.g., behavioral resistance vs. physiological tolerance vs. growth).

Larval anurans (tadpoles) are frequently infected by parasitic, larval trematodes (cercariae), which cause fitness-impairing ailments including kidney failure (Martin and
Conn 1990) and limb deformities (Johnson et al. 1999). Tadpoles span a broad spectrum of life history strategies, ranging from “fast-paced” species, which develop rapidly and metamorphose at a small size, to “slow-paced” species, which develop slowly and metamorphose at much larger sizes. Tadpole-infecting cercariae are exclusively aquatic and, therefore, tadpole developmental rate should be strongly correlated with parasite exposure. Furthermore, the eggs of many species with fast-paced tadpoles are laid in ephemeral water that is unlikely to be inhabited by snails, the first host in the trematode life cycle, making fast-paced tadpoles highly unlikely to be exposed to cercariae. Like many animals, tadpoles can resist cercarial infections with immunological responses (Kiesecker 2002). Tadpoles also utilize behavioral resistance, which can consist of avoidance (Hart 1994, Rohr et al. 2009) of infected snails and/or cercariae, as well as a stereotyped “anti-parasite behavior,” in which rapid swimming behaviors can dislodge cercariae and prevent infections (Johnson et al. 2001, Taylor et al. 2004, Koprivnikar et al. 2006). It is less clear, however, which classes of responses contribute to tolerance of trematode parasites in tadpole hosts.

Because length of larval period should result in predictable exposure to trematode cercariae, pace of life should result in consistent differences in resistance and tolerance to cercariae. Fast-paced tadpoles, which rarely encounter cercariae, might have some level of behavioral resistance, but probably invest little in the cells and tissues necessary to maintain immunological resistance and tolerance (Lee 2006). Behavioral resistance carries the advantage of being relatively cheap compared to immunological resistance because it requires no specialized tissues and is inducible. Thus, tadpoles only need to engage these behavioral responses in the presence of cues from parasites. Likewise, if
behaviors can also contribute to tolerance, behavioral tolerance could be relatively cheaper than immunological tolerance, which does require specialized cells and tissue (Raberg et al. 2009). However, fast-paced tadpoles’ encounters with cercariae should be relatively rare compared to those of slow-paced tadpoles, which might prevent the selective pressures necessary for the evolution of these responses from occurring in fast-paced species. Conversely, slow-paced tadpoles should regularly encounter cercariae and therefore are more likely to experience selective pressure for coping with cercariae. Because behavioral resistance could be energetically expensive or conspicuous to predators where parasites are abundant, slow-paced tadpoles might instead invest in immunological resistance and tolerance. Indeed, tadpoles tend to reduce their behavior in the presence of predators, even when parasites are also present (Skelly and Werner 1990, Thiemann and Wassersug 2000, Szuroczki and Richardson 2012).

Very little experimental work has addressed resistance and tolerance as separate, measurable responses to infectious disease (but see Raberg et al. 2007 and Ayres and Schneider 2008), despite the important implications for disease ecology. Disease models frequently assume random transmission probabilities (Grenfell and Keeling 2007), but tolerant hosts may prove to be “superspreaders” (Lloyd-Smith et al. 2005) and sources of spillback, in the case of invasive species (Lee and Klasing 2004). Such work is especially timely in amphibian systems given their precipitous decline (Stuart et al. 2004) and the role of infectious diseases in these declines (Lips et al. 2006, Rohr et al. 2008). Here, I investigated: 1) whether parasitic, larval trematodes (cercariae) can detect the least resistant tadpole host species, 2) a hypothetical framework for how host life history impacts the utilization of inflammation and thus, resistance and tolerance, 3) whether a
common anesthesia technique used in experimental infections immunocompromises tadpoles, 4) the relationship between tadpole host life history, tolerance, and behavioral resistance to cercarial infection, and 5) how tadpole behavior affects trematode infection location.

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CHAPTER 1: Do parasitic trematode cercariae demonstrate a preference for more susceptible host species?

Note to Reader:
This chapter has been previously published: Sears, B.F., Schlunk, A., Rohr, J.R. Do parasitic trematode cercariae demonstrate a preference for more susceptible host species? PLoS ONE 7(12):e51012. See Appendix A for the PDF of the published, open-access document.
CHAPTER 2: The economy of inflammation: when is less more?

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This chapter has been previously published: Sears, B.F., Rohr, J.R., Allen, J.E., Martin, L.M. The economy of inflammation: when is less more? Trends in Parasitology 27(9):382-387. See Appendix B for the PDF of the published document and Appendix C to see the permission from the publisher.
CHAPTER 3: No effects of two anesthetic agents on circulating leukocyte counts or resistance to trematode infections in larval amphibians

Note to Reader:
This chapter has been previously published: Sears, B.F., Snyder, P.W., Rohr, J.R.. No effects of two anesthetic agents on circulating leukocyte counts or resistance to trematode infections in larval amphibians. Journal of Herpetology 47(3):498-501. See Appendix D for the PDF of the published document and Appendix E to see the permission from the publisher.
CHAPTER 4: Host life history predicts behavioral resistance and tolerance to parasites in a tadpole-trematode system

AUTHORS
BF Sears, Snyder PW, Rohr JR

ABSTRACT
Host responses to parasites can be broadly categorized as contributing to resistance, which reduces the intensity of infection, and/or tolerance, which mitigates deleterious effects of infection. Tadpoles use both immunological and behavioral modes of resistance and tolerance to cope with parasitic larval trematodes (cercariae). Because cercariae are aquatic, differences in the duration of the aquatic stage of tadpole species should result in differential exposure to cercariae and thus, differential selection on resistance and tolerance. We exposed seven tadpole species to cercariae and recorded tadpole behavior (behavioral resistance), attempted but unsuccessful infections (behavioral plus immunological resistance), and tadpole mass change as a function of parasite exposure (tolerance) for both benzocaine-anesthetized and non-anesthetized tadpoles. Variation in parasite-induced behaviors and tolerance were predictable based on life histories of tadpole species, with rapidly-developing species exhibiting greater increases in behavioral resistance but poorer tolerance than slowly-developing species. These results suggest that species with short larval periods, which experience comparatively few trematode exposures, might utilize inducible, behavioral resistance more than immunological resistance. This trade-off could
result in the allocation of resources to metamorphosis-promoting traits (i.e., growth and development) rather than immunological traits in rapidly-developing, poor tolerance tadpoles, which would consequently reduce exposure to aquatic trematodes.

INTRODUCTION

Organisms can vary widely in their life histories. Divergence among classical life history traits, including longevity and the timing of reproduction, can affect – or be affected by – hosts’ responses to parasites (Zuk and Stoehr 2002). Broadly, host response strategies to parasites have been categorized into resistance and tolerance. Resistance reduces the intensity of an infection, whereas tolerance mitigates the deleterious effects of parasites (Miller et al. 2005). Resistance and tolerance can be achieved through multiple mechanisms, including immunological (Raberg et al. 2009), behavioral (Sears et al. 2013b), and chemical means (Kreil 1994). Given that the costs and benefits of a particular response can be dependent on life history (Ricklefs and Wikelski 2002), it might be possible to make predictions about relative investment in resistance and tolerance based on life history differences among hosts species.

Tradeoffs between life history traits and immunological responses have been previously hypothesized (Lee 2006; Schmid-Hempel and Ebert 2003; Sears et al. 2011). “Pace of life,” the correlation between development, longevity, and reproductive rate (Ricklefs and Wikelski 2002), can result in selection for different suites of parasite-responsive traits (e.g., Cronin et al. 2010). “Fast-paced” species, which develop and reproduce rapidly but also have high mortality, might use constitutive responses because they simply lack time to invoke more long-term, inducible responses (Lee 2006). Conversely, “slow-paced” species, which take longer to mature, reproduce slowly, but have greater longevity, could reap more benefits from specific, inducible responses.
Such parasite-specific responses could carry the benefit of causing less immunopathology, or collateral self-damage, than more general mechanisms of resistance (e.g., immunological memory vs. inflammation, respectively; Graham et al. 2005). In addition to the prioritization of constitutive versus inducible responses, life histories could predictably influence the prioritization of immunological resistance versus tolerance (Sears et al. 2011). However, despite these and other hypotheses regarding immunology and life history, it is poorly understood how non-immunological resistance and tolerance might vary with life history.

Indeed, many mechanisms of resistance are independent of immunological responses, including behavior that prevents infection or reduces its intensity. For instance, animals can avoid infected conspecifics (Kiesecker et al. 1999) or microhabitats where infections could be contracted (Hutchings et al. 2001; Kiesecker and Skelly 2000; Rohr et al. 2009). Furthermore, hosts might prevent or reduce infections with behaviors that physically remove parasites, such as grooming of ectoparasites. Costs of immunological responses, including development and usage costs (e.g., Martin et al. 2007; Kopp and Medzhitov 2009), could be mitigated by using behavioral resistance. However, behavior is not without its own costs and repeated use should accrue costs to host fitness in a manner similar to immunopathology. Behavioral resistance can result in increased metabolism (Giorgi et al. 2001), decreased foraging rate (Hutchings et al. 2001), and reduced brood size (Vandame et al. 2002). Given that tolerance of infection is often measured as the ability to maintain mass or fecundity despite infection, these costs of behavioral resistance might act in concert with parasites that tend to exert similar costs (e.g., parasite-induced weight loss or fecundity reduction). Thus, behavioral resistance could be traded-off against tolerance, with robust behavioral resistance predicting poor tolerance of infection.
Anuran larvae (tadpoles) are ideal for addressing relationships among pace of life, behavior, resistance, and tolerance because species differ in their life history, susceptibility to parasites, and behavior. Tadpoles can differ dramatically in their rates of development, ranging from two weeks for the fastest species to three years for the slowest (Lannoo et al. 2005). In experimental infections, rapidly-developing tadpoles are less resistant to trematode infections than slower-developing species (Johnson et al. 2012; Rohr et al. 2010; Sears et al. 2012). This correlation between developmental rate and resistance suggests that rapidly-developing tadpoles invest heavily in different life history traits, such as growth, which are traded off against parasite resistance. In the presence of trematode cercariae (a larval stage of parasitic trematodes), species’ tadpoles also differ in their use of a form of behavioral resistance, which consists of rapid swimming with many directional changes (Taylor et al. 2004) and reduces trematode infection relative to anesthetized (i.e., non-motile) conspecifics (Daly and Johnson 2011; Koprivnikar et al. 2006). However, there is wide variation in the utilization of parasite-responsive behavior. For example, the rapidly-developing tadpoles of *Bufo* spp. undertake more behavior in response to trematodes than those of other taxa (Johnson et al. 2001; Taylor et al. 2004) but slower-developing *Rana* spp. are less active in the presence of cercariae than in their absence (Thiemann and Wassersug 2000). Exposure to cercariae should be strongly correlated with length of larval period because cercariae are almost exclusively aquatic; thus, length of larval period should result in differential selection on parasite response strategies. Behavioral resistance might represent a practical defense for rapidly-developing tadpoles that is low-cost because it does not need to be maintained in the absence of trematodes and requires no specialized cells or tissues.

We conducted a laboratory experiment using tadpoles and parasitic trematode cercariae to test whether parasite-coping strategies for were predictable based on pace of life. We exposed
tadpoles of seven anuran species, which differ considerably in their life histories (Table A1; listed from fast to slow development): *Scaphiopus holbrookii* (Eastern spadefoot toad), *Osteopilus septentrionalis* (Cuban treefrog), *Gastrophryne carolinensis* (narrowmouth toad), *Hyla femoralis* (pinewoods treefrog), *Pseudacris ocularis* (little grass frog), *Hyla gratiosa* (barking treefrog), and *Rana catesbeiana* (bullfrog), to a range of armatae trematode cercariae doses and quantified their behavioral responses and resulting infection intensity as measures of resistance. Because every tadpole did not become infected (some were completely resistant at the focal doses and exposure durations), but cercariae can wound hosts in the absence of successful infection (i.e., a cost of exposure; Rohr et al. 2010), tolerance was quantified as mass change in response to cercarial dose. We hypothesized that trematode-coping strategies in anuran tadpoles are aligned along a life history axis, with rapidly-developing species investing heavily in behavioral resistance and more slowly-developing species investing more heavily in tolerance, whereby resources are allocated to mitigating the costs of exposure and infection rather than resisting the infection altogether.

**MATERIALS AND METHODS**

**Natural history**

The first host in the life cycle of trematodes is a mollusc, which sheds free-swimming cercariae that detect hosts using physical and chemical cues (Combes *et al.* 1994). The armatae cercariae used in this experiment are shed from freshwater snails (*Planorbella trivolvis*), attach to tadpoles’ skin, and use a pointed stylet and proteolytic enzymes to encyst subcutaneously as metacercariae (Schell 1970). Because of this traumatic infection process, cercariae can wound the host even in the absence of successful encystment, which can reduce fitness (Rohr *et al.*
Once an infected tadpole is consumed by the appropriate definitive host, the metacercariae excyst and develop into adult trematodes, which pass eggs into the feces of the definitive host. Ciliated miracidia hatch from the eggs, which in turn infect molluscs.

