Natural stressors and disease risk: does the threat of predation increase amphibian susceptibility to ranavirus?

N.A. Haislip, J.T. Hoverman, D.L. Miller, and M.J. Gray

Abstract: Emerging infectious diseases have been identified as threats to biodiversity, yet our understanding of the factors contributing to host susceptibility to pathogens within natural populations remains limited. It has been proposed that species interactions within communities affect host susceptibility to pathogens, thereby contributing to disease emergence. In particular, predation risk is a common natural stressor that has been hypothesized to compromise immune function of prey through chronic stress responses possibly leading to increased susceptibility to pathogens. We examined whether predation risk experienced during the development of four larval anuran species increases susceptibility (mortality and infection) to ranaviruses, a group of viruses responsible for amphibian die-offs. Using controlled laboratory experiments, we exposed each species to a factorial combination of two virus treatments (no virus or virus) crossed with three predator-cue treatments (no predators, larval dragonflies, or adult water bugs). All four amphibian species reduced activity by 22%–48% following continuous exposure to predator cues. In addition, virus exposure significantly reduced survival by 17%–100% across all species. However, exposure to predator cues did not interact with the virus treatments to elevate mortality or viral load. Our results suggest that the expression of predator-induced plasticity in anuran larvae does not increase ranaviral disease risk.

Key words: disease ecology, emerging infectious disease, inducible defense, parasite, phenotypic plasticity, trophic interactions, *Lithobates clamitans*, *Lithobates sylvaticus*, *Pseudacris feriarum*, *Hyla chrysoscelis*, ranavirus, frog virus 3, *Anax* sp., *Belostoma flumineum*.

Résumé : Bien qu'il soit établi que des maladies infectieuses émergentes constituent une menace pour la biodiversité, la compréhension des facteurs qui contribuent à la réceptivité des hôtes aux pathogènes dans les populations naturelles demeure limitée. Il a été proposé que les interactions d'espèces au sein d'une communauté auraient une incidence sur la réceptivité des hôtes aux pathogènes, contribuant ainsi à l'émergence de maladies. Le risque de prédation, en particulier, est un facteur de stress naturel dont il a été postulé qu'il compromet la fonction immunitaire des proies par le biais de réactions de stress chronique qui entraîneraient possiblement une réceptivité accrue aux pathogènes. Nous avons tenté de déterminer si le risque de prédation auquel sont exposées quatre espèces d'anoures au stade larvaire durant leur développement accroît leur réceptivité (mortalité et infection) aux ranavirus, un groupe de virus responsable de cas de mortalité massive chez les amphibiens. Dans le cadre d'expériences contrôlées en laboratoire, nous avons exposé chaque espèce à une combinaison factorielle de deux traitements viraux (pas de virus et virus) et trois traitements de signaux de prédation (pas de prédateur, larves de libellule et punaises d'eau adultes). L'activité des quatre espèces des amphibiens a diminué de l'ordre de 22 % à 48 % à la suite d'une exposition continue à des signaux de prédation. De plus, l'exposition aux virus a entraîné une réduction significative de la survie, de l'ordre de 17 % à 100 %, pour toutes les espèces. L'interaction de l'exposition aux signaux de prédation avec l'un ou l'autre des traitements viraux ne s'est toutefois pas traduite par une augmentation de la mortalité ou de la charge virale. Nos résultats portent à croire que l'expression de la plasticité induite par les prédateurs chez les larves d'anoure n'entraîne pas une augmentation du risque de maladies ranavirales.

Mots-clés : écologie des maladies, maladie infectieuse émergente, défense inductible, parasite, plasticité phénotypique, interactions trophiques, *Lithobates clamitans*, *Lithobates sylvaticus*, *Pseudacris feriarum*, *Hyla chrysoscelis*, ranavirus, virus 3 de la grenouille, *Anax* sp., *Belostoma flumineum*.

