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*Original Contribution*

# Natural Stressors and Ranavirus Susceptibility in Larval Wood Frogs (*Rana sylvatica*)

Brooke C. Reeve,[1] Erica J. Crespi,[2] Christopher M. Whipps,[1] and Jesse L. Brunner[1,2]

[1]*Department of Environmental and Forest Biology, State University of New York College of Environmental Science and Forestry (SUNY-ESF), Syracuse, NY*
[2]*School of Biological Sciences, Washington State University, P.O. Box 644236, Pullman, WA 99164*

**Abstract:** Chronic exposure to stressors has been shown to suppress immune function in vertebrates, making them more susceptible to pathogens. It is less clear, however, whether many natural stressors are immunosuppressive. Moreover, whether stressors make disease more likely or more severe in populations is unclear because animals respond to stressors both behaviorally and physiologically. We tested whether chronic exposure to three natural stressors of wood frog tadpoles—high-densities, predator-cues, and low-food conditions—influence their susceptibility to a lethal ranavirus both individually in laboratory experiments, and collectively in outdoor mesocosms. Prior to virus exposure, we observed elevated corticosterone only in low-food treatments, although other treatments altered rates of growth and development as well as tadpole behavior. None of the treatments, however, increased susceptibility to ranavirus as measured by the proportion of tadpoles that became infected or died, or the time to death compared to controls. In fact, mortality in the mesocosms was actually *lower* in the high-density treatment even though most individuals became infected, largely because of increased rates of metamorphosis. Overall we find no support for the hypothesis that chronic exposure to common, ecologically relevant challenges necessarily elevates corticosterone levels in a population or leads to more severe ranaviral disease or epidemics. Conditions may, however, conspire to make ranavirus infection more common in metamorphosing amphibians.

**Keywords:** crowding, immunosuppression, nutritional stress, predator cue, ranavirus, stress

## Introduction

Prolonged environmental stress is widely thought to suppress immune function, decreasing resistance to and increasing the severity of infectious disease, and thus elevating the risk of disease outbreaks (Pickering and Pottinger 1989; Sheridan et al. 1994). Stress has therefore been implicated as a driver of disease emergence in wildlife (Carey et al. 1999; Acevedo-Whitehouse and Duffus 2009; Martin et al. 2010). In light of the growing impacts of emerging infectious diseases on wildlife populations (Daszak et al. 2001; Plowright et al. 2008), there is a need to relate environmental conditions—both natural and anthropogenic—to stress responses and disease susceptibility.

Stress can be defined in ecological terms as a physical or behavioral state engaged to endure, avoid, or recover

*Correspondence to:* Jesse L. Brunner, e-mail: jesse.brunner@wsu.edu

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from adverse condition (Martin 2009). Physiologically, this state is partially mediated by glucocorticoids, the peripheral hormones of the neuroendocrine stress axis (hypothalamic–pituitary–interrenal [HPI] axis in amphibians). In the short term, glucocorticoids induce nutrient mobilization, increased heart rate, intensified reflexes, redistribution of leukocytes to peripheral tissues, and increased innate immune responses (Sapolsky et al. 2000; Dhabhar 2002; Hopkins and DuRant 2011). These responses enhance an organism's ability to deal with changes in environmental conditions and improve wound healing and infection resistance (Webster Marketon and Glaser 2008; Dhabhar 2009). In contrast, prolonged elevation of circulating glucocorticoids reduces numbers of circulating leukocytes, decreases cytokine production, and suppresses cell-mediated immunity (Elenkov and Chrousos 1999; Haddad et al. 2002; Dhabhar 2009). Elevated levels of plasma glucocorticoids are also associated with increased disease severity in many host–pathogen systems (Sheridan et al. 1994; Pickering and Pottinger 1989).

Natural environmental challenges, such as predation risk or competition, can chronically elevate glucocorticoid levels (Boonstra et al. 1998), and have therefore been implicated as possible drivers of disease emergence in wildlife (Bosch et al. 2001; Gray et al. 2009). Still, there is a need to explicitly link variation in glucocorticoid levels caused by environmental challenges to differences in disease susceptibility. Natural variation in food resources or conspecific density, for instance, may cause significant, if subtle increases in circulating glucocorticoids that adjust energy balance in animals (Sapolsky et al. 2000), but have no negative effects on the immune system. Alternatively, animals may have adapted to retain immune function in the face of common environmental challenges or modify their behavior to alter exposure (Wingfield et al. 1983; Sapolsky et al. 2000; McEwen and Wingfield 2003). In other words, what seems ''stressful'' a priori may not necessarily have deleterious effects on animals in the wild.

Understanding the relationship between environmental challenges, glucocorticoid responses, and disease susceptibility is a pressing issue for amphibians as emerging fungal and viral infections are leading to mass mortality events, local extinctions, and species declines (Collins and Storfer 2003; Gray et al. 2009; Teacher et al. 2010). As a result, studies have recently focused on how natural and anthropogenic environmental challenges influence disease in amphibians, with mixed results (Echaubard et al. 2010; Kerby et al. 2011; Koprivnikar 2010; Raffel et al. 2010; Haislip et al. 2012). While the negative effects of glucocorticoids on the tadpole immune system are well documented in the context of metamorphosis (Rollins-Smith 1998), and experimental elevation of glucocorticoids resulted in higher trematode burdens in gray tree frogs in one study (Belden and Kiesecker 2005), the links between environmental challenges, stress responses, and individual- and population-level susceptibility to disease are far from clear.