For this experiment, cercariae were obtained from *P. trivolvis* collected from a wetland in Tampa, Florida (28.164581,-82.31202). Snails were inspected under a dissecting microscope for the presence or absence of free-swimming armatae cercariae (Schell 1970). These cercariae have been identified as belonging to the family Plagiorchiidae, subfamily Reniferinae (Sears and Rohr 2013); although they have not been identified to species, reniferin trematodes are highly conserved in their host use, infecting freshwater snails, tadpoles, and water snakes as their first intermediate, second intermediate, and definitive hosts, respectively. Infected snails were housed in artificial spring water (ASW; Cohen *et al.* 1980) and fed frozen spinach *ad libitum*.

Tadpoles were collected from wetlands in Tampa, FL and all collection sites were sampled for snails to prevent prior trematode infection from influencing behavioral and immunological responses. Tadpoles exposed to zero cercariae (n=84) were verified to be trematode-free after the experiment, indicating that they were not naturally infected prior to collection. Prior to experimentation, tadpoles were housed in 37 liter aquaria filled with ASW, which was constantly cycled through a carbon filter. Tadpoles were fed frozen spinach *ad libitum*.

**Behavioral resistance**

Tadpoles were divided into two anesthesia treatments: 0.001% benzocaine (anesthesia) and ASW (control). The concentration of benzocaine used here has been demonstrated to have no side-effects on white blood cell counts and does not affect encystment success by armatae cercariae (Sears *et al.* 2013a). Prior to parasite exposure, tadpoles were weighed and placed individually in
plastic specimen cups with 30mL of either 0.001% benzocaine solution or ASW for 10 minutes (modified from Koprivnikar et al. 2006; Fig. A1). This exposure to benzocaine is sufficient to induce a minimum of 10 minutes of immobility once tadpoles are transferred from the solution to water. After anesthetic or ASW exposure, tadpoles were then collected in a small net, rinsed with ASW to remove any residual anesthetic, and placed individually in a plastic cup with 30 mL of ASW and 0, 10, 15, 20, or 30 cercariae (n=6 tadpoles per cercarial dose for *O. septentrionalis, G. carolinensis, H. femoralis, H. gratiosa, and R. catesbeiana*; n=3 tadpoles per dose for *P. ocularis* and *S. holbrooki*). Cercariae were collected directly from a specimen cup containing infected snails in ASW using a micropipette and dissecting microscope. Because hours-old cercariae are more infective than freshly-shed or several-hours-old cercariae (Fried et al. 1997), only 1- to 6-hours-old cercariae were used. Each tadpole was exposed to the assigned cercarial dosage for 10 minutes. Tadpoles were videotaped from above using a fixed-mount camera during the exposure period.

After cercarial exposure, tadpoles were rinsed with ASW to remove any attached cercariae that had not yet penetrated the tadpole’s skin. The rinse water was conserved for quantification of those cercariae. Tadpoles were then transferred to a 1 L aquarium. For all tadpole species except *O. septentrionalis, G. carolinensis, and P. ocularis*, after the tadpole was transferred to its 1 L aquarium, both the cercarial exposure water and rinse water were pooled and stained with several drops of Lugol’s iodine, which killed and stained any remaining cercariae. The number of cercariae remaining was subtracted from the total cercariae administered to calculate the number of “attempted infections.”

After parasite exposure, tadpoles were maintained in 1 L of ASW, fed frozen spinach *ad libitum*, and monitored for mortality daily. Water was changed four days after cercarial exposure.
Seven days after cercarial exposure, tadpoles were weighed and euthanized in a 0.1% benzocaine solution. Tadpoles not surviving through this seven day period were weighed and preserved immediately after death. Three of the six tadpoles from each cercarial dosage were preserved in 70% ethanol, whereas the other three were stored in RNA Later (Ambien, Inc.) for utilization in a separate study. RNA Later-preserved tadpoles thus contributed behavior and attempted infection data, but not infection intensity data.

*Quantifying metacercarial cysts (resistance)*

To make encysted metacercariae visible, ethanol-preserved tadpoles were cleared according to Hanken and Wassersug (1981). In brief, vials containing tadpoles received daily additions of hydrogen peroxide (30%) in 1% increments of the total ethanol volume, until all color was bleached. Once colorless, specimens were transferred to a glycerol/KOH solution to clear until transparent. Metacercariae were counted in cleared specimens at 100x magnification under a compound microscope.

*Cercarial infectivity*

Exposure of tadpoles to cercariae for 10-20 minutes has resulted in metacercarial infections in other tadpole-trematode systems (Daly and Johnson 2011; Koprivnikar et al. 2006). However, we anticipated that 10 minute exposures in our experiments might be too short a duration to consistently result in infections. If cercariae were unable to infect tadpoles because they are incompatible hosts, this phenomenon could appear to demonstrate absolute resistance in tadpole hosts. To confirm that cercariae were capable of infecting tadpoles, a separate cohorts of *O. septentrionalis* and *G. carolinensis* tadpoles (n=30) were exposed to cercariae from the same population of infected snails for 24 hours. Among this cohort, 33% of *O. septentrionalis* and
50% of *G. carolinensis* became infected. Thus, we are confident that the cercariae used in this experiment can successfully infect tadpoles, given sufficient time.

*Statistical analyses*

We conducted two tests for a phylogenetic signature in our traits using the fitContinuous function in the geiger package of R statistical software (see App. B for details). Regardless of the test, there was no indication of a phylogenetic signature. For all tested traits, sample-size-corrected Akaike information criteria was lowest for the white-noise or non-phylogenetic model of evolution. These findings indicated that a statistical correction for phylogeny was unnecessary.

All behavioral analyses were performed in JWatcher (Blumstein et al. 2006) by a single observer. Swimming behavior was scored as either normal (slow pace, no directional changes; Appendix C), angled (swimming faster than normal and at an angle to the container with few directional changes; Appendix C), or evasive (explosive swimming with many, rapid directional changes; Appendix D). Normal and angled swimming were scored as continuous states within JWatcher whereas evasive maneuvers were scored as discrete events because of their very short duration. We tested for the effects of species and cercarial dose (a continuous predictor) on the proportion of time spent swimming (normal plus angled; arcsine square-root transformed), the proportion of time spent angled swimming (arcsine square-root transformed), and the number of evasive events per minute (log transformed).

We used analysis of variance (ANOVA) to test whether species or anesthesia treatment affected resistance in two ways: 1) the attempted infections and 2) the number of successful metacercarial infections. In non-anesthetized tadpoles, the number of cercariae attempting infections should be governed by tadpoles’ attractiveness to cercariae (Sears et al. 2012) in
conjunction with the efficacy of tadpole behavior at preventing contact with cercariae. That is, both unattractive and highly active species should have fewer attempted infections by cercariae than attractive and/or less active species. Fisher’s post-hoc tests were used to detect treatment- and species-level differences. We used general linear models (GLM) to test for effects of anesthesia, species, and cercarial dose (a continuous predictor) on tolerance (log-transformed percent mass change per day). We used daily percent mass change to control for size differences between species and different durations of tadpole survival.

We hypothesized that larval period duration (days to metamorphosis) of the tested species would be 1) a negative predictor of the slope of the relationship between cercarial exposure and the amount of parasite-responsive behaviors. Thus, rapidly-developing species should exhibit larger increases in behavior in the presence of trematodes than slowly-developing species. 2) Larval period should be a positive predictor of tolerance (the slope of the relationship between cercarial exposure and mass change). Thus, slowly-developing species should exhibit less mass loss in response to cercarial exposure. Although immunological tolerance can be traded-off against immunological resistance (Graham et al. 2010; Raberg et al. 2007), we predicted that those tadpoles using resistance would use behavioral resistance, so we did not predict any significant trends between immunological resistance and larval period duration.

To test these two hypotheses, we regressed the rank order of larval period duration of the tested species (based on minimum time to metamorphosis [Table A1]) against each species’ slope coefficients for behavior and tolerance, weighting by the inverse of the standard errors of the slope; therefore, more weight was given to slopes with less error. Thus, these analyses use the species as the replicate and incorporate the error of the slope parameter. The rank order of time to metamorphosis is a useful proxy for selective pressures of trematodes because longer
larval periods might equate to greater risk of exposure to aquatic cercariae and thus, stronger
selective pressures for adaptations to aquatic parasites. Furthermore, a previous study found that
metamorphic traits (time to and size at metamorphosis) were better predictors of resistance and
tolerance of trematode infections than traits of amphibian reproduction (egg size, clutch size) and
adult life history (maximum age and size, age at maturity; Johnson et al. 2012). All statistical
analyses were performed in Statistica v9 (StatSoft 2009).

RESULTS

Resistance to trematode cercariae

Both cercarial dose \( (F_{1,128}=31.8, P<0.0001) \) and the species x cercarial dose interaction
\( (F_{5,128}=14.2, P<0.0001) \) were significant predictors of evasive behavior, with the rate of evasive
maneuvers per minute increasing significantly as a function of cercarial exposure in \( R. \)
catesbeiana, \( H. \) femoralis, and \( O. \) septentrionalis (Fig 1A). Larval duration was not a significant
predictor of the relationship between cercarial dose and evasive maneuvers (Fig 1A), but evasive
maneuvers were rare relative to overall swimming and angled swimming. Larval period duration
was a significant, negative predictor of the relationship between cercarial dose and total time
spent swimming (normal + angled) as well as angled swimming alone (Fig. 1B,C), with faster-
developing species exhibiting a greater increase in angled swimming as a function of cercarial
dose.

Only three of the seven species exposed to cercariae became infected: \( O. \) septentrionalis,
\( H. \) gratiosa, and \( H. \) femoralis. Among these three species that were infected, species and the
species x anesthesia interaction were significant predictors of infection intensity (species:
\( F_{6,158}=25.2, P<0.001 \); interaction: \( F_{6,158}=3.13, P<0.01 \) ) and prevalence (species: \( F_{6,158}=16.9, \)
Both prevalence and intensity patterns were driven by *H. femoralis*, which, when anesthetized, carried significantly higher infection intensities than *H. gratiosa* or *O. septentrionalis* and were universally infected when anesthetized (Fig A2, A3).

**Attempted infections by cercariae**

A higher proportion of cercariae attempted to infect anesthetized than non-anesthetized conspecifics ($F_{1,148}=5.78, P=0.017$). There was a significant interaction between anesthesia treatment and host species for the proportion of cercariae attempting to infect tadpoles ($F_{3,148}=4.88, P=0.003$); more cercariae attempted to infect both anesthetized *H. femoralis* ($P<0.001$) and *S. holbrookii* ($P=0.029$) relative to non-anesthetized conspecifics, whereas anesthetized and non-anesthetized *H. gratiosa* and *R. catesbeiana* were equally subject to infection attempts. Thus, within the anesthetized treatment, there were interspecific differences in attempted infections, but not among species within the control treatment (Fig 2).

**Tolerance to parasite exposure**

In general, hosts suffered mass loss after cercarial exposure and the number of cercariae to which a host was exposed was inversely associated with mass change (Table 1). Furthermore, species identity was a significant predictor of mass change post-infection, as was the interaction between species and cercarial dosage ($F_{6,301}=26.96, P<0.001$; $F_{6,301}=3.745, P=0.001$; Table 1). There was no significant effect of anesthesia alone, but there was a significant interaction between cercarial dose and anesthesia that resulted in anesthetized, but not control animals, losing significantly more mass when exposed to parasites than when unexposed ($F_{1,301}=7.65, P=0.006$; Fig 3A). There was a significant interaction between species and use of angled swimming behaviors (log angled bouts/min) on mass change ($F_{6,158}=2.66, P=0.012$; data not shown); generally, angled swimming was positively correlated with tolerance, but tolerance was
negatively correlated with angled swimming in *O. septentrionalis* and *H. gratiosa*. As postulated, the duration of the larval period was a significant positive predictor of tolerance of cercarial dose, with slowly-developing species exhibiting higher tolerance than rapidly-developing species \( (F_{1,5}=5.45, P=0.033; \text{Fig } 3\text{B}). \) Differences in swimming behaviors among species also correlated to variation in species’ tolerance to exposure. The relationship between cercarial dose and time spent swimming was a significant negative predictor of tolerance (Fig 4A), as was the relationship between cercarial dose and time spent angled swimming (Fig 4B). The relationship between cercarial dose and evasive behaviors, however, was not a significant predictor of tolerance to cercarial exposure (Fig 4C).

**DISCUSSION**

Among seven larval amphibian species, normal, angled, and evasive behaviors tended to increase as a function of exposure to armatae trematode cercariae, and both normal and angled swimming were negatively correlated with the duration of the larval period of the host species (Fig 1B,C). Thus, consistent with our hypothesis, rapidly-developing host species exhibited greater increases in parasite-responsive behaviors as a function of parasite dose than slowly-developing host species. Evasive behavior, however, did not significantly co-vary with larval duration (possibly because of the relative rarity of this behavior; Fig 1A). Other researchers have examined evasive behavior (Taylor et al. 2004), general activity (Daly and Johnson 2011; Koprivnikar et al. 2006), or both activity and avoidance responses of tadpoles to trematode cercariae (Rohr et al. 2010), but none have individually examined separate behaviors which collectively comprise parasite-responsive behaviors. Thus, our findings are novel, suggesting that tadpoles’ behavioral resistance against cercariae is comprised of many behaviors which
increase in the presence of cercariae and that these behaviors carry costs and benefits that affect their utilization in a life history-dependent manner.