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Introduction

Recognizing that disease dynamics are embedded within complex ecological systems, there have been repeated calls for integrating ecological principles into disease studies (Ostfeld et al. 2008). In particular, studies are needed that address whether basic species interactions affect the susceptibility of hosts to pathogens, thereby contributing to disease emergence. One common ecological stressor is the threat of predation, which causes prey to adaptively alter their behavior, morphology, and life-history traits (Lima and Dill 1990). Moreover, predation risk has a diversity of effects on prey physiology including increased production of glucocorticosteriods, elevated respiration, and increased heart rate (Steiner and Van Buskirk 2009; Hawlena and Schmitz 2010). Should the risk of predation extend for days or weeks (i.e., chronic risk), prey may experience reduced growth, development, body condition, reproduction, and immune function (Boonstra et al. 1998; Rigby and Jokela 2000; Stoks et al. 2006; Hawlena and Schmitz 2010). Thus, a constant threat of predation could increase the susceptibility of prey to pathogen infection by suppressing the immune system, and contribute to the emergence of infectious diseases. To date, few studies have examined whether the threat of predation increases prey susceptibility to pathogens (Griffin 1989; Boonstra et al. 1998; Rigby and Jokela 2000; Kerby et al. 2011). If predation risk contributes to disease emergence, disease outbreaks may be significantly associated with shifts in predator abundance.

Amphibians are experiencing population declines and species extinctions (Daszak et al. 1999; Carey 2000; Collins and Storfer 2003). While there are many hypotheses for amphibian declines, emerging infectious diseases are certainly playing a role (Wake and Vredenburg 2008; Collins and Crump 2009). A group of viruses within with the genus *Ranavirus* has caused amphibian die-offs in wild populations on five continents (Gray et al. 2009). In North America, for example, this pathogen has been associated with nearly 50% of all reported mortality events (Green et al. 2002; Muths et al. 2006). Although ranaviruses have been linked to numerous die-off events, the mechanisms responsible for disease emergence in wild populations remain unclear.

The natural stress of predation has been proposed as a driver of disease emergence in amphibian populations (Gray et al. 2009). The larvae of many amphibian species adaptively respond to predator cues by reducing activity levels and forming larger tail fins and smaller bodies (Lawler 1989; Werner and Anholt 1996; Relyea and Werner 1999; Van Buskirk 2001; Relyea 2001*a*, 2001*b*; 2002*a*, 2002*b*; Schoeppner and Relyea 2005). Recent research suggests that these responses are linked to endogenous corticosterone levels (Fraker et al. 2009; Hossie et al. 2010; Middlemis-Maher 2011). Similar to many vertebrates, activation of the hypothalamic–pituitary–interrenal (HPI) axis in amphibians in response to stress results in context-dependent changes in corticosterone levels (Denver 1997). In response to acute predator stress (i.e., minutes), tadpoles reduce corticosterone production, which promotes reduced activity levels as a defense (Fraker et al. 2009; Middlemis-Maher 2011). In contrast, chronic exposure to predation risk leads to an increase in corticosterone production, which appears to facilitate morphological responses to predators (Middlemis-Maher 2011). From a disease perspective, chronic exposure to corticosterone is known to have immunosuppressive effects in amphibians (e.g., decreased lymphocyte production, destruction of T-cell lymphocytes; Tournefier 1982; Ducoroy et al. 1999). However, recent research has demonstrated that ranavirus infection can activate the HPI axis of Wood Frog (*Lithobates* (= *Rana*) *sylvaticus* (LeConte, 1825)) tadpoles 4–5 days after infection (Warne et al. 2011). It was hypothesized that glucocorticoids play a permissive role in the adaptive immune response through antibody production (Dhabhar 2009; Warne et al. 2011). This suggests that the presence of predator stress may actually benefit amphibian larvae by activating the HPI axis. To our knowledge, only one study has examined whether exposure to aquatic predators increases susceptibility to ranavirus (Kerby et al. 2011). Cues from the dragonfly common green darner (*Anax junius* (Drury, 1773)) increased ranavirus associated mortality and infection prevalence in the larvae of Eastern Tiger Salamanders (*Ambystoma tigrinum* (Green, 1825)) (Kerby et al. 2011). Although this study provides preliminary evidence that predators may increase the susceptibility of amphibians to ranavirus, studies that test additional amphibian species are needed to develop generalizations.

Our main objective was to determine if the chronic stress of predation increases mortality of anuran larvae exposed to ranavirus. Tadpoles of four anuran species were exposed to ranavirus and chemical cues generated from two species of aquatic insect predators that differed in their level of risk posed to amphibians. We hypothesized that (*i*) the combination of predator cues and ranavirus would reduce activity, growth, and development to a greater degree than either factor alone, (*ii*) as predator risk increased, tadpole susceptibility to ranavirus (as indexed by viral load and mortality) would increase, and (*iii*) anuran species that exhibited stronger responses to predation would experience greater susceptibility to ranavirus infection when exposed to both factors.