The objectives of the current study were to determine (1) whether three common environmental challenges—low food, high density, and predator cues—alter wood frog tadpole (*Rana sylvatica*) stress levels as measured by growth (development rates and size), whole-body concentrations of corticosterone (the primary glucocorticoid in amphibians), and behavior; and (2) whether chronic exposure to these challenges make tadpoles more susceptible to ranavirus infection. Tadpoles were challenged for several weeks and then exposed to a ranavirus—an often lethal emerging pathogen—in laboratory and mesocosm experiments. This study is the first to relate larval amphibian corticosterone concentrations to ranavirus infection susceptibility with multiple natural challenges, and among the first to do so at both the individual- and the population-level.

## Methods

In April 2010, wood frog egg masses were collected from vernal pools at the SUNY-ESF Adirondack Ecological Center (AEC) in New York and transported to the Syracuse campus. Tadpoles were reared to feeding stages (stage 25; Gosner 1960) in aged tap water, at which point they were fed algae discs (Hikari, Kyorin Co., Ltd., Japan) ad libitum. These experiments used an FV3-like virus (AEC37) isolated in a previous study (Brunner et al. 2011) from a wood frog tadpole die-off at the AEC and passed twice from the original animal. This research was approved by SUNY-ESF's Institutional Animal Care and Use Committee, protocol 2009–2011.

### Laboratory Experiment

Experimental tanks were 80 × 43 × 12 $cm^3$ semi-opaque, plastic containers filled with aged tap water. Tadpoles were maintained at ~18°C to prevent rapid development (Moore 1939) and a 13:11 h light:dark cycle. Tadpoles were fed algae discs at a rate of ~10% of the average body mass per tadpole per day (Werner 1992), recalibrated weekly, which is effectively ad libitum.

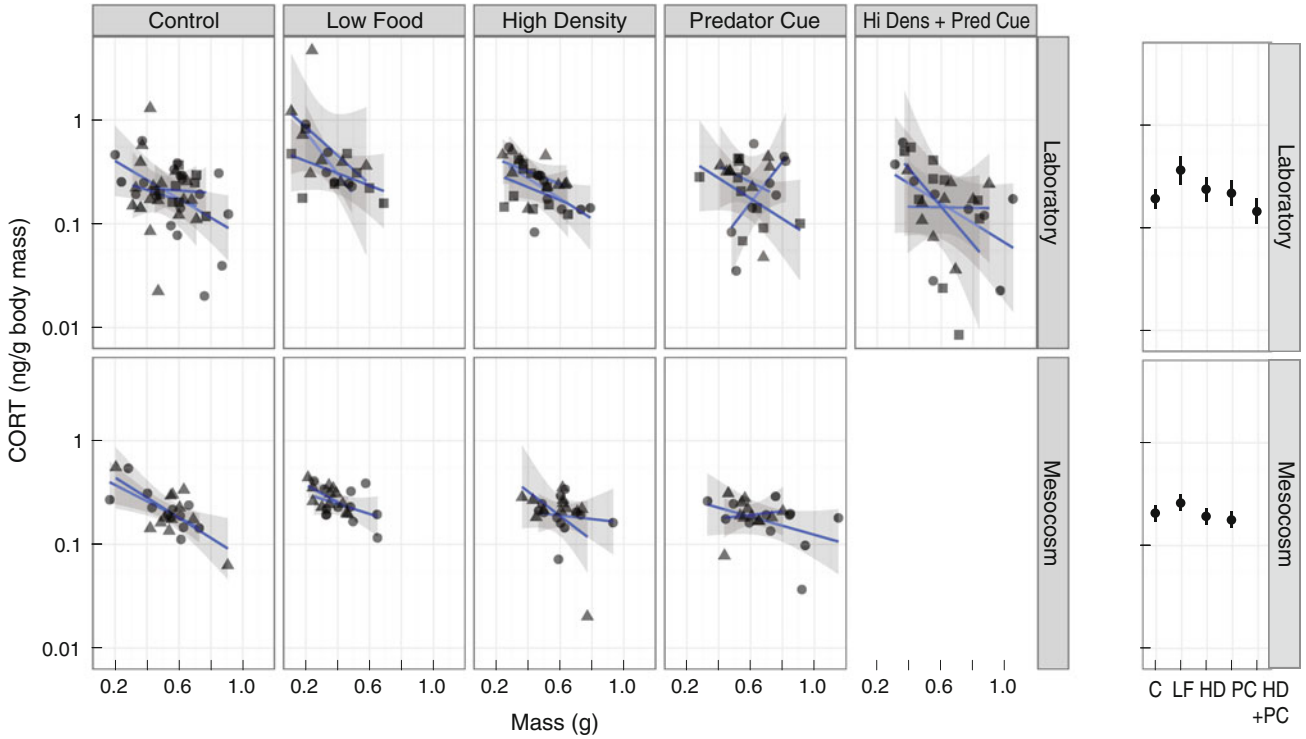


**Figure 1.** Relationship between log-transformed whole-body corticosterone (CORT) concentration (ng $g^{-1}$ body mass) and body mass after 21 days (lab) or 14 days (mesocosm) of exposure to experimental treatments. Each point is an individual. *Shapes* represent replicates within the treatment. *Lines* are linear regressions fit to each replicate and the *shaded areas* are their associated 95% confidence intervals. The marginal figures show the mean corticosterone concentration for each treatment adjusted for mass with 95% confidence intervals.