Although researchers have demonstrated that behavior is effective at reducing cercarial infections in several tadpole species (Daly and Johnson 2011; Koprivnikar et al. 2006), our design prevented most cercarial infections. Our data on attempted infections, however, is consistent with these previous studies and suggests that behavior of rapidly-developing species (H. femoralis, S. holbrookii) was indeed effective at limiting the number of cercariae making contact with tadpoles (Fig 2). There was no indication that behavior affected infection attempts for less-active, slow-paced species (H. gratiosa, R. catesbeiana; Fig 2). There was no difference in attempted infections among control animals (i.e., all species were contacted by equal numbers of cercariae), but interspecific differences did emerge among anesthetized animals, with significantly more cercariae attempting to infect H. femoralis and S. holbrookii than H. gratiosa and R. catesbeiana. These differences among anesthetized species can be interpreted as differences in the attractiveness of each species to cercariae. Indeed, Sears et al. (2012) demonstrated that cercariae tend to be attracted to the least resistant host species; in that experiment, the fastest-paced species (Bufo terrestris) was both the most attractive and most susceptible to infection. Thus, in the current experiment, the high rate of attempted infections in anesthetized H. femoralis and S. holbrookii suggests that these host species are more attractive to cercariae than H. gratiosa and R. catesbeiana. A similar pattern was not observed when these species were not anesthetized, indicating that their anti-parasite behaviors were effective at counteracting their inherent attractiveness to cercariae.

In accordance with our second prediction, we demonstrated a positive correlation between larval duration and tolerance to trematode exposure (Fig 3B). This pattern, coupled with
the negative relationship between behavior and larval duration (Fig 1B,C), suggested a negative relationship between the utilization of parasite-responsive behaviors and tolerance. Therefore, we also tested the correlations between behavior-cercarial dose relationships and the mass change-cercarial dose relationship. When species identity was removed, total time spent swimming as well as time spent angled swimming were significant, negative predictors of tolerance to parasite exposure (Fig 4A,B). Although behavior was generally negatively related to tolerance across species in this experiment, behavior can contribute to tolerance of infection within species. In *H. femoralis*, behavior (as compared to the absence of behavior in anesthetized conspecifics) resulted in significantly more trematode infections in the tail than in the head, increasing tolerance of the infections relative to the anesthetized, predominately head-infected tadpoles (Sears et al. 2013b). Because so few tadpoles were infected in the present study, however, we are unable to assess behaviorally-influenced encystment location as a mechanism of behavioral tolerance within species.

A shortcoming of including exposure dose rather than patent infections in a reaction norm for tolerance is the inability to assess tolerance to actual infections. However, parasite dose is a useful component of tolerance when working with trematode cercariae because cercarial-induced wounds can be costly, even in the absence of infection (Rohr et al. 2010). Indeed, despite the general absence of successful infections, anesthetized, parasite-exposed tadpoles gained mass more slowly than non-parasite-exposed tadpoles (Fig 3A). The absence of this trend among non-anesthetized tadpoles might be due to the higher contact rate of cercariae with some species when anesthetized (Fig 2). Given that the chief mechanisms of tolerance might be resource-intensive immunological processes such as wound-healing (Allen and Wynn 2011) and regulation of self-damaging inflammation (Raberg et al. 2009; Sears et al. 2011), it is not
surprising that wounds in the absence of infection should invoke costs among cercariae-exposed tadpoles. Because wound-healing and anti-inflammatory responses can take days to manifest, such processes might not be feasible for rapidly-developing tadpoles, for which these days could constitute a substantial fraction of the larval period. Thus, poor tolerance in these species might maximize fitness by relieving them of the developmental costs of tolerance and allowing tadpoles to achieve metamorphosis as rapidly as possible. Even in the absence of cercariae, minimizing delays to metamorphosis promotes fitness (Blouin 1992; Chelgren et al. 2006); when cercariae are present, rapid metamorphosis would also reduce aquatic cercarial exposure, which could further prevent loss of fitness.

Ultimately, better tolerance of trematode parasites in slowly-developing tadpoles might be explained by the stronger selective pressures imposed in these species because they are more likely to encounter cercariae. Rapidly-developing tadpoles tend to inhabit ephemeral wetlands which are snail-free, and thus cercariae-free. Slowly-developing species, however, are more likely to inhabit permanent, snail-inhabited water. Furthermore, even if rapidly- and slowly-developing tadpoles occupied the same habitat where cercariae were present, rapidly-developing species would have reduced cercarial exposure by virtue of their short larval periods. These disparities in life history and likelihood of parasite exposure probably result in selection among slowly-developing tadpoles for tolerance and against parasite-induced behavior because it would become too expensive in terms of energetic costs and/or behavioral conspicuity to predators to utilize strong behavioral resistance.

Given that parasitism is a nearly universal phenomenon, most hosts likely balance the cost of coping with parasites against other life history investments (e.g., Sheldon and Verhulst 1996; Graham et al. 2010). However, we have only recently begun to appreciate the relationship
between pace-of-life syndromes and defense against parasitism (Johnson et al. 2012; Martin et al. 2007). These results provide further evidence that the life history of hosts can predict investment in behavioral and immunological resistance and tolerance.

ACKNOWLEDGMENTS
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LITERATURE CITED


TABLES AND FIGURES

Table 4.1. Statistical results for the effects of anesthesia, tadpole species, and cercarial dose on tadpole mass change per day (log transformed).

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Fig 4.1. Relationship between cercarial dose and 3 swimming behaviors in 7 tadpole species: A) anti-parasite evasions per minute, B) total time spent swimming, and C) angled swimming. Species are arranged from fast to slow developmental rate, left to right. Each point is the standardized slope (± 1 SE) of the behavior as a function of exposure to 0-30 trematode cercariae. Asterisks indicate slopes that are significantly different from zero. Statistics are for the regression between the rank order of the pace-of-life of the species and the slope of each behavior as a function of cercarial dose, weighting by the inverse of the standard error of each slope parameter.
Fig 4.2. Mean proportion of cercarial dose attempting to infect tadpoles (i.e., not recovered after exposure to tadpole hosts) of 4 anuran species. Species are arranged by developmental rate, from slow (left) to fast (right). Asterisks indicate significant differences within a species between anesthesia treatments, whereas anesthetized species not sharing a letter are significantly different. There were no significant differences between species in the control treatment so no letters are shown. Error bars represent standard error.
Figure A:

- Mass change/day (back-transformed %)

- Benzocaine
- Control

$F_{1,301} = 7.65, p = 0.006$

Figure B:

- Tolerance to cercarial exposure

- Species: S. holbrookii, O. septentrionalis, G. carolinensis, H. femoralis, P. ocularis, H. gratiosa, R. catesbeiana

$F_{4,5} = 5.45, p = 0.033$
Fig 4.3. Tolerance of 7 anuran species exposed to trematode cercariae. A) Tolerance to cercarial exposure based on anesthesia treatment. Each point is the average (±1 SE) of the percent daily mass change of all 7 species. B) Tolerance to cercarial exposure based on species. Each point is the standardized slope (± 1 SE) of mass change as a function of exposure to 0-30 trematode cercariae. Species are arranged by developmental rate, from slow (left) to fast (right). Asterisks indicate slopes that are significantly different from zero and statistics listed are for the regression between the rank order of the developmental rate of the species and the slope of mass change as a function of cercarial dose, weighting by the inverse of the standard error of each slope parameter.
Fig 4.4. Tolerance to cercarial exposure as a function of rate of change in 3 swimming behaviors among 7 tadpole species: A) total time spent swimming, B) angled swimming, C) anti-parasite evasions per minute. Species are arranged from slow to fast developmental rate, left to right. Tolerance is defined as the slope for the relationship between cercarial exposure and mass change. Each point is the standardized slope (±1 SE) of mass change and each anti-parasite behavior (±1 SE) as a function of exposure to 0-30 trematode cercariae.
CHAPTER 5: Infection deflection: hosts control parasite location with behavior to improve tolerance

Note to Reader:

This chapter has been previously published: Sears, B.F., Snyder, P.W., Rohr, J.R. Infection deflection: hosts control parasite location with behavior to improve tolerance. Proceedings of the Royal Society B 280:20130759. However, the publisher does not permit the reproduction of their full print-set version of the article.

AUTHORS

Sears, B.F., Snyder, P.W., Rohr, J.R.

ABSTRACT

Anti-parasite behaviour can reduce parasitic infections, but little is known about how such behaviours affect infection location within the host’s body and whether parasite distribution ultimately affects tolerance of infection. To assess these questions, we exposed both anesthetized (no behaviour) and non-anesthetized *Hyla femoralis* tadpoles to plagiorchiid cercariae (larval trematodes) and quantified resistance, tolerance (relationship between mass change and infection intensity), and encystment location. Non-anesthetized tadpoles had significantly more infections in their tail region than anesthetized tadpoles, which had the majority of their infections in the head. This pattern indicates that parasites preferred to infect the head, but that hosts shunted
infections to the tail when possible. Furthermore, there was a significant effect of encystment location on tolerance, with head-infected tadpoles having poorer tolerance to infection than tail-infected tadpoles. Variance partitioning suggests that, among infected tadpoles, behaviour contributed more to tolerance than resistance. These results suggest that, in addition to using behaviour to resist parasites, *H. femoralis* tadpoles also use behaviour to enhance infection tolerance by deflecting infections posteriorly, away from their vital sensory organs. These findings highlight the need to assess how widespread and important behaviour is to the tolerance of infections.

INTRODUCTION

Nearly every organism has at least one parasite, but hosts vary in their strategies for coping with infections. These strategies can be broadly characterized as belonging to one of two categories: 1) resistance, which entails preventing or reducing infections, and 2) tolerance, which entails mitigating deleterious effects of infection as a function of infection intensity (e.g., weight loss). Although immunology is a frequent subject of resistance and tolerance research, these parasite-coping strategies can also be mitigated behaviourally. Animals can avoid infected conspecifics (Kiesecker *et al.* 1999) or microhabitats where infections could be contracted (Hutchings *et al.* 2001; Kiesecker & Skelly 2000; Rohr *et al.* 2009). Furthermore, hosts might prevent or reduce infections with behaviors that physically remove parasites, such as grooming (i.e., removal) of ectoparasites (Currie & Stuart 2001; Mooring *et al.* 2004).

Tadpoles are susceptible to infection by cercariae, a motile, free-living, aquatic larval stage of parasitic trematodes. Tadpoles are capable of behavioural resistance to these parasites, both avoiding cercariae (Rohr *et al.* 2009) as well as exhibiting stereotyped anti-parasite behaviour,
characterized by rapid swimming with many directional changes (Taylor et al. 2004). Both of these behaviours can reduce prevalence (Koprivnikar et al. 2006) and intensity of infection (Daly & Johnson 2011) relative to tadpoles that have been anesthetized, suggesting that these behaviours are successful forms of resistance. Although a few taxa of trematodes specialize in infecting the inguinal region of tadpoles (e.g., *Echinostoma* spp., *Riberoia ondatrae*; Taylor et al. 2004), many are generalists in their distribution on the bodies of tadpoles and encyst subcutaneously on tadpole hosts. This poses an interesting scenario in which all infections by a generalist trematode might not be equal: infections near vital organs might be much more costly than infections far from vital organs and therefore result in relatively poorer tolerance.

If the location of parasites on the host’s body affects tolerance to infection, selective pressures might exist for hosts not only to avoid infection, but to prevent infection from occurring in the most expensive body locations. Using behaviours to prevent infections is often referred to as behavioural resistance and thus, using behaviour to minimize infections in the most costly body locations might naturally be referred to as behavioural tolerance. The relative strength of behavioural resistance versus tolerance could be measured by quantifying both the body location and cost of infections for hosts that can and cannot exhibit behaviours. For example, a host exhibiting behavioural resistance would only use behaviours to reduce infections and would not reduce the relative probability of infections in the most costly body locations. Assuming the same exposure to parasites, a host exhibiting only behavioural tolerance would have the same number of infections when they can and cannot exhibit behaviours, but would have fewer infections in the most costly body locations when exhibiting behaviours. Statistically, multiple regression models should be able to partition the independent contributions of behavioural resistance and tolerance to fitness. These models would have the number of
parasites resisted (behavioural resistance) and the proportion of parasites in the least costly body location (behavioural tolerance) as predictors of a fitness proxy.

In this experiment, we exposed anesthetized and non-anesthetized tadpoles of *Hyla femoralis* to plagiorchiid, armatae cercariae (encystment generalists) and recorded encystment location, resistance, and tolerance of infection (mass change) in each treatment. We tested two predictions based on the hypothesis that hosts should avoid infection in costly body regions: 1) infections in the head region of tadpoles, because of the proximity to vital sensory organs, should result in poorer tolerance than infections in the tail, which lacks vital organs and is lost during metamorphosis, and 2) tadpoles capable of exhibiting behaviour should, therefore, attempt to minimize infections in the head. Conversely, we predicted that cercariae would predominantly infect the head of anesthetized tadpoles, which might facilitate completion of the parasite’s life cycle by making tadpoles vulnerable to predation by the parasite’s definitive host.