Materials and methods

Study animals and virus isolate

We used four species of larval anurans (Green Frog, *Lithobates* (= *Rana*) *clamitans* (Latreille in Sonnini de Manoncourt and Latreille, 1801); *L. sylvaticus*; Upland Chorus Frog, *Pseudacris feriarum* (Baird, 1854); Cope's Gray Treefrog, *Hyla chrysoscelis* Cope, 1880) for the experiments. Egg masses were collected for each species from single populations at breeding sites near Knoxville, Tennessee, USA, from January to July 2009 within 48 h of deposition, rinsed with sterile water, and transported to the University of Tennessee Joe Johnson Animal Research and Teaching Unit (JARTU). The egg masses were placed outdoors in 300 L wading pools filled with aged tap water to develop. These pools were covered with 60% shade cloth to prevent the colonization of aquatic insects or other amphibians. After hatching, the tadpoles were fed rabbit chow (Purina, St. Louis, Missouri, USA) and ground TetraMin® (Tetra, Blacksburg, Virginia, USA) ad libitum until they were used in the experiments. Although vertical transmission of ranaviruses is not known to occur (Gray et al. 2009), we used a random sample of 10 tadpoles from each species to confirm the absence of rana-

virus (see Molecular analyses below); all pre-experiment tadpoles tested negative.

We used larval dragonflies (genus *Anax* Leach, 1815) and adult giant water bugs (*Belostoma flumineum* Say, 1832) collected from local ponds as predators in the experiments. Once collected, invertebrates were rinsed with sterile water and placed into tubs containing aged tap water at JARTU. Five weeks before the start of the experiments, the predators were housed individually in 2 L plastic tubs filled with 1 L of aged tap water and fed 1 tadpole per week. These common tadpole predators represent two levels of risk (Relyea 2001*a*). Aeshnid dragonflies are generally high-risk predators owing to their high prey-capture efficiency and short handling time. In contrast, giant water bugs pose a lower risk owing to their poor prey-capture efficiency and long handling time. Consequently, tadpoles tend to exhibit stronger antipredator responses to chemical cues released during predation by dragonflies compared with giant water bugs (Relyea 2001*b*).

We used a ranavirus that was isolated by the University of Georgia Veterinary Diagnostic and Investigational Laboratory (VDIL) in 2006 from a morbid juvenile American Bullfrog (*Lithobates catesbeianus* (Shaw, 1802)) housed at a ranaculture facility in southern Georgia, USA (Miller et al. 2007). Preliminary molecular analyses suggest that the isolate is similar to *frog virus* 3 (FV3; GenBank accession No. EF101698, Miller et al. 2007); FV3 is the type species for *Ranavirus* (Chinchar et al. 2009). We have found that the tadpole species used in our experiments can display signs of disease from this isolate within 1–5 days after exposure and experience mortality within 5–21 days after exposure (Hoverman et al. 2010, 2011). The isolate was cultured at the VDIL using the same protocol described in Hoverman et al. (2010). An aliquot of the stock viral solution was titrated at the VDIL to determine the number of plaque forming units (PFUs) per millilitre. Following titration, the virus was sent overnight to the University of Tennessee and stored at –80 °C until used in the experiments. The virus was on the third passage since original isolation.

Experimental trials

A separate experiment was conducted for each of the four species and all experiments were conducted under identical laboratory conditions (23 °C and a 12 h day : 12 h night photoperiod) in JARTU. Each experiment was a factorial combination of two virus treatments crossed with three predator treatments. The virus treatments included a no-virus control and a virus exposure of 10^3 PFUs·mL^{-1}. The predator treatments were a no-predator control and predator cues from either *Anax* or *Belostoma*. Each treatment was replicated five times for a total of 30 experimental units. All experiments were conducted at a common shelf height. Each experiment lasted 29 days and was divided into before virus exposure (days 1–8) and after virus exposure (days 9–29). The predator treatments were initiated at the start of the experiment and lasted all 29 days. The virus treatments were initiated on day 8 to allow tadpole time to respond to the predation treatments. Virus exposure lasted 4 days.