Tadpoles were weighed, assigned to one of 18 groups of 20, and relocated to an experimental tank. Each tank was randomly assigned one of four natural environmental challenges (low food, high density, predator cue, or high density plus predator cue), or to one of two control groups (one exposed to ranavirus and one that was not), with three replicate groups per treatment. At this point mean body mass was similar among groups (mean = 0.062 g, $F = 0.296$, $P = 0.586$). Tadpoles in the low food treatment were fed ~5% of the average body mass per tadpole twice a week (rather than ~10% daily) for the first three weeks of the experiment, then every 3 days for the last five weeks because some of the smallest individuals had died, apparently of starvation. Similar food restriction in stage 25 *R. pipiens* increased glucocorticoid levels (Glennemeier and Denver 2002).

High-density treatments tanks had an additional 40 tadpoles to triple their density (and were provided with triple the food to maintain per capita food levels), which equates to 174 versus 59 $m^{-2}$ in all other treatments. While tadpole population densities can reach 400 $m^{-2}$ in nature, morphological and behavioral differences have been noted between tadpoles raised at 25 and 75 $m^{-2}$ (Relyea 2002). High densities can increase corticosterone levels in tadpoles (Glennemeier and Denver 2002; Rot-Nikcevic et al. 2005) and has been hypothesized to be immunocompromising (Gray et al. 2009).

Predator cues were generated by feeding wood frog tadpoles to a dytiscid beetle larvae (Dytiscidae) or dragonfly larvae (families Libellulidae, Aeshnidae, and Cordululiidae), each in 400 mL of water. Water from multiple predators was pooled within 24 h of prey consumption and 400 mL added daily to each predator tank (similar to Fraker et al. 2009). The cue concentrations in the tanks were ~1.5–4.0 mg of consumed tadpole $L^{-1}$, within the range that has previously produced behavioral and morphological responses in larval anurans (Relyea 2002; Van Buskirk 2001).

When the majority of the tadpoles in a replicate had reached Gosner stages 33–35 (21 ± 1 days in all treatments except the low-food treatment, which took 40 days) 20 haphazardly selected tadpoles in each tank were euthanized

**Table 1.** The Proportion of Early Mortality (Before Virus Exposure), Mean Mass in Grams, and Mean Gosner Stage and Their Associated Standard Errors for Each Treatment in the Laboratory and Mesocosm Experiments.

| | Laboratory experiment | | | | | Mesocosm experiment | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Control | Low food | High density | Predator cue | Density + predator | Control | Low food | High density | Predator cue |
| Mortality (pre-RV) | 0.063 | 0.218 | 0.006 | 0.050 | 0.011 | 0.219 | 0.513 | 0.077 | 0.319 |
| Mass | $0.54 \pm 0.02^{a}$ | $0.36 \pm 0.03^{b}$ | $0.47 \pm 0.03^{a}$ | $0.60 \pm 0.03^{a}$ | $0.62 \pm 0.04^{a}$ | $0.53 \pm 0.02^{a}$ | $0.39 \pm 0.02^{a}$ | $0.60 \pm 0.03^{a}$ | $0.66 \pm 0.04^{a}$ |
| Stage | $33.0 \pm 0.3^{a}$ | $31.4 \pm 0.8^{a}$ | $32.8 \pm 0.3^{a}$ | $33.6 \pm 0.2^{a}$ | $33.4 \pm 0.4^{a}$ | $34.8 \pm 0.6^{ab}$ | $32.3 \pm 0.7^{a}$ | $37.0 \pm 0.5^{b}$ | $35.0 \pm 0.3^{a,b}$ |

The mass and stage means were measured from the tadpoles that were euthanized for corticosterone analysis just prior to ranavirus exposure. Treatments within each experiment that do not share letters have significantly different treatment means based on post-hoc Tukey tests.

with an overdose of MS-222 (Finquel®, Argent Chemical Laboratories, Redmond, WA) followed by severing the spinal cord, weighed, and then frozen at −20°C until the corticosterone assay. These stages were selected because corticosterone has yet to increase as it will during metamorphosis (Rollins-Smith 1998), so we can look for elevated corticosterone before it surges for other reasons, and because tadpoles at these stages are highly susceptible to ranavirus infection (Warne et al. 2011).

The remaining 20 tadpoles in each replicate were weighed and staged, and then exposed as a group to $1.25 \times 10^4$ plaque forming units (pfu) of ranavirus $mL^{-1}$ in 1 L. We used water-bath exposures to ensure consistency in exposure and because ranaviruses are naturally transmitted through the water (Brunner et al. 2007). Control tadpoles were mock-exposed in water. After the 24 h the tadpoles were individually housed in 500 mL containers with clean, aged tap water and the experimental treatments were continued. Approximately, 30 mL of predator cue water was added daily to each container in the predator cue treatment. Two additional tadpoles were added to each high-density container in order to maintain their threefold greater density (equivalent to 275 tadpoles $m^{-2}$). These extra tadpoles were marked with fluorescent visible implant elastomer (VIE; Northwest Marine Technology, Shaw Island, WA) while anesthetized to distinguish them from the unmarked focal animals. Containers and water were changed every 3 days, at which point the extra tadpoles were removed, euthanized, and replaced to preclude the possibility that they would become infected and shed virus into the containers. Tadpoles were checked daily and dead animals frozen at −80°C until virus screening. Those that survived 21 days post-virus exposure were euthanized with an overdose of MS-222 and frozen at −80°C.

