Escape from the pond: stress and developmental responses to ranavirus infection in wood frog tadpoles

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Summary

1. Animal populations exhibit considerable variation in their susceptibility to infection by emerging diseases, yet it is poorly understood how environmental and intrinsic factors contribute to these patterns. Considering that intrinsic factors (e.g. life history stage, nutritional state) can impact immune function, knowledge of the physiological mechanisms that mediate susceptibility to infection may improve our understanding of the emergence of disease in natural populations.

2. Ranavirus outbreaks have been associated with die-offs of amphibians worldwide. While the ecological factors associated with epidemics have been widely studied, little is known about how physiological factors mediate amphibian responses to ranavirus infection.

3. The neuroendocrine hypothalamus-pituitary-interrenal axis (HPI) is a physiological system central to coordinating energy balance and development. It is known to both stimulate and inhibit immune function in vertebrates in different contexts. We hypothesized that the HPI axis would also mediate responses to ranavirus infection. We used wood frog (\textit{Rana sylvatica}) larvae and ranavirus isolated from recent die-offs of local wood frog populations to examine the physiological responses to infection.

4. In addition to increasing odds of death with increasing doses of virus in an LD\textsubscript{50} study, we saw a 1-7-fold increase in the odds of death with each increase in Gosner stage at the time of infection.

5. We then examined the HPI stress response of prometamorphic tadpoles exposed to a lethal dose of ranavirus. Infected tadpoles exhibited significantly elevated corticosterone levels, more rapid developmental changes, and a greater decrease in body weight relative to controls over 6 days after exposure.

6. Although elevated corticosterone mobilizes resources and enhances immunity, its acceleration of metamorphosis may be maladaptive in response to ranavirus infection, because it can draw energy away from expensive immune responses. These findings provide insight into how the balance of energy between development and immune function may contribute to patterns of ranavirus infection in pre-metamorphic amphibians.

Key-words: ranavirus, emerging disease, dose effects, corticosterone, stress response, development, wood frog

Introduction

Emerging diseases, such as chytridiomycosis and ranavirus infection, cause mass mortality and even extinctions in amphibian populations worldwide (Carey 2000; Lips \textit{et al.} 2006; Gray, Miller & Hoverman 2009; Kilpatrick, Briggs & Daznak 2010). While it is known that amphibian populations vary in their susceptibility to infection (Carey, Cohen & Rollins-Smith 1999; Brunner, Richards & Collins 2005; Cotter \textit{et al.} 2008; Lips \textit{et al.} 2008; Garner \textit{et al.} 2009), the underlying causes of this variation are unclear. Environmental factors such as pollution and environmental change are often invoked to explain these differences because they can directly or indirectly decrease the immune competence
of amphibians (Carey 2000; Rohr et al. 2004; Lips et al. 2006; Gray, Miller & Hoverman 2009; Kilpatrick, Briggs & Duffus 2010). A potentially important, but poorly understood factor underlying such variation is how amphibian immune function varies with intrinsic (e.g. nutritional state) and extrinsic factors (e.g. pollution) (Maniero & Carey 1997; Carey 2000; Demas 2004; Chinchar et al. 2009). Without this knowledge it is difficult to generalize how amphibians will respond to disease under differing environmental stressors and climate change scenarios.

The neuroendocrine hypothalamus-pituitary-interrenal (HPI) axis is likely central to understanding how amphibian immunocompetence varies with intrinsic and extrinsic factors (Rollins-Smith 2001; Denver 2009a). While the HPI axis and the broader neuroendocrine system translate environmental and intrinsic information into developmental responses (reviewed in Denver 2009b), stress induced activation of the HPI axis also likely modulates immune function (Rollins-Smith 2001; Denver 2009a). During the initial stages of an immune response, inflammatory cytokines activate the HPI axis, stimulating the release of corticotropin-releasing factor in the brain and glucocorticoids in the periphery, which act to increase metabolism, release glucose from stores, and suppress the thyroid and gonadal axes (Haddad, Saadé & Safieh-Garabedian 2002). Glucocorticoids also are critical, permissive factors that stimulate B-cell and antibody production in vivo (Dhabhar 2009). Although chronic activation of the HPI axis is immunosuppressive, recent studies have shown that acute stress and elevated glucocorticoid levels imposed immediately before a challenge enhance inflammatory cytokine processes as well as lymphocyte proliferation and distribution (Haddad, Saadé & Safieh-Garabedian 2002; Demas 2004; Viswanathan, Daugherty & Dhabhar 2005; Dhabhar 2009). Beyond the first few hours or days of an acute immune response, however, elevated glucocorticoids provide negative feedback on both the HPI axis and the immune system. Assuming that an initial infection has cleared this immunosuppressive effect helps return an animal to a homeostatic state (Haddad, Saadé & Safieh-Garabedian 2002; Dhabhar 2009). Thus, actions of the HPI axis help to modulate an immune response to infection and balance resource allocation to meet both short-term and long-term survival needs.