The experimental units were 11.7 L plastic tubs filled with 7 L of aged tap water. Tadpoles that were at Gosner (1960) stage 30 were used to reduce possible confounding effects of development on response variables (Haislip et al. 2011). We randomly assigned 10 tadpoles to each experimental unit. An additional random sample of 10 tadpoles was euthanized in benzocaine hydrochloride (1 g·L^{-1}) and weighed to the nearest 0.1 mg. The mean mass of these tadpoles was used to calculate growth of all tadpoles surviving at the end of the experiment (discussed later).

We used the addition of predator cues, generated during tadpole predation, to simulate predator presence in the experimental units (Fraker 2008; Schoeppner and Relyea 2005). Predator cues were generated for each predator in four 11.7 L tubs that were independent of the experiment. Each tub was filled with 10 L of water and the predator was housed within a cage constructed of a 250 mL plastic cup with window screen covering the opening. The predators were fed a known quantity of live tadpole biomass (400–500 mg) every day of the same anuran species being tested. Every day, water from the predator cue generation tubs was mixed and added to the appropriate experimental units to achieve a final concentration of 0.57 mg of consumed tadpole biomass·L^{-1}, which was within the cue range (0.071–3 mg·L^{-1}) known to elicit antipredator responses in anuran larvae (Van Buskirk 2001; Relyea 2002*a*; Schoeppner and Relyea 2008). A similar amount of aged tap water was added to the no-predator treatment to equalize disturbance.

The virus treatment was applied after 8 days of predator-treatment application to allow adequate time for responses to predator cues. This duration was reasonable considering that morphological and behavioral responses from predator cues have been observed as early as 6 days following exposure (Relyea 2003). For the no-virus treatments, 411 μL of virus-free media (Eagle's Minimal Essential Media (MEM)) was added to the tubs. The virus treatments received 411 μL of MEM containing ranavirus, which resulted in a final concentration of 10^3 PFUs·mL^{-1}. This concentration is within the range of doses used in other studies (10^2–10^6 PFUs·mL^{-1}; Bollinger et al. 1999; Brunner et al. 2005; Pearman and Garner 2005) and is an environmentally relevant concentration (Rojas et al. 2005; Schock et al. 2008). The exposure duration lasted 4 days, which has been shown to initiate infection in the species used in our study (Haislip et al. 2011; Hoverman et al. 2011). After 4 days, the water was changed and virus was not added again.

Every 2 days, tadpoles were fed ground TetraMin® at a ration of 6% of their body mass (Relyea 2002*c*). The food ration was calculated from the mean mass of 10 tadpoles that were independent of the experiment but reared under identical conditions as the control treatment (i.e., without virus and predators). Before each feeding, the nonexperimental tadpoles were blotted dry on a paper towel and weighed. The food ration was adjusted every other day to compensate for mortality by calculating the rations needed according to how many tadpoles were present in each experimental unit.

Water was changed every 4 days to maintain water quality. To reduce the likelihood of virus contamination during the water changes, no-virus treatments were handled first followed by virus treatments. Also, we used new nets, changed gloves, and rinsed all surfaces with 0.75% Nolvasan® for at least 1 min to prevent cross-contamination (Bryan et al. 2009). Predator cues were added after each water change.

Data collection and tadpole observations

We observed tadpole activity every day during the experiment using scan sampling (Altmann 1974). Tadpoles were considered active if they were moving in the water column or displaying tail movement. Percent activity was calculated as the number of active tadpoles divided by the total number of tadpoles present in the tub. Activity was measured 10 times per tub on each observation day, with tub observations separated by <5 min. The mean of the 10 observations was used as the response variable for each tub.