## Mesocosm Experiment

The laboratory experiments examined the effects of the three challenges on individual wood frog tadpoles. In order to determine whether individual-level responses to these challenges predict those of a whole population, particularly in light of how these challenges might influence tadpole movements, contacts, and thus ranavirus transmission, we replicated the basic design of the laboratory experiments in mesocosms.

On 2 June 2010 tadpoles were transported to the Collins Field Station at Vassar College, Poughkeepsie, New York and housed in eight black, 1,135 L cattle tanks (Rubbermaid® 300 gallon Livestock tanks, Newell Rubbermaid, Atlanta, GA) with a white marble chip substrate (to facilitate visual observations) and well water to a depth of 10 cm (∼350 L). Water was continuously pumped through ultraviolet sterilizers (Turbo Twister 2X, Coralife, Wisconsin, USA) using Eheim pumps with coarse pre-filters (EHEIM GmbH, Germany) to clarify the water and inactivate pathogens. Mesh barriers were used to prevent tadpoles from being sucked into the pumps. The tanks were sheltered from direct sunlight and rain by tarps, but were otherwise exposed to the elements. Ambient temperatures during the experiment were greater than in the laboratory (mean average daily temperature at the nearby Duchess County Airport weather station = 23°C, mean maximum daily temperature = 30°C).

Pairs of mesocosms were randomly assigned to control, high density, food limitation, or predator cue treatments. There were 80 tadpoles in each of six tanks (density = 31 $m^{-2}$) and 340 tadpoles in the two high-density tanks (density = 133 $m^{-2}$). Prior to their addition, a sample of 15 tadpoles from each treatment group was

staged and weighed. Neither body mass (mean = 0.14 g, $F = 0.011$, $P = 0.917$) nor stage (mean $\approx$ 27, range = 25–34, $F = 0.100$, $P = 0.752$) differed between replicates.

Tadpoles were fed algae disks at a rate of 10% of the average body mass per tadpole, per day. The low food mesocosms were fed the control ration every third day. Predator cues were generated by feeding wood frog larvae to five individually housed dragonfly larvae (Aeshnidae and Corduliidae) in mesh bags in each tank. The cages were checked daily and those in which the tadpole had been consumed had a new tadpole added. To establish conditions of chronic stress, these manipulations continued for 14 days before sampling for glucocorticoids and were maintained for the entire duration of the experiment.

When the majority of the tadpoles had reached Gosner stage 33–35 (mean = 35, range = 28–41; Table 1), 14 days after treatments were initiated, 12 tadpoles were removed from each tank, euthanized, and frozen for corticosterone analyses. The 12 tadpoles taken from the low-food mesocosms were less developed than those from the high density mesocosms, but no other between treatment comparisons were significant (Table 1). By this time many tadpoles had died (13% in high density, 22% in control, 32% in predator cue, and 51% in low food tanks; Table 1). To equilibrate densities, tadpoles were moved between tanks in the same treatment and then extra tadpoles from the same population held in outside mesocosms were added until each replicate had a density of 60 tadpoles per tank (25 $m^{-2}$), or 300 (117 $m^{-2}$) in the high-density tanks. Since these extra tadpoles were added late in the experiment, they were marked with VIE so that they could be distinguished from the original tadpoles that had been exposed to the treatments for the duration of the experiment. Only the original, ''focal'' animals were included in the analyses.

After tadpoles were removed for corticosterone analysis, ranavirus epidemics were initiated by introducing into each tank 12-infected conspecifics, which had been marked with a unique VIE tag and exposed to a high dose of ranavirus ($2.5 \times 10^5$ pfu $mL^{-1}$) via water bath for 24 h prior. This was meant to simulate the early stages of epidemics in vernal pools, emphasizing transmission by direct contact while minimizing stochastic loss of the infection that might result from fewer infected individuals being introduced (Brunner et al. 2007).

Mesocosms were checked three times a day for mortality and metamorphosing animals. Tadpoles found dead were removed and frozen at −80°C. Metamorphosing tadpoles (forelimb emergence; Gosner stage 42) were removed, euthanized with an overdose of MS-222, and frozen. The experiment continued until no tadpoles were left in a tank, or until no deaths had been observed for 10 days, at which point the remaining tadpoles were euthanized and frozen for virus screening.

Twenty days after the initiation of stress treatments (6 days after the addition of infected conspecifics) remote surveillance cameras (LHU31 4501, Lorex, Plainfield, IN, USA) were placed above each mesocosm to record the movements of the tadpoles. The paths of 20 haphazardly selected tadpoles in each tank were tracked frame-by-frame (every 0.5 s) for 5 min using ImageJ (Rasband 1997) with the manual-tracking module. These tracks were used to estimate the average velocity of tadpoles in each mesocosm as a surrogate for movement and contact rates. Unfortunately, it was not possible to distinguish in these videos the original focal from the extra tadpoles that had been added later, but the majority of tadpoles in each tank were there from the start of the experiment and so most tracks should be from focal tadpoles.