**MATERIALS AND METHODS**

The armatae cercariae used in this experiment were obtained from the trematode’s first intermediate host, *Planorbella trivolvis*, which were collected from a wetland in Tampa, FL (28.163914,-82.311829). ITS sequences from these cercariae (accession number) had 100% alignment with *Renifer aniarum* and *Lechriorchis tygarti* (Sears et al. 2012), both of which belong to the subfamily Reniferinae within the family Plagiorchiidae. All reniferin trematodes utilize water snakes as definitive (final) hosts and the planorbid snail-tadpole-water snake life cycle represented here is considered typical for the group (Byrd 1935). Infected snails were housed in artificial spring water (ASW; Cohen et al. 1980) and fed frozen spinach *ad libitum*. Tadpoles of *H. femoralis* were obtained from a snail-free wetland in Tampa, FL (28.068317, -
82.167983). Prior to experimentation, tadpoles were housed in 38 liter aquaria filled with ASW, which was constantly cycled through a carbon filter. Tadpoles were fed frozen spinach *ad libitum*.

Tadpoles were divided into two anesthesia treatments: 0.001% benzocaine (anesthesia) and ASW (control). A higher concentration of benzocaine than that used here has been demonstrated to have no side-effects on measured tadpole immunological responses and does not immunosuppress tadpoles in a manner that affects encystment success by armatae cercariae (0.005%; Sears et al. in press). Furthermore, in that experiment, benzocaine did not result in significantly different distribution of metacercariae on tadpoles’ bodies than control tadpoles (Table S1-4; Fig. 5.4). Immediately prior to parasite exposure, tadpoles were randomly assigned to an anesthesia treatment, weighed, and then placed individually in plastic specimen cups with 30mL of either 0.001% benzocaine solution or ASW for 10 minutes (modified from Koprivnikar et al. 2006). This exposure is sufficient to induce immobility for a further 10 minutes, after which tadpoles fully recover mobility, including foraging behavior. After anesthesia or ASW exposure, tadpoles were then individually collected in a small net, rinsed with ASW, and placed individually in a plastic cup with 30mL of ASW and 0, 10, 15, 20, or 30 cercariae (n=3 tadpoles per cercarial dose). Cercariae were collected directly from a specimen cup containing infected snails in ASW using a micropipette and dissecting microscope. Because hours-old cercariae are more infective than freshly-shed or several-hours-old cercariae (Fried et al. 1997), only 1- to 6-hours-old cercariae were used. Each tadpole was exposed to the assigned cercarial dosage for 10 minutes, rinsed with ASW to remove any attached cercariae that had not yet penetrated the tadpole’s skin, and transferred to a 1 L aquarium.
After parasite exposure, tadpoles were maintained in 1L of ASW, fed frozen spinach *ad libitum*, and monitored for mortality daily. Water was changed 5 days after exposure. Eleven days after cercarial exposure, tadpoles were weighed and euthanized in a 0.1% benzocaine solution. Tadpoles not surviving through this eleven day period were weighed and preserved immediately after death. To make encysted metacercariae visible, ethanol-preserved tadpoles were cleared according to Hanken & Wassersug (1981). In brief, 30% hydrogen peroxide was added daily, in 1% increments of the total ethanol volume, into vials containing tadpoles until all color was bleached. Once colorless, specimens were transferred to a glycerol/KOH solution to clear until transparent. Metacercariae (the encysted form of cercariae) were counted and Gosner stage (Gosner 1960) assessed in cleared specimens at 100x magnification under a compound microscope. Cyst location was categorized as either in the “head” (anterior to the eyes), “body” (immediately behind the eyes to the base of the tail), or “tail” (posterior to the base of the tail).

Ideally, measures of tolerance should be reliable proxies for lifetime fitness (Raberg et al. 2009); therefore, we measured mass change as a proxy for tolerance because mass of juvenile frogs at metamorphosis is predictive of adult frog fecundity (Semlitsch et al. 1988; Smith 1987). The proportions of cercariae infecting individual tadpoles were arcsine square root-transformed prior to analyses. We used analysis of variance (ANOVA) to test whether anesthesia treatment affected the proportion of cercariae that successfully encysted controlling for cercarial dose (continuous predictor) and Gosner developmental stage. Encystment locations (head, body and tail) were not independent because they were regions of the same host. Hence, a within-subjects ANOVA was used to test for differences in encystment location as a function of anesthesia treatment controlling for cercarial dose (continuous predictor) and Gosner developmental stage.
Fisher’s Least Significant Difference multiple comparison tests were conducted for post-hoc analyses.

Leung et al. (2010) revealed a positive relationship between metacercarial intensity and trematode recruitment to a particular host body location in the cockle Austrovenus stutchburyi. If the same pattern occurred for the focal trematode and host in this study, then it could have implications for the effect of cercarial encystment location on host mass gain. Consequently, we tested for an association between metacercarial intensity (log + 1) and the proportion of metacercariae in each body region (arcsine square root transformed). The above analyses were performed in Statistica v9 (StatSoft 2009).

For the tadpoles that were infected (because uninfected tadpoles cannot exhibit tolerance), we partitioned the variance in mass change that was unique to and shared among Gosner stage, number of cercariae resisted (resistance), and proportion of metacercariae in the tail (behavioural tolerance) using the ‘hier.part’ function in the ‘hier.part’ package of R. This allowed us to estimate how much of the host responses contributed to resistance versus tolerance. To evaluate whether each variable accounted for greater unique variation than expected by chance, we conducted a randomization test (1000 randomizations) using the “rand.hp” function in the ‘hier.part’ package of R (Mac Nally 2002).

RESULTS

Anesthesia did not affect mass change in the absence of cercariae (F1,9=0.017, P=0.90). Gosner stage and infection intensity were also unaffected by anesthesia treatment (F1,15=0.079, P=0.78; F1,15= 1.85, P=0.19, respectively), but anesthesia did increase infection prevalence (F1,27=5.47, P=0.027). Encystment location did not affect Gosner stage at the end of the
experiment ($F_{1,15}=0.055, P=0.91$) and Gosner stage was not a significant predictor of mass loss ($F_{1,15}=0.013, P=0.90$). Metacercarial intensity was not significantly associated with the proportion of metacercariae in any body region ($F_{1,17}<0.391, P>0.54$). Hence, given the range of encysted cercariae in this study, there was little evidence that other cercariae influenced the body region in which any individual cercariae chose to encyst.

Anesthesia, however, significantly affected encystment location (encystment location*anesthesia: $F_{2,32}=4.328, P=0.02$; head: $F_{1,17}= 4.53, P=0.048$; body: $F_{1,17}= 0.067, P=0.79$; tail: $F_{1,17}=5.20, P=0.035$). Anesthetized tadpoles had significantly more metacercarial infections in the head than tail, whereas non-anesthetized tadpoles had predominately tail infections; anesthesia did not affect encystment in the midbody region (Fig. 1b). Posthoc multiple comparison tests revealed that the proportion of metacercariae in the head when anesthetized and the proportion in the tail when not anesthetized were not significantly different from one another (LSD: $P<0.05$) but were significantly different from all other treatments (Fig. 1b). This indicates that cercariae preferred to infect the head of hosts, but that host behaviour tended to shunt cercariae away from the head.

Importantly, infections in the head were significantly more costly, resulting in more mass loss, than infections in the tail (proportion in head*proportion in tail: $F_{1,15}= 5.239, P=0.037$; Fig. 2). Specifically, there was a significant negative relationship between tadpole mass change and the proportion of infections in the head ($R=-0.570, df=1,17, P=0.011$; Fig. 2a), but mass change was not related negatively with the proportion of tail infections ($R= 0.484$; Fig. 2b). Hierarchical partitioning indicated that, among infected tadpoles, encystment location, but not number of parasites or Gosner stage, contributed significantly to tolerance of infection (location, $z=1.74$; Gosner, $z=-0.21$; parasites, $z=-0.58$; Fig. 3).
DISCUSSION

Consistent with our first hypothesis, we found that head infections were more detrimental to hosts than tail infections; or, in other words, hosts were more tolerant to tail than head infections. Furthermore, in keeping with our second hypothesis, we found that non-anesthetized tadpoles were capable of preventing these costly infections in the head and had infections concentrated in their tail region. In contrast, parasite encystment location was concentrated in the head of anesthetized tadpoles. Given the absence of an effect of anesthesia on mass change in tadpoles not exposed to parasites, we can affirm that the effect of anesthesia on mass change in parasite-exposed tadpoles was not mediated by chemical side-effects of benzocaine exposure (see also Sears et al. in press). Our results suggest that although anti-parasite behaviour in *H. femoralis* can improve resistance to parasites by reducing prevalence of infection (Fig. 1a), among infected tadpoles, behaviour also plays a crucial role in tolerance to infection, deflecting infections to the less-costly tail region (Fig 2b, 3). This behavioural adaptation is at odds with the apparent preference of parasites to infect hosts’ heads, which might constitute a Red Queen-type “arms race” between host and parasite (Van Valen 1973).

There are several non-exclusive mechanisms by which head infections might have compromised tolerance of *H. femoralis* tadpoles. A decline in feeding activity could result if parasite encystment interfered with musculature or mouthparts (e.g., Venesky et al. 2009), altered chemosensory detection of food, or comprised a stressor sufficient to reduce feeding behavior (Wingfield et al. 1998). Furthermore, an immune response in the head might be more expensive due to inflammatory damage of nearby organs (Sears et al. 2011) or parasite proximity to the brain could have affected metabolism or host behaviour (i.e., Poulin 2013).
Although we cannot rule out the possibility that anesthesia with benzocaine might locally immunocompromise hosts, resulting in more head than tail infections, an analysis of tadpoles that were anesthetized, allowed to recover mobility, and immediately infected (Sears et al. in press) indicates that metacercariae do not encyst significantly more often in any body region of benzocaine-anesthetized tadpoles as compared to non-anesthetized tadpoles (Table S1-4; Fig. S1). The reduction of tolerance associated with head infections relative to tail infections might facilitate parasite transmission, thus explaining the apparent preference of cercariae for the head of tadpole hosts. First, if the head is easier to penetrate than that the tail, cercariae might prefer the head simply because they have a limited lifespan. Similarly, Taylor et al. (2004) suggested that certain locations of a moving host might be more difficult to infect than others. Second, head infections could alter host traits that facilitate transmission to the definitive host. For instance, if olfaction or activity levels are modified by head infections, predator avoidance by tadpoles might be compromised, making them more vulnerable to predation by the parasite’s definitive host, water snakes. Additionally, any interference of head infections with foraging could result in a decline in body condition that might make the tadpole slow to respond to predation attempts. Conversely, by shunting infections to the tail, tadpoles could avoid the compromising effects of infection, improving their likelihood of avoiding predation as well as their fitness post-metamorphosis. Future work will address by which mechanisms trematode infections reduce fitness in tadpole hosts and whether susceptibility to predation is affected by parasite encystment location.

To our knowledge, this is the first description of behavioural tolerance to a parasite. Nevertheless, this phenomenon might be widespread. That is, many hosts that cannot entirely avoid infections might shunt parasites to parts of their body that minimize damage or might
preferentially remove parasites from parts of their body where the parasites are most costly. This pattern was observed in our own results, with behaviour decreasing prevalence but not intensity of infection and simultaneously increasing tolerance to infection. Asymmetry in location of infections among hosts is not uncommon, especially among motile parasites such as trematodes and ectoparasites (e.g., Floriao et al. 2011; Peoples & Poulin 2011). Although some of this asymmetry might be parasite-mediated, how host behaviour or parasite preference influences this asymmetry is unclear (Shaw et al. 1998). For example, bonobos direct most of their allogrooming to the face of conspecifics (Franz 1999), perhaps because ectoparasitic infections of the eyes, nose, or ears are more costly than on the body (though this has not apparently been quantified). Consequently, grooming, like the anti-cercarial behaviours of H. femoralis, might serve to both resist and tolerate infections. We certainly need a better understanding of where on the body certain parasites cause the greatest harm and how widespread and important behaviour is to both resistance and tolerance of infections. Moreover, given that species vary in their resistance and tolerance to infection (e.g., Johnson et al. 2012; Rohr et al. 2010; Sears et al. 2011), future studies should utilize variance partitioning to investigate what portion of observed resistance and tolerance is comprised by behaviour.

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Figure 5.1. Anesthesia affected a) prevalence of infection and b) encystment location of trematode parasites. a) Significantly more anesthetized tadpoles were infected by plagiorchiid cercariae than non-anesthetized tadpoles. b) Significantly more parasites encysted in the head of anesthetized tadpoles but the tail of control (non-anesthetized) tadpoles. Posthoc multiple comparison tests revealed that the proportion of metacercariae in the head when anesthetized and the proportion in the tail when not anesthetized were not significantly different from one another (LSD: $P<0.05$) but were significantly different from all other treatments. Error bars denote standard error.
Fig 5.2. Tolerance of tadpoles to the proportion of metacercariae encysted in a) the head or b) the tail. Anesthetized and control treatments are included in both figures. Dotted lines denote 95% confidence bands.
Fig 5.3. The percentage of explained variance in tadpole mass change explained by developmental stage (Gosner stage), parasite resistance (number of cercariae resisted), and parasite tolerance (proportion of cysts in tail) independently (solid bars) and jointly (open bars) as determined by hierarchical partitioning. Independent contributions are unique to the variance of a particular variable; joint contributions are correlated among all variables. Variables marked with asterisks accounted for a significant amount of variance based on 1000 randomizations.
APPENDIX A: Do parasitic trematode cercariae demonstrate a preference for more susceptible host species?