The timing of infection during development, however, could influence amphibian immune function because the HPI axis also plays an important role in coordinating metamorphosis (Denver 2009a). Particularly, during later tadpole stages, beyond hind limb growth and after maturation of the thyroid axis, environmental stressors are known to accelerate development rates (Denver 2009a). This developmental plasticity is thought to be adaptive because this process allows individuals to escape adverse conditions in the aquatic environment and increase survival (Denver 1998; Boorse & Denver 2004). Metamorphosis is an energetically costly endeavour, however, that entails massive cellular turnover, and tissue restructuring of both the soma and immune system (Denver 1997; Rollins-Smith 1998). Consequently, if a persistent pathogen infection occurs during late stages of metamorphosis, there may be a conflict in allocation between development and an energetically costly immune response (Lochmiller & Deerenberg 2000; Demas 2004; Martin, Weil & Nelson 2008). This energetic trade-off could have profound effects on amphibian immunocompetence and disease epidemiology (Carey, Cohen & Rollins-Smith 1999; Harp & Petranka 2006; Cotter et al. 2008; Gervasi & Foufopoulos 2008).

Indeed, the epidemiology of reported ranavirus outbreaks in which late-stage larval amphibians nearing metamorphosis were predominately affected (Green, Converse & Schrader 2002; Duffus et al. 2008; Gray, Miller & Hoverman 2009), suggests that environmental and intrinsic factors may be important drivers of its emergence. Ranaviruses (family Iridoviridae) are directly transmitted, often lethal viruses of ectothermic vertebrates that cause mass die-offs in amphibians worldwide (Green, Converse & Schrader 2002; Chinchar et al. 2009; Gray, Miller & Hoverman 2009). While the adult amphibian immune system produces robust responses to ranavirus infection that includes both the innate immune system (macrophages, neutrophils and antimicrobial peptides in the skin), and the adaptive immune system (CD8 T cells and antibodies) (Chinchar et al. 2004; Maniero et al. 2006; Morales & Robert 2007; Robert & Ohta 2009), larval amphibians may be more susceptible to infection. This is because functionally, amphibians have two immune systems. Adults have a unique, more diverse ensemble of lymphocytes, antibody repertoire, and cell types not found in tadpoles (Flajnik et al. 1987; Rollins-Smith 1998). Further, many components of the larval immune system such as circulating lymphocytes are down regulated or cleared during metamorphosis, presumably to avoid recognizing and attacking newly developed adult tissues (Rollins-Smith, Barker & Davis 1997; Rollins-Smith 2001). Because of potential energetic trade-offs and the intense restructuring of the immune system, metamorphosis can have profound, but little explored effects on the immunocompetence and the subsequent epidemiology of disease outbreaks in amphibians (Rollins-Smith 1998; Carey, Cohen & Rollins-Smith 1999; Harp & Petranka 2006; Cotter et al. 2008).

Here, we present experimental data that provides basic insight into the role that the HPI axis plays in mediating ranavirus infection in wood frog tadpoles, Rana sylvatica (Fig. 1; nomenclature following (Hillis 2007). The specific goals of this study were twofold. First, we conducted an LD50 experiment to establish the patterns of mortality imposed by ranaviruses isolated from recent die-offs. Second, we profiled the changes in development stage, body condition and glucocorticoid content of tadpoles exposed to a lethal dose of this ranavirus during 6 days of infection to characterize the relationship between HPI activity and resource allocation patterns during infection.