## Corticosterone Radioimmunoassay

Whole-body corticosterone concentrations of the focal tadpoles from both experiments were analyzed by radioimmunoassay (RIA) following the methods of Ledon-Rettig et al. (2009). In brief, tadpoles were homogenized, lipids extracted by a series of purifications in ethyl acetate, and corticosterone isolated by thin layer chromatography with a final ether extraction. After drying under nitrogen flow, samples were resuspended in phosphate-buffered saline, 400 µL of which was added into the RIA. We obtained estimates of corticosterone content from an average of 10 tadpoles from each laboratory tank and 12 tadpoles from each mesocosm, which were analyzed in two separate sets of assays. For the laboratory experimental samples, the RIA inter-assay coefficient of variation (CV) among five assays was 15.7% and the mean intra-assay CV was 2.4%. For the mesocosm experiment samples, the RIA inter-assay CV among three assays was 12.7% and the mean intra-assay CV was 6.8%. All corticosterone estimates were adjusted for body weight of the tadpole and reported in ng $g^{-1}$ body mass.

## Virus Screening

Livers were dissected from tadpoles in the laboratory experiment and digested in cell lysis buffer (0.1 M NaCl,

0.05 M Tris–HCl, 0.001 M EDTA) with Proteinase K. Genomic DNA was then extracted using a salt extraction protocol (Sambrook and Russell 2001) and tested for ranavirus DNA using polymerase chain reaction (PCR) with primers specific to *Ranavirus* major capsid protein (MCP 4/5; Mao et al. 1999). PCR products were visualized on a 1.5% agarose gel stained with 0.1 μg/mL ethidium bromide. Each extracted DNA sample was screened twice by PCR; negative and inconsistent samples were run a third time.

We used the DNeasy Blood and Tissue kits to extract DNA from the livers of the mesocosm tadpoles (Qiagen, Valencia, CA). Samples were diluted to 20 ng DNA $\mu L^{-1}$ with elution buffer using measurements from a NanoDrop 1000 (Thermo Fisher Scientific) and then screened in duplicate 20 μL reactions using a quantitative Taqman realtime PCR (qPCR) reaction with primers and probe internal to MCP 4/5 (Brunner and Collins 2009). Negative samples from both the laboratory and mesocosm experiments were rerun in duplicate with qPCR. Any sample that tested positive in at least two reactions was scored as ranavirus-positive.

### Statistical Analyses

Whole-body corticosterone concentrations and tadpole average velocities were natural log-transformed to achieve normality. We used linear models to determine whether pre-exposure mass, velocity, prevalence of infection, and case mortality (the proportion of infected animals that died) varied among treatments. Pearson's correlation coefficients were calculated to test for correlations between stage, mass, and corticosterone concentrations. Because tadpoles within a tub or mesocosm are not independent, we used linear mixed models to determine differences in corticosterone concentrations, mass, and stage across treatments, with tub or mesocosm as the random effect (random intercepts) using the lmer() function in the lme4 package (Bates et al. 2011) in R 2.11.1 (R Development Core Team 2011). The variation explained by these random effects were very small (e.g., $s < 0.0001$ for corticosterone in both experiments) so we used Tukey contrasts for pairwise comparisons of adjusted means among treatments. We used Kaplan–Meier curves, Cox proportional hazard models (survival package; Therneau et al. 2011), and, in the case of the mesocosm experiment where tadpoles were both dying and metamorphosing, competing risks models (cmprsk package; Gray 2010) to test for treatment effects on rates of mortality and metamorphosis.

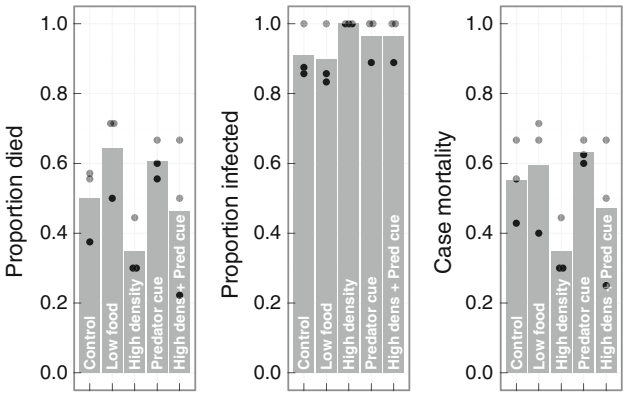


**Figure 2.** Proportion of tadpoles that died following ranavirus exposure in the laboratory experiment, the proportion that became infected, and the proportion of the infected animals that died (case mortality). *Bars* show the mean values and the points show the values for individual replicates.

## Results

### Laboratory Experiment

Corticosterone varied among treatments ($F_{1,4} = 4.94$, $P < 0.001$), with concentrations two to three times higher in the low-food treatment compared to all other treatments (all $P < 0.05$, except compared to high density where $P = 0.091$; Fig. 1). The low-food treatment had more than twice the pre-virus exposure mortality (30%) and significantly lower body masses relative to all other treatments (Table 1), suggesting our manipulation was very strong. Final mass was negatively correlated with corticosterone ($r = -0.44$, $t = -6.34$, $P < 0.001$; Fig. 1); smaller animals, like those in low-food treatment, had higher levels of corticosterone. The difference among treatments in corticosterone concentrations was largely accounted for by the masses of individual animals: in the full model with treatment and mass the effect of mass is strongly significant ($F_{1,4} = 41.35$, $P < 0.001$), but the treatment effect was no longer significant ($F_{1,4} = 1.71$, $P = 0.150$).