We monitored tadpole survival daily in each experimental unit and dead individuals were removed. The experiment was terminated 3 weeks after exposure, which is sufficient duration to observe mortality following ranavirus exposure (Hoverman et al. 2010). For each experimental unit, we calculated the proportion of individuals that survived until the end of the experiment as our survival response variable. All surviving individuals were euthanized, weighed, and staged (Gosner 1960). We calculated daily growth rate as the difference between mean mass at the beginning of the experiment (based on the initial sample of 10 tadpoles) and the mean mass of the surviving individuals in each tub divided by the duration of the experiment. For the virus-free control treatments, two tadpoles that survived the entire experiment and all individuals that died during the experiment were necropsied to test for infections. All control individuals tested negative for ranavirus infection. For each experimental tub within the virus treatments, two tadpoles that died during the experiment were randomly selected and necropsied to estimate viral load. Although we did not test all individuals, mortality rates from ranaviral disease closely track infection rates in larval amphibians (Haislip et al. 2011; Hoverman et al. 2011). Sections of the pronephros (kidneys) and liver were removed, placed in a 1.5 mL microcentrifuge tube, and frozen at –80 °C until processing. Gloves were changed and a different set of sterile instruments was used for necropsy between each individual to prevent cross-contamination. Individuals that metamorphosed prior to the end of the experiment were deemed survivors and removed from the experimental unit. Metamorphs were removed after approximately 20% tail resorption to reduce the risk of drowning in the tubs. Because metamorphs were not included for the full duration of the experiment, they were not used in the analysis of growth or viral load.

Molecular analyses

Viral load has been used as an index of susceptibility to iridoviruses (e.g., Goldberg et al. 2003, Inendino et al. 2005), and was estimated using quantitative polymerase chair reaction (qPCR). We pooled the liver and kidney sample from a given individual and extracted genomic DNA (gDNA) using the DNeasy blood and tissue kit (Qiagen Inc., Valencia, California, USA). We used the Qubit™ fluorometer and the Quant-iT™ dsDNA BR assay kit to quantify the concentration of gDNA ($\mu g \cdot \mu L^{-1}$) in each sample (Invitrogen Corp., Carlsbad, California, USA). This allowed us to standardize the amount of gDNA used in the PCR reaction. We used the *Taq*Man qPCR assay following the methods of Picco et al. (2007). A SmartCycler® (Cepheid, Sunnyvale, California, USA) thermal cycler was used for the qPCR. In each run of the qPCR, four controls were included as follows: a ranavirus-negative tadpole sample, a negative DNA-grade water sample, a ranavirus-positive tadpole sample, and a cultured virus sample. For each sample, we recorded the cycle number at which the sample crossed the fluorescent threshold level (i.e., CT value) and declared infection when the sample CT was <30). Given that a standard quantity of gDNA was used for each reaction, the CT values were presumed to reflect a qualitative measure of viral load. A standard curve was obtained from a qPCR conducted with a gradient of known concentration of cultured virus ($PFU \cdot mL^{-1}$) and the CT value of each sample was used to calculate viral load. Viral load was averaged within each experimental unit prior to analyses.

Data analyses

We conducted repeated-measures analysis of variance (ANOVA) to test the effects of treatments on activity before and after virus exposure (Sokal and Rohlf 1995). Main effects were virus treatment, predator treatment, and time. The before-exposure analysis included the first 8 days of the experiment and provided behavioral evidence of predator-cue effectiveness. The after-exposure analysis included the remaining days of the experiment. However, the data for the after-exposure analysis were truncated when mortality rates exceeded 20% to avoid an increase in sample variation associated with fewer individuals available for calculating activity. Whenever an interaction of main effects occurred, analyses were separated by predator or virus levels. In several analyses, we detected significant time by treatment interactions. Inasmuch as the main-effect responses associated with predator and virus treatments were of greatest interest, we focus on the presentation of these results.

We used a two-way ANOVA to test for differences in predator and virus effects on growth and development. Given that no tadpole of *L. sylvaticus* that were exposed to ranavirus survived until the end of the experiment, we could not analyze growth or development for the virus treatment. Thus, we used a one-way ANOVA to test for predator-cue effects on growth within the no-virus treatment. We also used a one-way ANOVA to test for differences in $\log_{10}$-transformed viral load among predator treatments; a virus effect was not included in the model because all unexposed tadpoles tested negative for ranavirus. For all tests, if a significant predator effect existed, post hoc comparisons among the three predators treatments were conducted at a Bonferroni-corrected $\alpha = 0.017$. Normality was met in all cases (Shapiro–Wilks' test, $P > 0.11$).