Note to Reader:
This chapter has been previously published: Sears, B.F., Schlunk, A., Rohr, J.R. Do parasitic trematode cercariae demonstrate a preference for more susceptible host species? *PLoS ONE* 7(12):e51012. See Appendix A for the PDF of the published, open-access document.
Do Parasitic Trematode Cercariae Demonstrate a Preference for Susceptible Host Species?

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Abstract

Many parasites are motile and exhibit behavioral preferences for certain host species. Because hosts can vary in their susceptibility to infections, parasites might benefit from preferentially detecting and infecting the most susceptible host, but this mechanistic hypothesis for host-choice has rarely been tested. We evaluated whether cercariae (larval trematode parasites) prefer the most susceptible host species by simultaneously presenting cercariae with four species of tadpole hosts. Cercariae consistently preferred hosts in the following order: Anaxyrus (= Bufo) terrestris (southern toad), Lithobates (= Rana) sphenocephala (southern leopard frog), and Osteopilus septentrionalis (Cuba tree frog). These host species varied in susceptibility to cercariae in an order similar to their attractiveness with a correlation that approached significance. Host attractiveness to parasites also varied consistently and significantly among individuals within a host species, if inheritable, this individual-level host variation would represent the raw material upon which selection could act, which could promote a Red Queen "arms race" between host cues and parasite detection of those cues. If, in general, motile parasites prefer to infect the most susceptible host species, this phenomenon could explain aggregated distributions of parasites among hosts and contribute to parasite transmission rates and the evolution of virulence. Parasite preferences for hosts belong the common assumption of disease models that parasites seek and infect hosts at random.

Introduction

Parasitism is the most common consumption strategy among heterotrophs [1] and parasitic associations undeniably involve some of the most intimate co-adaptations between organisms. Generalist parasites, capable of infecting multiple host species, might possess adaptations that extend beyond single host-species interactions to include traits affecting discrimination among host species. Indeed, generalist parasites frequently demonstrate behavioral preferences for particular host species when presented with multiple suitable host species. A number of characteristics can influence these preferences, including host abundance [2], reproductive status [3], or species identity [4,5,6]. Several proximate explanations have been offered for these preferences, such as reduced host vigilance against ectoparasites during reproduction [3] or parasite preference for a particular host species as the result of historical sympathy [2]. At the ultimate level, these preferences are presumed to be adaptive decisions that maximize parasite fitness, but it remains unclear and nearly un-investigated whether patterns of host selection by parasites are attributable to host competence. The competence of a host to a parasite can be influenced by several traits, including host survival post-infection and interactions between infected and uninfected hosts, but the first hurdle a parasite must surmount is to successfully infect a host, which requires a susceptible host. Given that hosts can vary widely in their susceptibility [7], an examination of whether generalist parasites infect the most competent hosts must begin with an examination of whether parasites select among hosts based on susceptibility to infection.

The larvae of digenetic trematodes, cercariae, are frequently generalists in their host use [8]. Cercariae actively swim through the water column, detecting hosts via physical and chemical cues [9,10,11,12,13]. Within a species of host, susceptibility to a particular species of cercariae may depend on a number of factors, including host genotype, habitat, or infection history [14,15,16]. Given that 1) the reproductive success of cercariae depends on their ability to successfully infect a host, 2) cercariae can often infect a range of host species, and 3) use chemical cues to detect hosts, they might demonstrate a preference to infect the most susceptible hosts available.

In this experiment, we tested whether a cercarial species local to Tampa, FL (Fig 1a) exhibits preferences for certain sympatric, simultaneously-presented tadpole species (Fig 1b: Anaxyrus terrestris, Lithobates sphenocephala, and Osteopilus septentrionalis) and, in independent trials, whether their preference for particular host species correlates with susceptibility of hosts to infection. We predicted that among-individual variation in attractiveness and susceptibility to cercariae would be greater than within-individual variation in these traits. Furthermore, because O. septentrionalis has been recently introduced to this locality (post-1997, [17,18]), we predicted that it would be the least-preferred host because cercariae are unlikely to have co-adapted to its cues.
Materials and Methods

Study System

Digenean trematodes typically have a three-host lifecycle, including a molluscan first intermediate host. We collected 
Panorpa triunguis snails in the Tampa, FL area in July of 2009 and screened them for trematode infections based on the presence or absence of free-swimming armate cercariae (Fig. 1a). These cercariae infect tadpoles by directly penetrating the skin and encysting subcutaneously as metacercariae. If an infected tadpole is eaten by the definitive host, typically a vertebrate, the metacercariae develop into adult trematodes in the host's intestine, from which they will shed eggs that hatch into miracidia, completing the lifecycle when they infect new snail hosts.

Tadpoles used in this experiment were collected in August and September of 2009 from wetlands in the Tampa, FL area. Both snails and tadpoles were collected in accordance with permit WXX08179 from the State of Florida Fish and Wildlife Conservation Commission. The species of tadpoles used here are frequently sympatric with trematode-infected P. triunguis, but all tadpoles were collected from snail-free habitat and were collected soon after hatching and maintained in the lab free of trematodes until we all four species were collected for the trials. Hence, it is unlikely that any of the tadpoles were infected before exposure to cercariae in this experiment. When used in trials, tadpoles ranged in Gosner developmental stage [19] from stage 28–37; we included Gosner stage as a covariate in our analyses (see “Statistical Analyses,” below) to control for within-species differences in size and potential effects of Gosner stage on host immunology (e.g., [20,21]). Tadpoles were maintained in artificial spring water [9] and fed frozen spinach ad libitum between experimental trials.

Parasite Identification

The cercariae used in this experiment are an armate morphotype, which occurs exclusively in the families Plagiocotyleidae and Telorchidae [22]. To identify cercariae more specifically, we sequenced DNA from the ITS1 region of armate cercariae from snails collected at the same site as those used in this experiment. DNA from approximately 100 cercariae was extracted using an UltraClean® Fecal DNA kit (Mo-Bio Laboratories). The primers BD1/4S and ITS2.2 from Wilson et al. [23] were used to amplify the ITS-1 and ITS-2 regions. PCR was carried out in a total volume of 25 μL, which consisted of 1 μL of each primer (0 μM), 10 ng DNA, and 1.25 μL PCR Master Mix (Promega Corporation). Reaction conditions were identical to Wilson et al. [23]. PCR product was viewed on an ethidium bromide-stained 1% agarose gel. DNA sequencing was conducted by Eurofins MWG Operon. Sequence results indicated 100% sequence similarity to both Rutilus amarum and Lefelewia tigrina, both of which belong to the subfamily Rutilusinae in the family Plagiocotyleidae [24]. Renifer trematodes are fairly conserved in their host use, utilizing freshwater snails as first intermediate hosts, tadpoles as second intermediate hosts [25,26], and snakes as definitive hosts [27,28]. Given that the sequenced cercariae were a close match to both of the above species, it likely exhibits a similar life history as other renifer species.

Parasite Preference for Host Species

Selection chambers were used to determine whether cercariae demonstrated a behavioural preference among four species of tadpoles (Fig. 1c). The basic design was as follows; four 9.5 mm holes were drilled at 90° angles to one another into a 20 mL scintillation vial. To prevent water leakage, holes were fitted with rubber grommets, each of which held one horizontal transfer
pipette. The tube and bulb of each transfer pipette were separated by Nitec mesh (11 μm), secured by silicone seals. The Nitec mesh allowed tadpole chemical cues to pass, but prevented the cercariae from infecting the amphibians. The bulb of each transfer pipette was cut to create flaps, allowing space for a tadpole to be inserted. The size of the bulb prevented tadpoles from most movement. The apparatus was filled with 5 mL artificial spring water ASW, a quantity that reached just above the rubber grommets, which prevented cercariae from travelling vertically. Thus, there was a central region from which four arms projected, each holding a different tadpole species. No negative control trials (i.e., empty arms) were conducted.

We allowed tadpoles to condition the water with chemical cues for 10 minutes, forming a gradient, then added approximately 30 cercariae (range: 31–35) to the center of the scintillation vial. The cercariae were allowed to swim freely for 30 minutes. After 30 minutes, each tube was sealed off from the central scintillation vial using a metal clamp. Tadpoles were removed from the apparatus and returned to individual 1-L cups. The water and cercariae from each arm and the central scintillation vial were pipetted separately into petri dishes. Cercariae were killed and stained with Lugol's iodine and counted at 40x magnification under a dissecting microscope. Cercariae which swam into a given arm were assumed to have selected the host at the distal end of the arm for infection.

Five selection chambers were run simultaneously on 10 days for a total of 50 trials over the course of the experiment. All trials were conducted under artificial, fluorescent light at 25°C and tadpoles were rotated among the chambers’ arms to prevent biased effects of any particular arm. All cercariae were more than one hour old and less than 12 hours old, as cercariae have a <24-hour lifespan and several-hour-old cercariae are more infective than new or old cercariae [29]. Two trials were excluded from the analyses because only one and two cercariae, respectively, made choices in these trials. Individual tadpoles were repeat-tested between one and five times (average use: 2.5 trials per tadpole) throughout the 10 days of trials. This allowed us to evaluate whether among-individual variation in attractiveness to cercariae was greater than within-individual variation in attractiveness. To calculate the proportion of cercariae making a choice among hosts, the number of cercariae remaining in the center chamber was subtracted from the initial number added to the apparatus. The number of cercariae in each arm was then divided by the total number of cercariae that entered any arm.

Susceptibility of Host Species

One week after the parasite preference trials were completed, we conducted a second experiment testing if host species differed in their susceptibility to parasites (i.e., the ability to prevent infection or limit the intensity of infection). The same tadpoles utilized in the parasite preference portion of the experiment (above) were isolated in individual plastic specimen cups with 25 ml of artificial spring water for experimental exposure to infective cercariae. The dose of infective cercariae was 20 cercariae for A. terrestris and 30 for H. squirella, L. sphenecephala, and O. septentrionalis. The differences in dose were due to variation in the output of cercariae by infected snails on the day of exposure. All of the cercariae penetrated the tadpoles within 12 h (i.e., none were found alive or dead at the bottom of the cups). After 48 h, tadpoles were euthanized in a 0.1% benzocaine solution and preserved in 70% ethanol. Tadpoles were then demembraned and cleared in order to make parasite cysts visible, as detailed by [30]. In brief, 30% hydrogen peroxide was added in increments of 1% of the total ethanol volume until the tadpole was colourless; specimens were then evenly distributed in a solution of 0.5% potassium hydroxide and glycerol until transparent. Metacercarial cyst quantification was performed under a compound light microscope at 100x. The majority of L. sphenecephala individuals used in the preference trials were damaged during the cleaning protocol and, thus, their metacercarial could not be quantified. We subsequently experimentally infected each L. sphenecephala tadpole to obtain susceptibility data, but lacked the susceptibility data from the same tadpoles used in preference trials with which to quantify the relationship between attractiveness and susceptibility.

Statistical Analyses

For the cercarial choice data, we conducted an analysis of variance (ANOVA; type II sums of squares) on the arcsine square-root-transformed proportion of cercariae that selected each tadpole species in each trial. In this analysis, we blocked by each of the 10 days we conducted host preference trials, weighted the analysis by the number of cercariae that made a choice in each trial, and tested for an effect of tadpole species and individuals nested within tadpole species. Block, host species, and individuals were all treated as random effects.

For the susceptibility data, we conducted an ANOVA on the arcsine-square-root transformed proportion of cercariae that encysted, controlling for individual Gosner developmental stage [19] and testing for differences among species. Gosner stage and frog mass were correlated (r = 0.570, P = 0.036), Gosner stage is independent of water and fecal loss, and there are well-documented changes in susceptibility to trematodes with Gosner stage independent of mass [21], so we chose to present the results with Gosner stage as the covariate. However, the results did not qualitatively change when mass was substituted as the covariate. We conducted Fisher’s least significant difference post hoc multiple comparison tests to determine which host species significantly differed in their attractiveness and susceptibility to the cercariae. We used regression analysis (one-tailed) to determine whether there was a positive relationship between host susceptibility and attractiveness to cercariae using the species as the replicate. Both cercarial choice and susceptibility analyses were performed using Statistica software v9.1.210 [31].

Results

Parasite Preference for Host Species

Parasite preference for hosts significantly differed between host species (F_{4,45} = 5.37, P = 0.003). Parasites demonstrated the strongest preference for A. terrestris, followed by H. squirella, L. sphenecephala, and O. septentrionalis (Fig. 2). Among species variation in host attractiveness to cercariae was greater than within-species variation in attractiveness, but individual identity within a species was also a significant predictor of attractiveness (F_{1,118} = 1.83, P = 0.003). That is, there was greater variation in attractiveness to cercariae among than within individuals of a species, but there were also significant and repeatable differences in the attractiveness of individual tadpoles.