Although low-food tadpoles were smaller, had higher corticosterone concentrations and died in greater numbers before pathogen exposure, we observed no differences among treatments in outcome of ranavirus exposure. Of the 129 exposed tadpoles, 123 (95%) tested positive, and 65 (50%) died during the 21-day experiment. Infection prevalence exceeded 83% in every tub and did not vary significantly between treatments ($F_{4,10} = 1.20$, $P = 0.371$), nor did case mortality ($F_{4,10} = 2.0$, $P = 0.172$) or time to death ($F_{4,10} = 0.81$, $P = 0.550$; Fig. 2).

### Mesocosm Experiment

As in the laboratory experiment, more tadpoles died prior to virus exposure in the low-food mesocosms (51%) than in other treatments (≤32%; Table 1), although their mass was only significantly smaller compared to high-density tadpoles ($P = 0.043$). The pattern of whole-body corticosterone concentrations was similar to that of the laboratory experiment (Fig. 1), although the laboratory data were more variable than the mesocosm data (mean residual SD = 0.177 among the mesocosms and 0.279 among the laboratory tubs; $t = 2.72$, $P = 0.013$). Tadpoles in the low-food treatment had slightly, but not significantly higher levels of corticosterone compared to controls ($F_{3,92} = 2.91$, $P = 0.098$). No other treatments differed in corticosterone. As in the laboratory experiment, (log-transformed) corticosterone concentrations were negatively correlated with mass ($r = -0.519$, $t = -5.90$, df = 94, $P < 0.001$) and again the effect of treatment on corticosterone was significant only when mass was not included in the model (treatment only: $F_3 = 2.91$, $P = 0.039$; treatment effect when mass was included in the model: $F_3 = 0.026$, $P = 0.994$). Thus, nearly all of the influence of treatments on whole-body corticosterone concentrations could be explained by their effects on tadpole mass.

We also observed significant differences in tadpole activity across treatments ($F_{3,75} = 13.7$, $P < 0.001$; Fig. 3).

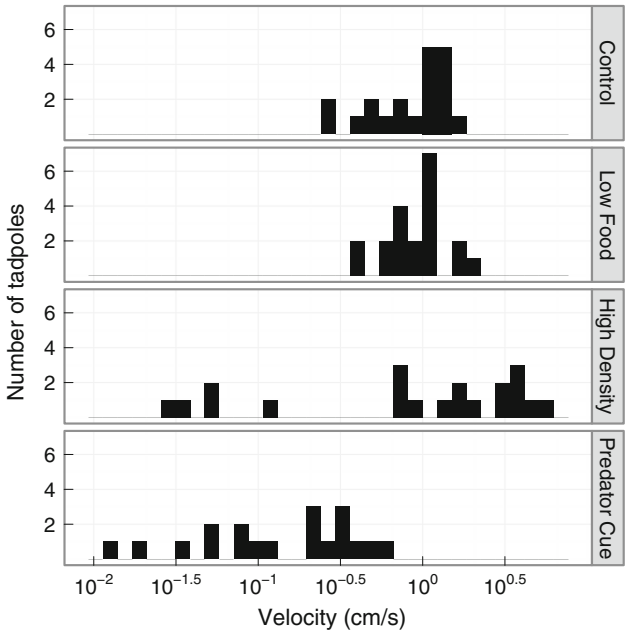


**Figure 3.** The distributions of average velocity (cm $s^{-1}$) over a 5-min period for 20 individual tadpoles from each treatment in outdoor mesocosms.

Control, low-food, and high-density tadpoles moved at similar average velocities, but the predator-cue tadpoles were more than five times slower than control tadpoles ($P < 0.001$). There was much more variation in tadpole velocities in the high-density treatment than among control tadpoles: the standard deviation in high-density treatments ($s = 1.77$ cm $s^{-1}$) was four times greater than that of the control ($s = 0.41$ cm $s^{-1}$).

As in the laboratory experiment, ranavirus prevalence was ≥90% across all treatments, although only 21% of infected tadpoles died in the mesocosms, compared to 45% in the laboratory experiment. Most tadpoles in the mesocosms metamorphosed and were euthanized, largely because of the high-density treatment. The rate of metamorphosis was three times greater and hazard of death was five times lower in the high-density treatment than the controls ($P < 0.001$, Fig. 4). Mortality was as low as 12% in the high-density tanks, compared to 37–51% in the other treatments (Fig. 4). The prevalence of infection, however, was similarly high in both the tadpoles and metamorphs in this treatment (prevalence = 0.975 and 0.920, respectively, $P = 0.056$).

## Discussion

We exposed wood frog tadpoles to environmental conditions expected to elicit a physiological stress response, specifically increased activity of the HPI axis and associated changes in feeding and movement rates. Because chronically high levels of corticosterone are immunosuppressive (Dhabhar 2009), we anticipated that our challenges would increase susceptibility of these tadpoles to ranavirus infection (Gray et al. 2009; Robert 2010). The results of our laboratory and mesocosm experiments, however, largely ran counter to these expectations.