We used nonparametric Kruskal–Wallis tests to assess predator cue and virus effects on the proportion of tadpoles surviving the experiment (arcsine-transformed). Nonparametric tests were used because the lack of mortality in some treatments resulted in non-normal distributions. For *L. sylvaticus*, we were not able to conduct statistical analyses of survival because all individuals in virus-exposed treatments died, while all individuals in the virus-free treatments survived. Thus, these results were qualitatively interpreted. All analyses were performed with SPSS version 16.0 (SPSS, Inc., Chicago, Illinois, USA) at $\alpha = 0.05$ for main tests.

Results

Tadpoles responded to the presence of predator cues by reducing activity levels (i.e., the percentage of individuals moving) before and after the addition of the virus. Before virus exposure, *Anax* and *Belostoma* cues reduced the activity of all species by 4%–18% and 5%–10%, respectively, compared with the controls (Table 1A; Fig. 1A; $P < 0.001$). Additionally, activity was lower for tadpoles exposed to *Anax* cues compared with *Belostoma* cues for *H. chrysoscelis* and *P. feriarum* ($P < 0.001$), but not the remaining species ($P \geq 0.145$). After virus exposure, activity was 4%–24% lower in the predator-cue treatments compared with the control for the four species (Table 1B; Fig. 1B; $P < 0.001$). Activity levels were lower for *P. feriarum* exposed to *Anax* cues compared with *Belostoma* cues ($P = 0.005$), but not for the other three species ($P \geq 0.064$). There was limited evidence that the addition of the virus affected tadpole activity levels (Table 1B). Activity for *H. chrysoscelis* was, on average, 3% lower in the virus-exposed treatment compared with the no-exposure treatment ($P = 0.037$). The opposite relationship existed for *L. clamitans*, where activity was 2% higher in the virus-exposed treatment ($P = 0.026$).

Virus exposure significantly reduced survival by 17%–100%, with survival lowest in tadpoles of *L. clamitans* and *L. sylvaticus* ($\chi^2_{[2]} \geq 12.6$, $P < 0.001$; Fig. 2A). However, exposure to predator cues did not decrease survival following virus exposure ($\chi^2_{[3]} \leq 0.6$, $P \geq 0.745$). For each species, there was no difference in viral load among the predator treatments within the virus treatment ($F_{[2,15]} \leq 1.3$, $P \geq 0.299$; Fig. 2B). However, mean viral load differed significantly among species ($F_{[3,41]} = 56.6$, $P < 0.001$). For individuals that survived until the end of the experiment, daily growth rate and developmental stage were largely unaffected by the virus ($F_{[1,24]} \leq 1.7$, $P \geq 0.207$), predator cues ($F_{[2,24]} \leq 2.6$, $P \geq 0.092$), or their interaction ($F_{[2,24]} \leq 2.3$, $P \geq 0.119$; Figs. 2C, 2D). The only exception was the tadpoles of *L. clamitans* that experienced 8% greater growth in the virus treatment compared with the no-virus treatment ($F_{[1,24]} = 12.1$, $P = 0.002$).

Discussion

Consistent with previous amphibian research, predator cues from *Belostoma* and *Anax* significantly reduced activity of all tadpole species (Relyea 2001*a*). Across all species, the greatest reduction in activity from predator cues occurred in *H. chrysoscelis* (12%–24%) and *L. sylvaticus* (10%–14%). Both species tend to breed in temporary or semi-permanent wetlands with relatively few aquatic predators and have developmental constraints to metamorphose before pond drying (Wellborn et al. 1996). Consequently, they typically display high activity levels in the absence of predators but respond strongly to the presence of predators (Relyea 2001*a*; Van Buskirk 2002). Although *L. clamitans* also displayed reduced activity with predators, the magnitude of the response was lower compared with *L. sylvaticus* and *H. chrysoscelis*. Low activity for *L. clamitans* has been reported (Thiemann and Wassersug 2000; Relyea 2001*a*), and usually is attributed to their association with permanent wetlands that contain more predators (Werner and McPeek 1994).