Susceptibility of Host Species

Independent of developmental stage (β = 0.059, F_{1,54} = 24.92, P < 0.001), host species also varied significantly in their susceptibility to infection (F_{4,55} = 7.35, P < 0.001; Fig. 3), with A. terrestris being most susceptible, followed by L. sphenecephala, H. squirella, and O. septentrionalis (Fig. 3). The relationship between a species’ attractiveness to cercariae and its susceptibility to cercariae was positive and approached significance (F_{1,1} = 1.22, P = 0.08).
Figure 2. Proportion (mean ± 1 SE) of experimentally-administered cercariae demonstrating a preference for a host species. Values for those species not sharing a letter are significantly different based on Fisher’s least significant difference tests.
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Figure 3. Proportion (mean ± 1 SE) of experimentally-administered cercariae successfully infecting each host species. Values for those species not sharing a letter are significantly different based on Fisher’s least significant difference test.
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Discussion

Cercariae discriminated among host species and tended to demonstrate a preference for the most susceptible host species but this relationship was not statistically significant. Tadpoles of *A. terrestris* were the most preferred and most susceptible to infection by cercariae, with *H. spinella, L. sphenochelys,* and *O. septentrionalis* exhibiting reduced attractiveness and susceptibility in the order listed (Figs. 2, 3). Although the tadpole species used here differ in size, the proximate driver of attractiveness is unlikely to be attributable to simple size differences. First, the most attractive species, *A. terrestris,* is also the smallest, with the same host species used in previous studies [32]. Individuals tested here were from the same location within species and were fed ad libitum. Finally, 98% (57/61) of the Cuban, Squirrel and Leopard frogs were below Gosner stage 42 and frogs don't begin losing mass until after stage 42 (see [33]). The toads were all the same Gosner stage (i.e. no variability). Therefore, it is unlikely that mass or a mass-by-Gosner stage interaction influenced the outcome of the trials.

Life history differences, however, may partially explain the variation in susceptibility among species. Adults of species with a short larval period, such as *A. terrestris,* tend to lay their eggs in ephemeral water bodies, including ditches and puddles [54] that rarely support permanent small populations (pers. obs.). The absence of snails, and therefore cercariae, might free tadpoles of the selective pressure necessary to maintain a robust immune response to trematodes, leading to increased susceptibility to parasitism. Indeed, other experiments have demonstrated that tadpole species with short larval periods are more susceptible to infection by cercariae than species with long larval periods [7,35]. Furthermore, life history differences can also be coupled with behavioural differences (e.g., [55]), which might have contributed to the attractiveness of *A. terrestris*.

We included O. septentrionalis in this study to explore whether its success as an introduced species might be attributable to cercarian in its non-native range failing to exhibit a preference that is proportional to their susceptibility, perhaps because of a lack of co-evolutionary history. Such a pattern would contribute to a commonly observed phenomenon, in which introduced species have few parasites in their non-native range, formalized in the "enemy release" hypothesis [37]. The trematodes used in this experiment are likely novel to O. septentrionalis; the only cercariae described from O. septentrionalis in its native range is *Metacercariae crassorum.* [38]. We can be confident that M. crassorum is not the parasite used in this experiment because 1) it is in a different family (Brachycercidae) than the species suggested by DNA sequencing and 2) the cercariae of M. crassorum are dissimilar from those used here; most notably, cercariae of M. crassorum are tailless [40], whereas ours possess a well-developed tail (Fig. 1A). O. septentrionalis was not significantly less attractive or more susceptible than every native species, but it was ranked lowest among host species for both measures (Fig. 2, 3). Future studies should investigate whether introduced species are less attractive to active, host-seeking parasites and vectors of parasites than native species and whether this contributes to enemy release.

In this experiment, individual hosts exhibited consistent variation in their attractiveness to cercariae. Consistent individual variation in a trait, in this case attractiveness to parasites, is the material upon which natural selection acts. If such a trait is heritable, then one might expect a Red Queen-type evolutionary arms race between host evasion of parasites through altered chemical cues and parasite counter-adaptations to remain capable of identifying and infecting the most susceptible hosts. The specific cues used by cercariae to locate tadpoles are not clear, but host peptide cues are frequently utilized by echinostomatid cercariae [41]. The chemical composition of tadpole skin might have wide variation, given interspecific differences in traits such as palatability [42], which is presumably governed by chemicals such as toxins in the skin [43]. If variation in cercarial host preference and resistance does correlate with tadpole susceptibility and length of larval period, as densed above, parasites should constitute a selective pressure affecting tadpole life history evolution at the ultimate level, as well as expression of chemical cues at the proximate level [44,45].

In summary, our results suggest that, among native host species, cercariae seem to select the most susceptible host species and exhibit consistent preferences for certain individuals within a species. If this occurs commonly in nature, epidemiological models could be underestimated parasite transmission rates for parasites that infect multiple host species (but see [46]). Non-random host usage could explain patterns of parasite aggregation among and within host species [47,48] and could be especially profound for those parasites that can actively seek out susceptible hosts. Effects of non-random host choice could also apply to parasites that manipulate vectors into seeking preferential hosts (see [44]). Given the role of infectious diseases in current amphibian declines [49,50], adjusting these models could be crucial for accurately predicting and mitigating amphibian declines.

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Author Contributions

Concepted and designed the experiments: AS JS. Performed the experiments: BS AS. Analyzed the data: BS JS. Contributed reagents/materials/analysis tools: JS. Wrote the paper: BS JS.

References

Trematode Cercariae Prefer Susceptible Hosts


APPENDIX B: The economy of inflammation: when is less more?

Note to Reader:
This chapter has been previously published: Sears, B.F., Rohr, J.R., Allen, J.E., Martin, L.M. The economy of inflammation: when is less more? *Trends in Parasitology* 27(9):382-387.

See Appendix B for the PDF of the published document and Appendix C to see the permission from the publisher.
In ecology, tolerance of parasites refers to host mitigation of the fitness costs of an infection. This concept of parasite tolerance contrasts with resistance, whereby hosts reduce the intensity of an infection. Anti-inflammatory cells and molecules have been implicated as mechanisms of parasite tolerance, suggesting that a major role of tolerance is in minimizing collateral damage associated with inflammation. A framework is proposed here in which the cost–benefit outcome of an inflammatory host-response is hypothesized to be dependent on host life-history, parasite virulence, and the efficacy of a current inflammatory or anti-inflammatory response. Testable predictions, both within and among host species, are presented for this hypothesis.

**Inflammation promotes resistance but reduces tolerance to parasites**

In modern immunology the term ‘tolerance’ refers to the ability of the immune system to avoid self-reactive components that can cause autoimmune disease (i.e. self-tolerance). More recently, parasite tolerance (Glossary) has been emphasized [1,2], with particular attention to the mechanisms by which hosts minimize the fitness costs of a particular parasite burden [3–6]. Parasites, a term which refers to any infectious organisms that exact fitness costs on a host, can be coped with in two ways: (i) resistance, whereby hosts reduce parasite burden by preventing infection or clearing parasites that do infect, and/or (ii) tolerance, whereby hosts mitigate the deleterious effects of a particular burden of parasites that have infected. Whereas resistance reduces the cost of an infection by minimizing parasite exploitation of host resources, tolerance can minimize costs by repairing tissue damage or immunopathology resulting from the resistance responses of the host. Ecologists increasingly appreciate that parasite tolerance is an important component of host–parasite interactions and disease outcomes [1,7], but mechanisms of parasite tolerance are still poorly understood relative to our understanding of resistance. However, a central theme that emerges from recent studies by both groups [3–6] is that inflammation is a major mediator of resistance but also a perpetrator of immunopathology and, therefore, a depressor of tolerance.

Inflammation is a particularly important mediator of resistance because of its rapid and broad efficacy in infections. The majority of immune responses begin with the induction and propagation of inflammation by a series of positive-feedback loops (Box 1). These processes thwart the establishment or persistence of diverse parasites (i.e. viral, bacterial, and eukaryotic), even novel parasites for which a host has no immune memory. However, immunopathology can be a major cost of infection [8], the pathogenicity of a parasite, also defined as ‘virulence’ [9,10], can be due to both host and parasite [11]. For example, intestinal helminths can cause tissue damage directly by burrowing into the epithelium, but the host can also cause damage by launching an inflammatory response in the mucosa. For this reason, high levels of inflammation can eventually lead to downregulation of inflammatory cells and cytokines (Box 1). Given both the protective and self-damaging capacity of inflammation, a central issue that remains to be reconciled is: how does a host determine the magnitude and duration of an inflammatory response?
Box 1. Activation and resolution of inflammation

Wounding results in immediate inflammation, which creates positive feedback loops that perpetuate and amplify inflammation (Figure 1a). First, the release of neurokinins recruits mast cells to the site of injury, which release the inflammatory cytokine TNFα. Simultaneously, PAMPs are phagocytosed by DCs and macrophages; macrophages kill the parasite with ROS and RNS (abbreviations: Figure 1 legend). DCs present parasite antigen to TH1 helper cells, including IFNγ, which induces hyperactivation in macrophages by secreting IFNγ. Macrophages, in turn, release IL-12, which promotes more TH1 activity. Hyperactivated macrophages also produce TNFα, which promotes IFNγ production by NK cells and further macrophage activation. Macrophages actively recruit neutrophils from circulation, which phagocyte small parasites or degranulate near larger parasites, releasing ROS.

However, high levels of inflammation eventually downregulate further inflammatory processes (Figure 1b). High levels of TNFα cause neutrophils to become apoptotic; if a macrophage ingests the apoptotic neutrophils, it induces a regulatory phenotype in the macrophages, which produces the anti-inflammatory cytokine IL-10. Similarly, high levels of ROS and LPS cause DCs to become apoptotic, which, when ingested by DCs, arrest maturation of the DC and cause it to produce TGFβ, which contributes to the induction of potentially anti-inflammatory Treg cells.

Figure 1. Regulation of inflammation. (a) Positive feedback promotes inflammation. (b) Negative feedback eventually suppresses inflammation, promoting parasite tolerance. PAMP-colored cells refer to inflammatory capabilities, with deep pink being more inflammatory than light pink; blue-colored cells and cytokines refer to anti-inflammatory cells, and cytokines that probably contribute to parasite tolerance, particularly those below the dashed line. Red arrows and outlines indicate downregulation of a cell type or cytokine. Abbreviations: DCs, dendritic cells; TNFα, tumor necrosis factor alpha; PAMPs, pathogen-associated molecular patterns; ROS, reactive oxygen species; RNS, reactive nitrogen species; TH1, type 1 T helper cell; DCs, dendritic cells; IFNγ, interferon gamma; IL-12, interferon-γ; IL-10, macrophages; NK, neutrophils; NK cells, natural killer cells; LPS, lipopolysaccharide; TGFβ, transforming growth factor beta. Treg, regulatory T cell.

response? Here, it is hypothesized that hosts use inflammation in a manner that minimizes infection intensity until the cost of the immune response is larger than the cost of the infection in the absence of inflammation. Thus, with information about hosts and parasites, the duration and magnitude of inflammatory responses - and when to favor tolerance over resistance - should be predictable.

The goals of this Opinion are threefold. First, in light of the costs and benefits of resistance versus parasite tolerance, the impact of host life-history upon inflammatory responses is outlined. Second, the costs and benefits of inflammation are reviewed; specifically, that parasite pathogenicity and the location of host infection are determinants of much of the variation in inflammation between...
hosts. Third, how a host might switch the character of its response from resistance to tolerance (or vice versa) is explored. Finally, relevant under-studied research areas are identified, particularly putative molecular and cellular mechanisms of parasite tolerance and shared mechanisms of self- and parasite-tolerance. The motivation is not to unequivocally support these hypotheses, but instead to promote ecological and evolutionary investigations of inflammation [12].

Ecology of resistance and tolerance
Host and parasite life-histories should impact strongly upon the balance of resistance and tolerance and, hence, variation in inflammation. Variations in life history will have consequences at three different time-scales (Figure 1): (i) before parasite exposure, (ii) at the time of exposure, and (iii) over the duration of an infection.

Before parasite exposure: host life-history
Life-history theory suggests that variation in the timing of and investment in reproduction is due to selective pressure to maximize the fitness of an organism (i.e., the production of offspring that produce offspring). Life-history 'strategy', which refers to a web of adaptations that dictates the allocation of reproductive effort over the lifetime of an organism [13], is a concept which has been applied to all multicellular taxa. Life-history strategies tend to fall along a fast to slow pace-of-life continuum, with fast-paced species maturing rapidly and reproducing prolifically, whereas slow-paced species are slower to mature and produce fewer offspring per unit time [14]. Because host life-history strategy will impact both parasite exposure and the resources available to mount immune responses, inflammatory responses are probably under evolutionary pressure such that the pace of life is an important determinant of the magnitude of inflammation.

A recurring observation is that fast-paced species tend towards inflammatory responses, whereas slow-paced species produce anti-inflammatory responses [15–17] (Figure 1a). These patterns could result from heritable differences, in which fast-paced hosts are genetically predisposed to respond with proinflammatory responses relative to slow-paced hosts, and/or they could be due to heritable behavioral differences that predispose hosts to be exposed to antigens in a particular order or with a particular frequency; in the latter case the immune cells of the host would be 'educated' to acquire parasite tolerance much as T and B cells are educated during the maturation of the immune system (e.g., [18]). Nonetheless, these events need not be exclusive; acquired parasite tolerance influenced by environmental exposures could eventually become heritable through genetic assimilation [19].