Only one treatment elicited increased activity of the HPI axis. In both the laboratory and mesocosm experiments the low-food treatment produced elevated corticosterone concentrations, slowed growth and development, and increased pre-infection mortality compared to the controls, consistent with other studies with tadpoles (e.g., Glennemeier and Denver 2002; Crespi and Denver 2005; Ledon-Rettig et al. 2009). However, while these low-food tadpoles were clearly nutritionally stressed, they were not more susceptible to ranavirus infections in terms of the proportion becoming infected, case mortality, or timing of mortality. While it is possible that pre-exposure mortality

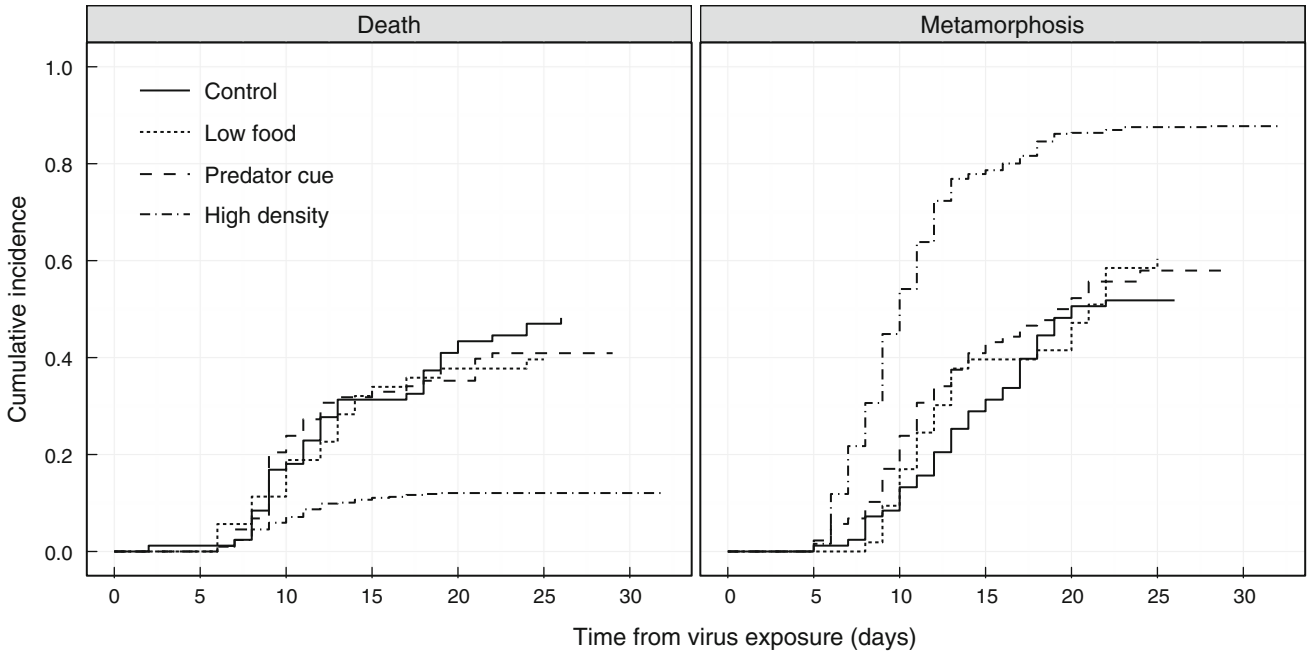


**Figure 4.** Cumulative hazard of death (*left*) or metamorphosis (*right*) for each treatment following virus exposure in outdoor mesocosms.

selectively eliminated tadpoles with the highest corticosterone levels, which might have showed increased susceptibility to infection, this suggests that starvation itself presents a greater risk to tadpoles than any associated immunosuppression. No other challenges increased susceptibility to ranavirus infection.

The apparent lack of immunosuppression is surprising given the well-documented observation in mammals and other vertebrates that nutrient restriction or alterations in energy balance signals associated with food restriction (e.g., glucocorticoids, leptin; Demas 2010) have negative effects on the immune system. For example, cell-mediated immunity was suppressed and lymphocyte numbers decreased in nutritionally stressed bobwhite chicks (Lochmiller et al. 1993) and young cotton rats fed a low-protein diet had fewer splenocytes (Davis et al. 1995). In addition, amphibian tadpoles given a higher quality diet had enhanced immune responses and higher survival when exposed to the fungal pathogen, *Batrachochytrium dendrobatidis* (Venesky et al. 2012).

There are several potential explanations for the lack of difference in susceptibility. First, it is possible that wood frog tadpoles mount weak, ineffective responses to ranavirus infection regardless of nutritive condition or glucocorticoid levels. Anurans do not have a fully functioning adaptive immune system until after metamorphosis (Rollins-Smith 1998). Yet amphibian larvae do respond to and clear ranavirus infections (Brunner et al. 2004; Robert 2010), and a sizeable fraction of the infected tadpoles in our experiments survived for several weeks (Figs. 2, 4), which suggests a moderately robust response in at least some tadpoles. Alternatively, the response to ranaviruses might involve components of the immune system that are not modulated by physiological stress. Again, the evidence does not support this hypothesis. While the response to ranavirus in *Xenopus laevis* tadpoles is largely innate, including up-regulation of inflammation factors and immunoglobin expression (Robert 2010; Chen and Robert 2011), innate immune functions are modulated by glucocorticoids (Chinenov and Rogatsky 2007) as well as other factors related to nutritional condition (Demas 2010). Perhaps, then, food restriction does not elicit a large enough elevation in glucocorticoids to be immunosuppressive, or maybe wood frogs have evolved mechanisms to avoid the immunosuppressive effects of this common environmental challenge.