**Materials and methods**

**SOURCE OF VIRUS AND HOSTS**

All wood frog tadpoles used in this study were collected as eggs from a single ephemeral pond on the grounds of the Vassar College...
Ecological Preserve, and maintained in captivity in accordance with Vassar College institutional animal care and use committee (09-04B). Prior to the experiments tadpoles were maintained in outdoor 50 gallon cattle tanks stocked with aged well-water and leaf detritus (a substrate typical to local ponds) and phytoplankton and zooplankton from nearby ponds. Four days prior to experiments, animals were transferred to Vassar College’s animal care facility and housed at room temperature, 12L:12D photoperiod, and fed ad libitum alfalfa pellets. During experiments the tadpoles were provided ad libitum alfalfa pellets and were transferred to new containers with clean water weekly.

We used two virus isolates from die-offs in two vernal pools in the Teatown Lake Reservation in the Hudson Valley, NY in the summer of 2008 (Brunner, J. et al. unpublished data). One virus was isolated from a recently dead wood frog tadpole, the other from a dead larval spotted salamander. The virus was grown in Flathead minnow cells for two passages from the original animals and titered by a plaque assay on Epithelioma papilloma cyprini cells. These two isolates are identical in c. 500 bp of sequence of the major capsid protein gene [amplified using primers MCP 4/5; (Mao et al. 1999)] and 100% similar to the type ranavirus, Frog Virus 3 (FV3).

**LD-50 Design and Exposure**

We measured the susceptibility of wood frog tadpoles to ranavirus using an LD50 design. Sixty tadpoles at Gosner stages 27–41 (Gosner 1960) were randomly assigned to one of five concentrations of virus (10-fold dilutions from 2.36 × 10^3 through 2.36 × 10^5 plaque forming units (pfu) mL^-1 for the wood frog isolate; 2.51 × 10^5 through 2.51 × 10^3 pfu mL^-1 for the spotted salamander isolate) created by serial dilutions in well-water from the Vassar Preserve. Each tadpole was weighed, staged and placed in individual 300 mL plastic containers with 200 mL of the appropriate concentration of virus for 24 h, after which they were transferred to and housed in individual 700 mL plastic containers with c. 450 mL of virus-free well-water in a room maintained at a temperature of 20 °C (range = 18–21 °C). To avoid cross-contamination clean plastic screen nets were used to transfer each animal and containers and equipment were disinfected with 10% bleach between uses.

Animals were visually examined daily for symptoms of infection (papules, lesions and altered behaviour), metamorphosis (defined as Gosner stage 42) and mortality. Dead animals were frozen at –80 °C and later screened for infection with standard PCR diagnostic tests (Greer & Collins 2007). Twenty-two days after exposure the remaining tadpoles were euthanized by exposure to an overdose of MS-222.

The probability of death in this LD50 study was analysed using logistic regression (glm function in R 2.10) with log(K0 [virus], virus isolate, and Gosner stage as independent predictors. The timing of mortality was analysed using parametric survival analyses (Surv function in R, 2.10) assuming a lognormal distribution of times to death (although results were qualitatively the same with a weibull distribution) and the same three predictors.**

**Stress Response to Infection**

Informed by these data, we then tested for stress axis and developmental responses to infection in tadpoles exposed to 2.36 × 10^3 pfu mL^-1 of the wood frog ranavirus isolate, a dose that was shown to cause 100% mortality within 7 days (Fig. 2). Tadpoles (n = 142) at the prometamorphic Gosner stages of 37–39 were paired by size (visually estimated) and then split into infected and control treatments. Control tadpoles were given an identical handling protocol, but exposed to a similar concentration of cell culture medium free of virus. All animals were exposed and housed as described above.

Paired infected and control animals were randomly selected and euthanized by 0.1% benzoic acid solution at days one through six after exposure and frozen at –20 °C. Body weights, length and Gosner developmental stage were measured both prior to and after treatment exposure. Whole-body corticosterone (CORT) concentration was later measured in tadpoles (n = 96) by radioimmunoassay (RIA).