Within species, fast and slow pace-of-life can also be assigned to individuals. Fast and slow paces are often assigned to males and females, respectively, owing to the generally shorter life spans of males and more frequent mating opportunities. Indeed, male mammals consistently exhibit a greater inflammatory bias than do non-pregnant females [15,20,21]. Pregnant females have an even more pronounced anti-inflammatory bias, characterized by a regulatory T cell (Treg) skew that could prevent maternal immune responses against the fetus [22]. Fast and slow pace of life can also be attributed to temperate and tropical individuals in broadly distributed species. Based on body size, clutch size, and average number of clutches per year, Martin et al. [23] describe neotropical populations of house sparrows (Passer domesticus) as slow-paced relative to temperate populations; neotropical house sparrows are also immunologically slow-paced, demonstrating stronger antibody-based responses to a novel antigen than temperate individuals [17]. If tropical animals are generally slow-paced and parasite-tolerant, it could explain the general tendency of tropical host species to harbor more parasites than temperate hosts [24].

Eco-evolutionary theory makes sense of these patterns. First, fast-paced species and individuals are relatively short-lived, and are therefore less likely to encounter a parasite repeatedly. This lower chance of re-exposure would minimize the relative value of investing in a developmentally expensive adaptive immune system [15]. Second, the short lives of fast-paced animals could prevent them from experiencing the long-term costs of inflammation that long-lived, slow-paced animals endure; strong inflammatory responses are more likely to be beneficial to fast-paced animals because cumulative damage is relatively less likely to compromise fitness in a short-lived organism. Third, the time required to involve the adaptive immune system in an infection (~10 days) could impart opportunity costs (e.g., missed matings) during an illness that are incompatible with a fast pace of life. In summary, we hypothesize that, for a fast-paced host, the benefits of a strong inflammatory response result in resistance-type responses to infection; for slow-paced hosts, the costs of inflammation outweigh the benefits of such a response.

Figure 1. Host and parasite characteristics influence the magnitude and duration of inflammatory responses. (a) Fast-paced hosts or those infected with virulent parasites (solid line) should use inflammatory responses that maximize the fitness of an infection (i.e., resisted) so as to survive to one or a few reproductive events. Chronic infections in a fast-paced host will probably slowly transition to tolerance-type responses. Slow-paced hosts or hosts infected with less virulent parasites (dotted line) probably utilize some resistance initially (due to positive feedback box 1), but quickly transition to tolerance to prevent the accumulation of immunopathology. Note also that the character of the constitutive defenses at the host (i.e., the immune system) also plays a role in different hosts, with slow-paced hosts maintaining a less inflammatory state in the absence of infection. (b) Hosts can adopt different strategies during chronic infections, depending on the intensity of infection. (i) A host that initially launches an inflammatory response will return to homeostasis if infection intensity is modest or the infection is cleared (dot-dash line), or the host can damp inflammation (i.e., faster tolerance; solid line). If inflammation fails to clear a high-intensity infection. (ii) A parasite that is initially tolerated, such as a helminth, can eventually elicit inflammation at high burdens (solid line) or damp inflammation (i.e., remain tolerated) at low densities (dot-dash line).
resulting in immune responses that instead promote tolerance to parasites.

At the time of exposure: costs of infection
Host life-history can be important to setting the threshold at which inflammation is used or avoided but, for an individual host, what determines whether a parasite is resisted or tolerated once an infection occurs? The answer probably lies in the potential cost of a specific infection, particularly parasite virulence and the location of the infection. Hosts probably avoid inflammation/resistance in favor of anti-inflammatory processes and tolerance when: (i) the tissue at the site of infection will be compromised by a strong inflammatory response, and/or (ii) when the cost of resisting is greater than the cost of tolerating (i.e., the parasite has low inherent virulence).

In particular tissues, inflammatory processes cannot be engaged continuously without compromising functionality (e.g., mucosal tissue, brain, and placenta); these tissues are subject to immunological privilege, in which immune responses are markedly downregulated and specific effector cells are excluded. Exclusion of immune cells from specific tissues was first described in studies of self-tolerance; however, immunological privilege also appears to extend to parasite-responsive immune cells (Table 1). In the mucosa, for instance, Treg cells produce anti-inflammatory cytokines, such as interleukin 10 (IL-10) and transforming growth factor beta (TGFβ), both of which depress effector T cell actions, including inflammation [25]. To maintain tissue integrity, parasitic infection could initially incite minimal inflammation followed by rapid transition to tolerance-type responses, mediated by cells with regulatory phenotypes (Figure 1b).

Parasite virulence, in the sense of pathogenicity [9], should also impact upon whether resistance or tolerance is favored. A less virulent parasite is, by definition, a lesser threat to host fitness than a virulent parasite, and hosts should engage immune responses appropriate to indicators of parasite virulence (Figure 1a) [26]. Hosts can use pathogen-associated molecular patterns (PAMPs) as traits that offer both specific information regarding a pathogen, as in more-or-less virulent genotypes, and taxonomic affiliation (i.e., bacteria versus helminths) that might reflect the replicative potential (virulence sensu lato) of the parasite [27,28] and, therefore, inform the host of the necessity of employing inflammation. For example, lipopolysaccharide (LPS; a bacterial cell-wall component) and flagellin (a bacterial flagellum protein) variants can offer fine-tuned information regarding the virulence of Salmonella enterica genotypes [29]. Similarly, exposed mannan on the cell wall of the commensal yeast Saccharomyces cerevisiae distinguishes it from the pathogenic yeast Candida albicans, which lacks mannan [30]. In addition, the accompaniment of PAMPs with danger-associating molecular patterns (DAMPs), such as necrotic cells, could indicate a more virulent parasite than PAMPs alone [31], informing the host that a stronger inflammatory response might be necessary to manage the infection.

Overall, PAMPs and virulence factors both appear to be integral determinants of the magnitude and duration of inflammatory responses because of their propensity to activate the immune system. The manner in which the immune system is activated then influences the recruitment and proliferation of T helper cell subsets, which impacts upon the inflammatory milieu of an immune response. Inflammatory, type 1 T helper cells (Th1) typically promote resistance to intracellular infections, which tend to have high virulence because they cause host cell damage and have high rates of replication. However, Th1 responses can induce marked pathology during infections (e.g., [32]), thereby compromising tolerance. On the other hand, Th2 cells, which are the hallmark response to metazoon infection [33], promote both tolerance and resistance to extracellular parasites. Resistance can occur by flushing out intestinal parasites or encapsulating worms in tissues. Similar mechanistic pathways also mediate tolerance by rapidly repairing damage caused by these metazoon parasites, such as sepsis-inducing breaches to the gut wall [34].

The ability of Th2 responses to promote both resistance and tolerance could help to explain why the predominance of Th2 responses in intestinal tissue, which is sensitive to inflammation. Th2-mediated responses are often associated with the development of fibrosis [35], indicating that the need for rapid repair can be costly.

### Table 1. Mechanistic and functional similarities of self-tolerance and parasite tolerance

<table>
<thead>
<tr>
<th>Description</th>
<th>Self-tolerance mechanisms</th>
<th>Parasite tolerance mechanisms</th>
<th>Refs.</th>
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<tbody>
<tr>
<td>Anergy of over-reactive lymphocytes</td>
<td>Binding of the receptors Fox and PD-1 induces energy in auto-reactive B and T cells</td>
<td>Binding of the receptors PD-1 and CTLA-4 mediated energy of repeatedly activated (exhausted) T cells</td>
<td>[42,46,47]</td>
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<td>Overactivation-induced cell death</td>
<td>Binding of the receptors Fox, PD-1, or CTLA-4 induces apoptosis</td>
<td>Binding of the receptors PD-1 or CTLA-4 induces apoptosis</td>
<td>[42,46,47]</td>
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<td>Reactive T cells become Treg cells</td>
<td>Generation of natural Treg cells in thymus from self-reactive lymphocytes</td>
<td>During infection, generation of inducible Treg cells from overstimulated T cells</td>
<td>[48]</td>
</tr>
<tr>
<td>Immunologically privileged sites (e.g., mucosa)</td>
<td>Expression of Fox-liganded in privileged tissue stimulates apoptosis of Fox-bearing lymphocytes, which enter privileged sites, preventing immunopathology</td>
<td>Treg-populated mucosal tissue, bias toward defense by antibodies (non-inflammatory) rather than effector cells (potentially inflammatory)</td>
<td>[25,42,48]</td>
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<tr>
<td>Treg cells</td>
<td>Decreased activity of self-reactive T cells</td>
<td>Decreased activity of parasite-responsive effector T cells</td>
<td>[26]</td>
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Abbreviations: Fox, ‘death receptor’ CD95; PD-1, programmed cell death-1 receptor; CTLA-4, inhibitory receptor CD152; Treg, regulatory T cells; Th, T helper cell.

* Anergy, loss of responsiveness.
During an infection: switching strategy from resistance to tolerance

A final factor that might influence the host inflammatory response involves the comparatively shorter generation times of parasites than hosts and the capacity of parasites to exhibit plasticity [36]. As an infection progresses from acute to chronic, or parasite strategies change (such as the switch from dormant to proliferative stages of Toxoplasma gondii [32]), it could sometimes be necessary for hosts to transition from a tolerant strategy back to a resistant one. Over the course of an infection, tolerance should manifests if the cost of minimizing burden (resistance) becomes greater than the cost of minimizing damage (tolerance); such a situation would probably arise if immunopathology is the chief cost of an infection. Indeed, a switch from resistance to parasite tolerance is typical of many, if not most, chronic intracellular infections such as toxoplasmosis and leishmaniasis. The acute phase of these two infections is characterized by IFN-γ-mediated activation of macrophages to resist infection. However, as the infection progresses, increasing amounts of IL-10 modulate this response [37], reflecting a more parasite-tolerant state. If tolerance is ineffective at minimizing the fitness cost of an infection, hosts can shift back to one of resistance (Figure 1b). Such is the case in toxoplasmosis, when the quiescent stage of the parasite periodically reactivates, requiring renewed host resistance [38].

Hosts can also adjust their immune responses during an infection contingent on the initial intensity of an infection. For example, rats infected with low numbers of Echinostoma trivolvis rapidly clear all worms, but rats infected with a high dose of E. trivolvis do not, maintaining nearly as many worms late in the infection as the initial inoculation [39]. Such a pattern suggests a shift from resistance in low-dose infections to tolerance in high-dose infections, perhaps based on balancing the benefit of clearance of low numbers of parasites against the cost of potential tissue damage in the intestine. Conversely, sheep seem to initially tolerate both low- and high-intensity infections of Fasciola hepatica, releasing the Th2-associated anti-inflammatory cytokines IL-4, 5, and 10. However, after 12 weeks of infection, heavily-infected sheep shift to inflammatory responses (by reducing IL-10 and TGFβ production) indicative of a shift to resistance [40].

Regulation of inflammation

At the molecular and cellular level, regulation of specific cytokines and particular T cell subsets can mitigate the induction, duration, and resolution of inflammation (Box 1). Th cells have particularly dynamic mechanisms for the downregulation of inflammation. When repeatedly activated, as occurs during an infection, T cells can become refractory to further activation by upregulating the expression of CTLA-4, a molecule that prevents a T cell from responding when it encounters antigen [41] (Table 1). Furthermore, if repeatedly stimulated, Th cells can differentiate into Treg cells [42] or produce downregulatory cytokines [37], which can shut down inflammatory cascades. Therefore, contrary to the many positive feedback loops that initially induce inflammation, long-term inflammation can eventually downregulate the progenitors responsible for its induction [43] (Box 1).

The subset of Th cells responding to a parasite can also play an important role in the degree of inflammation produced. A combination of host life-history and parasite identity are responsible for inducing different types of Th cells, as discussed above. The differing roles of Th cell types in an infection are further polarized by reciprocal negative regulation of Th1 and Th2 cells (Box 1). Likewise, Th17 and Treg cells could have functionally similar inflammatory/anti-inflammatory roles; Th17 cells incite very strong levels of inflammation mediated by the cytokine IL-17 whereas Treg cells are strongly anti-inflammatory and suppress other effector Th cell functions [44]. Th2 cells are also fundamentally anti-inflammatory, which is probably associated with their role in tissue repair, a process that requires an end to the inflammatory cascade [45].

In many ways, the mechanisms of both parasite tolerance and self-tolerance overlap substantially (Table 1). Treg cells play an integral role in promoting immune quiescence in both types of tolerance. Researchers are only beginning to understand mechanisms of parasite tolerance, but we, as researchers, could find other probable mechanisms of parasite tolerance by considering the self-tolerance literature.

Concluding remarks

In an ecological and evolutionary light, the magnitude and duration of inflammatory responses should be predictable based on the costs and benefits of the response in terms of host fitness, although so far these ideas have been only indirectly substantiated. First, the cost-benefit outcome of an inflammatory host response is probably dependent on host life-history, parasite virulence, and the efficacy of a current inflammatory or anti-inflammatory response. Second, whereas inflammation is a key component of resistance to parasitic infection, anti-inflammatory responses and the resolution of inflammation are probably key mediators of tolerance.