While we had expected predator cues and high densities to elicit stress responses as well, results from previous studies have been inconsistent. Glennemeier and Denver (2002) and Rot-Nikcevic et al. (2005) found that conspecific density alters corticosterone concentrations, but Belden et al. (2007), like us, did not detect any relationship between wood frog tadpole density and corticosterone levels in experimental mesocosms, or among natural ponds.

Similarly, the glucocorticoid responses to predators vary in the literature, possibly due to difference in experimental design. Fraker et al. (2009) showed reduced activity at 4 h in response to predator cues, in concert with reduced corticosterone concentrations, while Dahl et al. (2012) showed increased corticosterone levels within 24 h of predator exposure, but no predator-induced elevation of corticosterone 2 weeks later. Perhaps our tadpoles habituated to the predator cues over the 2 weeks before we measured corticosterone.

The effects of predators on disease susceptibility reported in the literature have also been mixed. Kerby et al. (2011), for instance, observed that tiger salamander larvae exposed to predator cues were more likely to succumb to ranavirus infection, and Belden and Wojdak (2011) showed that mortality was significantly increased when tadpoles were exposed to both predators and trematode parasites compared to parasites alone. By contrast, Raffel et al. (2010) found no difference in trematode burden between predator-exposed and non-exposed American toad tadpoles, and a recent study by Haislip et al. (2012) found that while predator exposure strongly influenced the behavior and survival of four species of anuran tadpoles, predator cues did not increase their susceptibility to a ranavirus. The differences in outcomes may again be a result of the timing and duration of exposure to predator cues. Both Kerby et al. (2011) and Belden and Wojdak (2011) introduced the predator cues at the same time as the pathogens, whereas we and Haislip et al. (2012) introduced predator cues for over a week before the virus exposure and, like Raffel et al. (2010), maintained the exposure throughout the experiment. Interestingly, this would seem to suggest that contrary to expectations from mammalian and avian systems, *acute* exposure to predators may increase susceptibility while chronic exposure has little effect in amphibian tadpoles.

We had also expected that, separate from individual-level susceptibility, behavioral responses to these challenges—changes in feeding and movement rates—would influence transmission dynamics in our mesocosm experiment. In particular, we expected predator-induced reductions in activity and movement that we and others have observed (e.g., Van Buskirk 2001; Raffel et al. 2010; Haislip et al. 2012) would reduce contact rates and thus transmission since ranavirus is spread by close contact (Brunner et al. 2007). Similarly, we expected that transmission would be highest in the high-density treatment where there were more than four times as many tadpoles crowded into the mesocosm because transmission of animal disease is often thought to be density-dependent (McCallum et al. 2001, but see Harp and Petranka 2006). With $\geq$90% infection prevalence across all treatments it is difficult to determine how or whether tadpole density-influenced ranavirus transmission. A separate experiment currently being analyzed will address this question.

The high-density mesocosms did see significantly lower mortality, but apparently not because they were less susceptible or less likely to become infected. Rather, proportionally fewer infected tadpoles died of ranavirus infection in the high-density mesocosms because they metamorphosed at a much greater rate. For logistical reasons metamorphosing tadpoles in the mesocosms were euthanized and thus censored (not counted as having died) in our analyses. We cannot be certain whether these individuals would have died soon after metamorphosis or, had they survived, whether they would have cleared the infection. Larval and metamorphosed tiger salamanders can maintain sublethal infections for several months (Brunner et al. 2004), so it is possible that these wood frog metamorphs would have survived with infections.

This finding illustrates how physiological and developmental responses to infection in tadpoles can influence ranavirus epidemiology. We previously showed that ranavirus infection activates the HPI axis, resulting in increased corticosterone concentrations and development rates, in late-staged wood frog tadpoles (Warne et al. 2011). In our mesocosm experiment, more tadpoles in the high-density treatment were prometamorphic (i.e., at later developmental stages, Table 1). They could therefore mount a stress-induced acceleration of metamorphosis, here caused by the stress of ranavirus infection rather than increasing density or water volume reductions that signal pond drying in temporary ponds (Denver et al. 2002). High-density conditions, which accelerate development, might then lead to a greater proportion of infected tadpoles metamorphosing and dispersing from a pond, which could initiate epidemics in following years (Brunner et al. 2004) and in nearby ponds. While we did not measure corticosterone after ranavirus exposure, this result highlights the need to examine how environmental conditions alter the stress response of anuran tadpoles to ranavirus infection.

In summary, chronic exposure to three ecologically important natural stressors did not make wood frog tadpoles more susceptible to ranavirus infection individually or as a population. Although we observed changes in growth, development and activity caused by our challenges, corticosterone was elevated only under low-food conditions, and even then the risks of infection or mortality were

no greater than in controls. It is possible that combinations of stressors or evolutionarily novel stressors (e.g., introduced predators, pesticides) elicit stronger physiological changes (e.g., increases in glucocorticoids) and are more immunosuppressive; most studies identifying immunosuppression or worsened disease outcomes from environmental factors have focused on anthropogenic stressors, particularly chemicals (Forson and Storfer 2006; Kerby et al. 2011; Nain et al. 2011). Yet our results caution against the common assumption that what seems ''stressful'' a priori necessarily increases physiological stress or susceptibility to disease in wild amphibians.

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