**Radioimmunoassay**

Whole-body corticosterone of each tadpole was measured by RIA following lipid extraction and thin-layer chromatography (TLC) to purify the samples. The procedures used followed those of Denver (1998), with some modifications. Briefly, total lipids were extracted by homogenization of each tadpole in 2 mL of ethyl acetate and the organic phase separated by centrifugation; the supernatant was isolated and dried by rapid evaporation. Steroids were fractionated on TLC plates (JT Baker Si250F, Phillipsburg, NJ, USA) placed in a mobile phase of toluene : cyclohexane (1 : 1), followed by chloroform : methanol (9 : 1) mobile phase (exposed twice). To determine recoveries and migration distance of CORT on the TLC plates samples were spiked, prior to loading on to TLC plates, with 3500 cpm tritiated CORT ([H]CORT; Perkin-Elmer, Waltham, MA, USA). Migration was measured in two extra tadpole samples, run on each TLC plate, by incrementally scraping 1-cm sections of the silica gel and determining the radioactive peak using a Beckman LS6500 scintillation counter. A 1 × 3 cm section of silica from each sample lane (corresponding to the [H] CORT peak) was scraped into a borosilicate tube, and the CORT extracted for 1 h with anhydrous ether : methanol (9 : 1). Samples were dried under nitrogen and resuspended in 0.5 mL of 0.2 mol L^-1 phosphate-buffered saline with 1% gelatin for RIA. Samples from each treatment and infection day were included on each TLC plate and RIA run. For the assay, recoveries were determined from 50 μL aliquots of each sample, while 400 μL of the sample was allocated to the assay. Serial dilutions for standard curves were performed in duplicate. Samples and standards were incubated for 3 h at 37 °C with 50 μL of antibody and 50 μL of 10 000 cpm tritiated CORT. Unbound steroid was separated using dextran-coated charcoal and the bound steroid

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decanted into scintillation vials. The inter-assay coefficient of variation between four runs was 8%, and mean intra-assay coefficient of variation was 1%.

The effect of ranavirus exposure on whole-body CORT (ng g\(^{-1}\) body weight) development and body weight were analysed by ANCOVA with treatment and day of infection as main effects, and Gosner stage and body length as covariates. The body condition of tadpoles was estimated as the least squares mean of body weight adjusted for snout-vent length (SVL) and developmental stage as covariates in an ANCOVA model (Packard & Boardman 1988; García-Berthou 2001). Because developmental stage could have a confounding affect on our measure of body condition through its influence on size, weight and development rate, we also controlled for stage (other than as a covariate in ANCOVA) by pairing control and treatment tadpoles by stage and approximate size at the start of the experiment, and then collected these pairs on days 1–6 after infection. Thus, all else being equal, differences in size, development rates, condition and CORT presumably should reflect the effects of infection. Post-hoc comparisons of treatment effects among animals sacrificed at different days were conducted using Tukey-Kramer's HSD test. Prior to these analyses, the data were tested for homogeneity of variance and confirmed to meet model assumptions. These analyses were performed in Minitab\textsuperscript{15} Statistical Software (Minitab Inc., State College, PA, USA).

**Results**

**Susceptibility to Ranavirus Infection**

Of the 53 tadpoles exposed to these FV3-like ranaviruses, 39 (74%) died. Twenty-three (59%) of these 39 dead tadpoles had at least one sign of infection (papules, oedema, skin sloughing, lethargy, or loss of balance) prior to death. Neither the probability nor the time to death depended on the identity of the virus isolate (\(z = 0.126, P = 0.900\) and \(z = 0.28, P = 0.781\), respectively), so these virus isolates were assumed to be the same for the following analyses. The best-fit logistic regression model of infection included log\(_{10}\)-dose of virus and Gosner stage at infection. In this model, the probability of death increased 16-fold with each 10-fold increase in dose of ranavirus (Fig. 2a, \(Z = 3.42, P < 0.001\)). The odds of death increased 1.7-fold with each increase in Gosner stage at the time of infection (Fig. 2b, \(Z = 2.68, P = 0.007\)). Metamorphosis during infection did not significantly increase the probability of death (best-fit model plus metamorphosis, \(Z = 0.006, P = 0.995\)), but only four tadpoles metamorphosed and so this parameter could not be estimated with confidence. The LD\(_{50}\) was 10\(^{-2.37}\) pfu mL\(^{-1}\) (95% confidence interval: 10\(^{-2.01}–10^{-2.73}\) pfu mL\(^{-1}\)). Death occurred more rapidly at higher doses (Fig. 2c, Cox proportional hazard = 2.66, \(P < 0.001\)), but the time to death did not change with Gosner stage at infection or metamorphosis.