Anax cues reduced activity more than *Belostoma* cues for *H. chrysoscelis* and *P. feriarum* before virus exposure and for *P. feriarum* after virus exposure. However, there was no difference between the *Anax* and *Belostoma* treatments for *L. sylvaticus* and *L. clamitans*. Previous research has demonstrated that *Anax* larvae are more lethal than *Belostoma*, and tadpoles reduce activity level to a greater degree with more lethal predators (Relyea 2001*b*). Although we did not find a consistent reduction in activity level associated with predator riskiness across the tested species, research in a diversity of systems has shown substantial species-level and population-level variation in predator-induced plasticity (Dodson 1988; Kohler and McPeek 1989; Azevedo-Ramos et al. 1992; Spitze 1992; Peckarsky 1996; Relyea 2001*a*). Regardless of the mechanism driving the differences in the relative magnitude of tadpole responses to the predators, the threat of predation was effective at reducing tadpole activity level.

Mortality from ranavirus occurred in all tadpole species in our experiment, providing additional evidence that ranaviruses infect multiple amphibian hosts (Duffus et al. 2008; Hoverman et al. 2011). Mortality was substantially higher for ranid tadpoles (*L. clamitans* = 62%, *L. sylvaticus* = 100%) than for hylid tadpoles (*H. chrysoscelis* = 17%, *P. feriarum* = 19%), which corresponds with die-off trends in wild populations (Green et al. 2002) and experimental exposure studies (Hoverman et al. 2011). However, exposure to predator cues and ranavirus did not synergistically increase mortality rates as predicted. Given that exposure to predator cues before and after ranavirus exposure reduced activity for all species, it was clear that our procedures for predator-cue generation and exposure were effective. Moreover, the cue concentration used in our experiment is known to cause behavioral and morphological responses in tadpoles and is considered ecologically relevant (Van Buskirk 2001; Relyea 2002*a*; Schoeppner and Relyea 2008). The lack of differences in viral load among predator treatments provides additional support that predators may not function as significant ecological stressors for ranaviral disease. If predator cues were suppressing immune function, we would have expected to observe higher viral loads (an index of virion density; Inendino et al. 2005; Cotter et al. 2008) in predator treatments. Larvae of *A. tigrinum* that were exposed to *Ambystoma tigrinum* virus (ATV) and dragonfly cues experienced about 20% greater mortality than larvae exposed only to ATV (Kerby et al. 2011). We did not include a salamander species and used a FV3-like ranavirus in our experiments. Therefore, the impact of predator-induced stress on susceptibility to ranavirus may depend on amphibian species and ranavirus isolate. Until additional studies can be performed, our results suggest that generalizations about greater likelihood of ranavirus emergence in predator-rich environments should not be made or be restricted to aquatic systems with larvae of *A. tigrinum* and ATV.

There was no evidence that exposure to the virus altered the magnitude of predator-induced defenses. This result suggests that virus exposure does not alter the ability of tadpoles to detect or respond to the presence of predators. Virus exposure did alter the behavior of *H. chrysoscelis* and *L. clamitans*, but the responses were in opposing directions and relatively minor (≤3% change). Whether such minimal changes induced by ranavirus exposure are biological relevant remains to be tested. For example, these behavioral

Table 1. Results of repeated-measures ANOVAs examining temporal patterns of percent activity for four tadpole species (Green Frog, *Lithobates* (= *Rana*) *clamitans*; Wood Frog, *Lithobates* (= *Rana*) *sylvaticus*; Upland Chorus Frog, *Pseudacris feriarum*; Cope's Gray Treefrog, *Hyla chrysoscelis*) exposed to predator cues before virus addition (A) and after virus addition (B).

	Hyla chrysoscelis			*Pseudacris feriarum*			*Lithobates sylvaticus*			*Lithobates clamitans*		
Effects	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
(A) Before virus addition.												
Predator	2, 27	79.1	<0.001	2, 27	64.3	<0.001	2, 27	61.2	<0.001	2, 27	16.7	<0.001
Time	7, 21	73.0	<0.001	7, 21	77.1	<0.001	7, 21	156.7	<0.001	7, 21	144.1	<0.001
Time × predator	14, 42	4.3	<0.001	14, 42	4.9	<0.001	14, 42	1.3	0.265	14, 42	2.8	0.005
(B) After virus addition.												
Predator	2, 24	118.3	<0.001	2, 24	89.2	<0.001	2, 24	51.2	<0.001	2, 24	16.6	<0.001
Virus	1, 24	4.9	0.037	1, 24	1.7	0.204	1, 24	0.1	0.767	1, 24	5.7	0.026
Predator × virus	2, 24	0.1	0.886	2, 24	0.6	0.555	2, 24	0.2	0.810	2, 24	0.3	0.740
Time	15, 10	38.7	<0.001	17, 8	14.7	<0.001	7, 18	17.9	<0.001	9, 16	23.1	<0.001
Time × predator	30, 20	2.1	0.048	34, 16	4.1	0.002	14, 36	1.8	0.070	18, 32	1.3	0.277
Time × virus	15, 10	2.5	0.071	17, 8	0.8	0.664	7, 18	2.8	0.039	9, 16	1.5	0.218
Time × predator × virus	30, 20	1.4	0.237	34, 16	1.3	0.314	14, 36	2.8	0.007	18, 32	1.2	0.338