Although some tolerance mechanisms might not be directly related to anti-inflammation, investigation of the instigators and resolution of inflammatory responses could open new doors to immunological understanding. Ecological immunologists can benefit from the mechanistic approach of classic immunology by better appreciating the nuances of immunological processes and the concomitantly nuanced costs of such processes; there is rarely a monolithic "immunocompetence" to be measured. Likewise, ecological thinking could lend immunology a context upon which to improve human health.

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APPENDIX D: No effects of two anesthetic agents on circulating leukocyte counts or resistance to trematode infections in larval amphibians

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No Effects of Two Anesthetic Agents on Circulating Leukocyte Counts or Resistance to Trematode Infections in Larval Amphibians

BRITTANY SEARS,1,2 PAUL SNIEDER,1,3 AND JASON ROHR1

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ABSTRACT.—Pharmacological anesthetics are used frequently in aquatic animal husbandry and research. Although several studies have investigated the effects of these anesthetics on larval immune responses in fish, none have assessed such effects in amphibians. To address this disparity, we exposed Osteopilus septentrionalis tadpoles to 0.008% benzocaine, 0.1% tricaine methanesulfonate (MS-222), or artificial spring water, and quantified circulating white blood cells and susceptibility to infection by larval trematodes. Exposure to neither MS-222 nor benzocaine elevated circulating white blood cells relative to water-exposed tadpoles. Furthermore, anesthetic treatment did not affect resistance to larval trematode infection. These results indicate that the anesthetics tested here probably do not affect tadpole immune function and should aid researchers in determining anesthetic usage.

Anesthetic agents are utilized in animal husbandry and research to reduce stress and pain as well as in disease research, where anesthesia can answer important questions about the efficacy of behavioral versus physiological defenses against pathogenaire. For these reasons, anesthetics are widely used in aquaculture and basic research. However, anesthesia could cause unwanted side effects that might confound its benefits. Some effects of anesthetics, especially recovery and health postanesthesia, are well documented (Cakir and Strauch, 2005) but sublethal effects of anesthesia, such as immunological changes, are also an important area of study. Anesthesia might reduce immune responses through reduced cellular function (e.g., Azuma et al., 2000) or, conversely, anesthesia might act as a transient stimulus and improve immune functions. Although long-term anesthesia reduces immune function, transient stressors (seconds–minutes) can augment or at least mobilize and redistribute immune responses (Sapolsky et al., 2000; Dhabhar and McEwen, 2001; Kuhlman and Martin, 2010). However, it is unclear whether anesthesia boosts, suppresses, or has no effect on immune function. Therefore, a careful assessment of the physiological effects of anesthesia can inform both husbandry and research.

Most anesthesia research on aquatic animals has been conducted on fish (e.g., Orton et al., 2002; Cuesta et al., 2003; Gomulka et al., 2008), despite the ubiquitous usage of anesthetics on amphibians. In fact, of 89 records obtained from the Web of Knowledge with the use of the search “amphib” AND “anesthet”*, none assessed immunological effects of anesthesia on amphibians. Understanding the effects of anesthetics on immune responses is particularly important to amphibian research because anesthetic agents are utilized to investigate experimentally antiparasite behavior of tadpoles in response to larval trematodes (e.g., Koprivnikar et al., 2006; Daly and Johnson, 2011). Anesthetic immunosuppression or immunoenhancement might confound research on the efficacy of behavior for preventing infections.

Here we report the effects of two anesthetic agents, tricaine methanesulfonate (MS-222) and benzocaine, on immune responses in tadpoles of the Cuban Treefrog, Osteopilus septentrionalis. We examine both a proxy for immune function, circulating white blood cells (WBC), as well as a functional measure, resistance to larval trematodes. We hypothesized that both anesthetic agents increase circulating white blood cells because anesthesia acts as a transient stressor. Given that larval trematodes can complete encystment in tadpoles 4–8 h postinfection (Fried et al., 1997; Holland, 2009), we predicted that brief anesthesia treatment would reduce successful trematode infection because the transient immune-enhancing effects of stress could improve tadpoles’ resistance to parasitic infections.

MATERIALS AND METHODS

Effects of Anesthetic Agents on Circulating White Blood Count (WBC).—Osteopilus septentrionalis tadpoles (Gosner stage 25–30) were collected in Port Charlotte, Florida in September 2009 and housed for 2 weeks prior to experimentation in 38-L aquaria of artificial spring water (ASW; Cohen et al., 1980). A 0.005% benzocaine solution was prepared as described by Vanable (1985). Preliminary trials indicated that 15 min of exposure to 0.005% benzocaine was sufficient to induce 10 min of anesthesia. MS-222 was prepared as a 0.1% solution in ASW. Solutions in ASW buffered the solution to pH 6.8. Ten-minute exposure to 0.1% MS-222 induced 10-min anesthesia. Although these concentrations differ, they are both common working concentrations recommended by published literature (e.g., Vanable, 1985; Azuma et al., 2000; Wright, 2001) and require similar induction times to induce 10 min of unresponsive anesthesia. Furthermore, although concentrations of benzocaine in excess of 0.005% produced almost immediate anesthesia, they also resulted in highly variable recovery times and, in one case, tadpole death (unpublished data).

Tadpoles were divided into three treatments: benzocaine-anesthetized (N = 32), MS-222-anesthetized (N = 29), and sham, ASW-exposed control (N = 36). Within the control treatment, five tadpoles comprised an unstressed control group and were sacrificed immediately after removal from aquaria and received no sham ASW exposure. These unstressed control animals served as a baseline for circulating lymphocyte levels prior to the transient stress of confinement and anesthesia. All other tadpoles received their respective exposures in 100-ml specimen cups with a 30-ml volume of benzocaine, MS-222, or ASW. After exposure, tadpoles were rinsed with ASW and placed into individually labeled 1-L plastic aquaria containing fresh ASW. Benzocaine, MS-222, and ASW solutions were reused within treatments.

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Table 1. Sample sizes of tadpoles per anesthesia treatment and hour sampled. n/a indicates that an unstrained control was not applicable for this treatment because a transient stressor, confinement to a specimen cup, was necessary to induce anesthesia.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0</th>
<th>2</th>
<th>6</th>
<th>12</th>
<th>24</th>
<th>48</th>
<th>72</th>
<th>168</th>
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<tr>
<td>Control</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>5</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Benzocaine</td>
<td>n/a</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>3</td>
<td>5</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>MS-222</td>
<td>n/a</td>
<td>3</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>4</td>
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</tr>
</tbody>
</table>

Tadpoles in each treatment were sacrificed by decapitation at 0, 2, 6, 12, 24, 48, 72, and 168 h postanesthetic exposure (Table 1). Blood was collected in heparinized capillary tubes (Fisher Scientific), transferred to glass microscope slides, and smeared into a thin film. Slides were fixed with methanol, then stained in benzidine followed by Giemsa according to Raffel et al. (2006). Coverslips were affixed to smears with adhesive (Permount®, Fisher Scientific) and slides were assigned random numbers by a technician naïve to the experimental design to prevent bias in blood cell quantification. Slides were examined on a light microscope at ×1,000 magnification under oil immersion. Circulating blood cells were quantified by counting all blood cells on a slide until 5,000 erythrocytes had been counted. Numbers of lymphocytes, thrombocytes, neutrophils, eosinophils, and basophils were identified according to Claver and Quaglia (2009) and counted and recorded as a proportion of 5,000 erythrocytes. We detected monocytes in only one sample (24-h control), and therefore did not include monocytes or macrophages in our analyses. During the counting process, it became apparent that eosinophils and basophils could not be reliably differentiated from one another, so counts for both blood cells were combined into an eosinophil/basophil category for analysis.

**Effect of Anesthetic Agents on Resistance to Trematode Infection.** —Because the presence or absence of changes in circulating WBC might not represent immune function and infection outcome accurately, we also assessed whether the administration of benzocaine or MS-222 could reduce resistance to trematode infection. *Ostertagia seminulans* tadpoles for this study were collected in Tampa, Florida in July 2010. We collected *Planorberla bimorbis* snails, a common host for trematodes, from a wetland in Tampa, Florida, and assessed infection status by the presence or absence of free-swimming armature cercariae, which infect tadpoles. Infected snails were housed in 1-L aquaria containing ASW; prior to experimental infections, snails were transferred to aquaria with fresh ASW in order to obtain freshly shed cercariae. Because cercariae only have a 24-h life span and several-hour-old cercariae are more infective than those that are young or old (Fried et al. 1997), we used cercariae that were 2–4 h old. Cercariae were obtained from infected snails’ water with the use of a micropipette under a dissecting microscope.

For experimental infections, tadpoles were separated into control, benzocaine, and MS-222 treatments (n = 13 per treatment). Benzocaine and MS-222 solutions were prepared and administered as described above. After anesthesia, tadpoles were rinsed with ASW and transferred to 100-ml specimen cups with fresh ASW; upon recovery of normal swimming behavior, 0, 15, or 30 cercariae were added to each tadpole’s container (n = 3, 5, and 5 per anesthesia treatment, respectively). After 24 h, cups were examined for any remaining cercariae and none were found. Tadpoles were then transferred to 1-L plastic containers with fresh ASW and maintained for 7 d, then euthanized in an overdose of 0.5% benzocaine and preserved in 70% ethanol. Specimens were cleared according to (Hanke and Wassersug, 1981) to make encysted metacercariae visible. Successful cercarial infection was detected by observation of metacercarial cysts under a light microscope at ×100 magnification; parasites were quantified as a proportion of the total cercariae administered (15 or 30).

**Statistical Analyses.** —The effects of handling stress on WBC in stressed versus unstrained ASW-exposed tadpoles were analyzed with the use of a one-way ANOVA on log-transformed WBC counts. Protozoan parasites of unknown species were detected in blood smears for 19 of the 118 tadpoles in the experiment; these tadpoles were excluded from analyses. Two slides that were unreliable because of poor staining were also excluded. After these exclusions, only one data point remained for 168 h postanesthesia and this sample was also excluded. WBC counts were then log-transformed and analyzed with the use of multivariate general linear models controlling for the researcher counting the cells and testing for an effect of treatment, time, and their interaction. The main and interactive effects of anesthetic treatment and cercarial dose on ascitic square-root-transformed trematode encystment were analyzed with the use of factorial ANOVA. All analyses were performed with the use of Statistics v6.1 (Statsoft, Inc., Tulsa, Oklahoma).

**RESULTS**

**Effects of Anesthetic Agents on Circulating White Blood Cells.** —Handling stress and confinement to specimen cups did not affect circulating WBC significantly (Fig. 1; Wilks’ F<sub>4,80</sub> = 0.624, P = 0.67). Neither anesthetic treatment nor time postexposure were significant predictors of circulating WBC (Wilks’ F<sub>6,144</sub> = 1.32, P = 0.23; Wilks’ F<sub>2,48</sub> = 0.47, P = 0.77, respectively; Fig. 2), nor was there a significant interaction between time and anesthetic.
least granulocytes (neutrophils, eosinophils, basophils), which are important to resistance against macroparasites (Janevsky, 2008), are unaffected by anesthesia. However, although WBC abundance was unaffected by anesthesia, we probably did not assess the functionality of nongranolocytes, nor how that function might translate to effects on resistance against non-trematode pathogens. For example, lymphocytes cannot be differentiated into subsets microscopically (e.g., B vs. T cells; effector vs. regulatory T cells). Similarly, one cannot microscopically assess the functionality of thrombocytes, which are typically considered clotting cells with a role similar to platelets in mammals, but which may assume immunological roles in nonmammalian vertebrates (Köllner et al., 2004).

Our failure to detect effects of anesthesia on immune function is good news to many aquaculturists and researchers using anesthetics, but some users may still prefer to use either MS-222 or benzocaine according to each chemical’s advantages and disadvantages. For example, MS-222 is soluble in water, whereas benzocaine requires dissolution in ethanol before becoming water-soluble—but benzocaine is far less expensive. Although some have reported benzocaine anesthesia to result in variable recovery times (Crook and Whitman, 2006), we found this not to be the case, in keeping with the findings of other researchers (Vanale, 1985; Cecala et al., 2007). In summary, we find both MS-222 and benzocaine to be safe, reliable agents of tadpole anesthesia, with no effects on the immune responses we quantified.

Acknowledgments.—We thank T. Raffel for assistance in identifying white blood cells as well as S. Reed, B. Bustamante, and S. Craig for quantifying white blood cells. Both snails and tadpoles were collected in accordance with permit WX8508179 from the State of Florida Fish and Wildlife Conservation Commission. Laboratory work was conducted according to Institutional Animal Care and Use Committee at the University of South Florida (W3867).

LITERATURE CITED
CAREY, Y., AND S. M. STRADEL. 2005. Tricaine (MS-222) is a safe anesthetic compound compared to benzocaine and pentoobarbital to induce anesthesia in Leopard Frogs (Rana pipiens). Pharmacological Reports 57:465–474.
NO IMMUNOLOGICAL EFFECTS OF ANESTHESIA


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University of South Florida
Google Scholar
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