**Stress and Developmental Responses to Infection**

In the stress experiment 37 of the 71 tadpoles exposed to ranavirus showed signs of infection by the fourth day after exposure, eight of which died on days 5 and 6 of exposure.

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Fig. 2. Dose-dependent effects of ranavirus infection on mortality rates in wood frog tadpoles. (a) The proportion of tadpoles that died when exposed to each of five doses of ranavirus (open symbols) as described by a best-fit logistic regression (solid black) with 95% confidence interval (dotted line). The grey lines show the predicted LD\(_{50}\) dose. (b) The LD\(_{50}\) odds of death as a function of Gosner developmental stage at infection. Grey symbols represent the number of tadpoles in the experiment at each developmental stage. (c) The number of tadpoles that died at each day of the experiment (solid symbols) or were euthanized (open symbols) after exposure to each dose of virus. Solid line is median time to death as predicted by a log-normal parametric survival model.
These eight dead animals were not analysed for CORT content. No control animals (n = 71) were symptomatic of viral infection, or died during the course of the stress experiment. Prior to infection control and infected animals did not differ in developmental stage (both groups Gosner stage = 37 ± 0.7, mean ± SD), SVL (both groups = 14.3 ± 0.1 mm) or body weight (control = 0.59 ± 0.16 g; infected = 0.56 ± 0.14 g). The control animals exhibited changes in CORT content, developmental stages, and body condition expected for late-stage tadpoles undergoing metamorphosis (Fig. 3). Infected tadpoles, however, had significantly higher whole-body CORT concentrations relative to control tadpoles (Fig. 3a, F1,87 = 8.59, P = 0.004; ANCOVA with stage post-infection as a covariate). Note that data for day-two after exposure were excluded from all analyses because body weights post-infection were not recorded. Infection also accelerated development rates, measured as the difference in Gosner stage from just before exposure to the time of sacrifice (Fig. 3b, F1,87 = 6.39, P = 0.01; ANCOVA with stage prior to infection as a covariate), and reduced body condition, measured as body weight adjusted for SVL (Fig. 3c, F1,87 = 11.94, P = 0.001; ANCOVA with after infection as a covariate). Developmental stage post-infection was also included as a covariate in the model but did not have a significant effect on body weight (F1,81 = 5.21, P = 0.3) or interaction with SVL. Note that SVL did not differ between treatments at any collection day after exposure, and did not change significantly from measurements prior to infection. Within any given day of infection, however, the differences between infected and control tadpoles in mean CORT content, developmental rates, or body weight adjusted for SVL were not significantly different (Tukey HSD test, P > 0.05). There were no interactions between treatment and days of infection in any of these models.

Discussion

Our LD50 study produced three notable results: (i) Wood frog tadpoles are highly susceptible to infection by FV3-like ranaviruses; (ii) the probability and rate of mortality increases significantly with the dose of inoculum (Fig. 2), which is consistent with previous studies (Brunner, Richards & Collins 2005; Duffus et al. 2008) and (iii) prometamorphic tadpoles (stages 35–40) infected shortly before metamorphic climax are more likely to die (Fig. 2b). This last result is consistent with the patterns of mortality observed in recent die-offs, in which late-stage larval amphibians were predominately affected (Green, Converse & Schrader 2002; Duffus et al. 2008; Gray, Miller & Hoverman 2009; Une et al. 2009), but it begs the question: Why are late-stage larvae that are nearing metamorphosis more susceptible to infection and mortality by ranavirus? Our second experiment in which we exposed late-staged tadpoles to a lethal dose of ranavirus offers some intriguing potential explanations for how interactions among physiological, immunological, and developmental states may influence the susceptibility of amphibians to emerging diseases.

In our second experiment, we observed a clear increase in whole-body CORT concentrations in infected tadpoles compared with uninfected-control tadpoles (Fig. 3a), which shows that the HPI axis is activated in response to ranavirus infection. The function of the HPI axis in this context is both
In addition to supporting immune function through energy mobilization, activation of the neuroendocrine stress axis also played a role in accelerating metamorphosis via corticotropin-releasing factor stimulation of the thyroid axis and synergistic effects of CORT and thyroid hormone on the breakdown of larval structures and tissue reorganization associated with metamorphosis (Denver 2009b). The control animals in our study, for example, exhibited a steady increase in CORT (Fig. 3a) that corresponded with their developmental progression through the late stages of metamorphosis (Fig. 3b); a pattern common to tadpoles approaching metamorphosis (Rollins-Smith, Barker & Davis 1997; Denver 1998). The infected tadpoles exhibited significantly faster development rates than control animals (Fig. 3b). This result suggests that the precocious elevation in CORT induced by ranavirus infection was also driving faster metamorphosis. Whether this interaction is an adaptive response to infection, or simply a by-product of elevated CORT is an open question. Amphibians adapted to temporary habitats do exhibit surges in CORT in response to environmental challenges such as pond drying and predators, which has been shown to speed development and hasten metamorphosis (Boorse & Denver 2004; Denver 2009a). Life history theory and empirical evidence demonstrate that with an increased risk of mortality in the larval environment, a faster metamorphosis at a smaller size can be adaptive (Wilbur & Collins 1973; Travis 1984; Morey & Reznick 2004).

Metamorphosis and immune responses to infection, however, are both energetically costly (Lochmiller & Deerenberg 2000; Demas 2004; Martin, Weil & Nelson 2008). Indeed, in addition to having elevated levels of CORT and faster development relative to control animals, infected tadpoles also had more dramatic decreases in body condition (Fig. 3c). While metamorphosing amphibians do not eat, the greater decrease in body condition of infected animals presumably reflected the extra nutritional costs of both faster metamorphosis and the mounting of an immune response to infection. One explanation for the observed epidemiology of ranavirus die-offs, in which late-stage tadpoles are predominately affected, may thus lie in energetic trade-offs between development and a costly immune response (Carey, Cohen & Rollins-Smith 1999; Harp & Petranka 2006; Gervasi & Foufopoulos 2008; Garner et al. 2009).

Because tadpoles undergoing metamorphosis have already committed substantial resources to development, the energetic costs of also simultaneously mounting a vigorous immune response to viral infection may simply be too much. Consequently, tadpoles may not have the energy stores or resources to sustain the immune response necessary to clear an infection and are thus overwhelmed by viral replication. If this hypothesis is correct, then tadpoles at a given stage that are in better condition should be more likely to survive a ranavirus infection compared with those in poor condition (Fig. 4). Although the HPI axis is central to such dynamics, the mechanisms by which it integrates intrinsic nutritional state information with environmental cues to counter...
pathogenic infections, however, remains unclear (Demas 2004; Nelson 2004; Hu, Crespi & Denver 2008).

There is an alternative explanation, however, for the increased susceptibility of late-stage tadpoles to ranavirus. As noted before, the extensive reorganization and replacement of larval tissues during metamorphosis extends to the immune system. During metamorphosis, some components of the larval immune system are down regulated or cleared, presumably to avoid these cells recognizing newly formed tissues as foreign (Rollins-Smith, Barker & Davis 1997; Rollins-Smith 1998). In fact, CORT itself leads to the apoptosis of lymphocytes during metamorphosis. Thus, while elevated CORT may normally be an adaptive stress response to an environmental challenge, the surge of CORT during metamorphosis may actually make tadpoles more vulnerable to infectious agents such as ranavirus. If this hypothesis is correct, then late stage tadpoles are inherently more susceptible to ranavirus infections.

The developmental state of the immune system and energetic trade-offs could both influence tadpole susceptibility, but they would have different consequences for amphibian ecology and evolution. If energetic trade-offs are a strong determinant of increased susceptibility, then at least those individuals in good condition would be likely to survive infections. Ranavirus outbreaks would then be another selective agent favouring larger animals with more rapid development. If instead, tadpoles immunocompromise themselves during metamorphosis, then selection might favour those tadpoles that delay their metamorphosis until after an epidemic. Regardless of which of these possible mechanisms dominate, our results strongly suggest a central, albeit potentially context-dependent, role for the neuroendocrine HPI axis in coordinating development, immune function, and energy use during ranavirus infection. Because pollution, habitat loss and climate change are imposing unprecedented levels of environmental stress on animals, a deeper understanding of the interactions between the neuroendocrine and immune systems is fundamental to our understanding and ability to conserve amphibians in the face of environmental change and emerging disease (Rohr et al. 2004; Forson & Storfer 2006; Garner et al. 2009; Kerby & Storfer 2009; Kilpatrick, Briggs & Daszak 2010).

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