Note: Degrees of freedom differ among species for some effects because experimental units were removed from the analysis owing to mortality.

Fig. 1. The effects of predator cues on the percent activity of tadpoles of Cope's Gray Treefrog (*Hyla chrysoscelis*), Upland Chorus Frog (*Pseudacris feriarum*), Wood Frog (*Lithobates* (= *Rana*) *sylvaticus*), and Green Frog (*Lithobates* (= *Rana*) *clamitans*) before (A) and after (B) the addition of the virus. Predator-cue treatments are no predator (NP), giant water bug (*Belostoma flumineum*) (B), and dragonfly *Anax* sp. (A). Open circles represent the no-virus treatment and closed circles represent the virus treatment. Data (least-squares means ± 1 SE) were averaged across time.

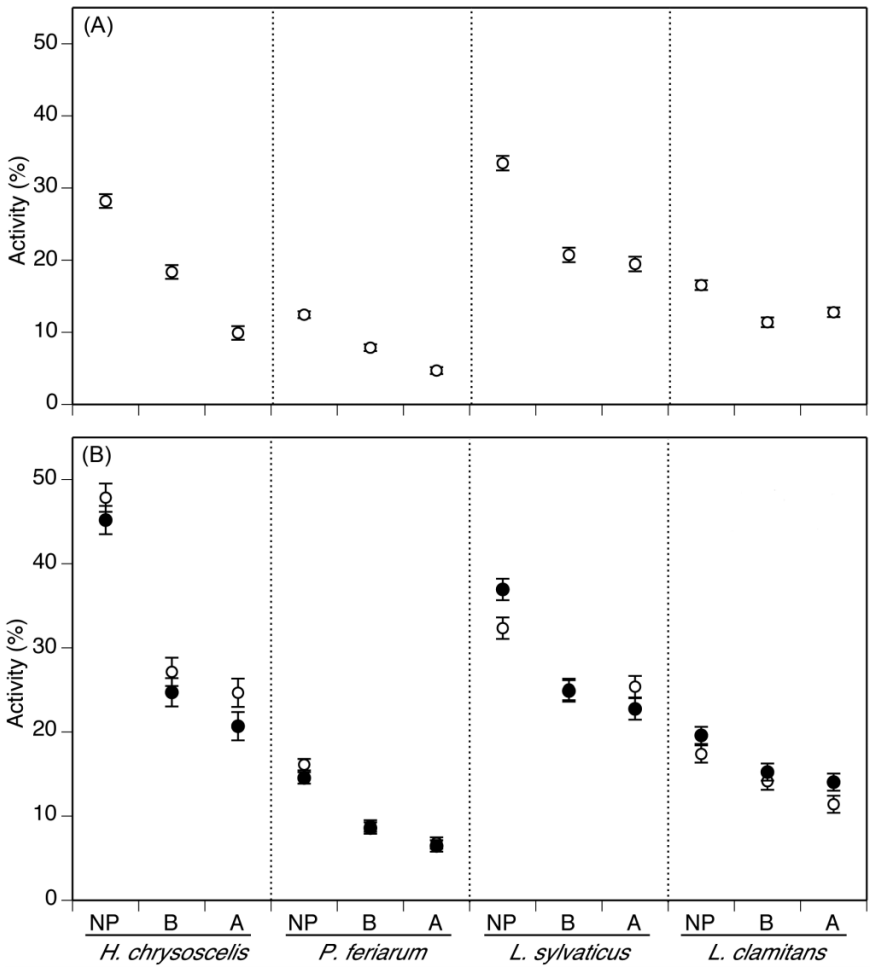
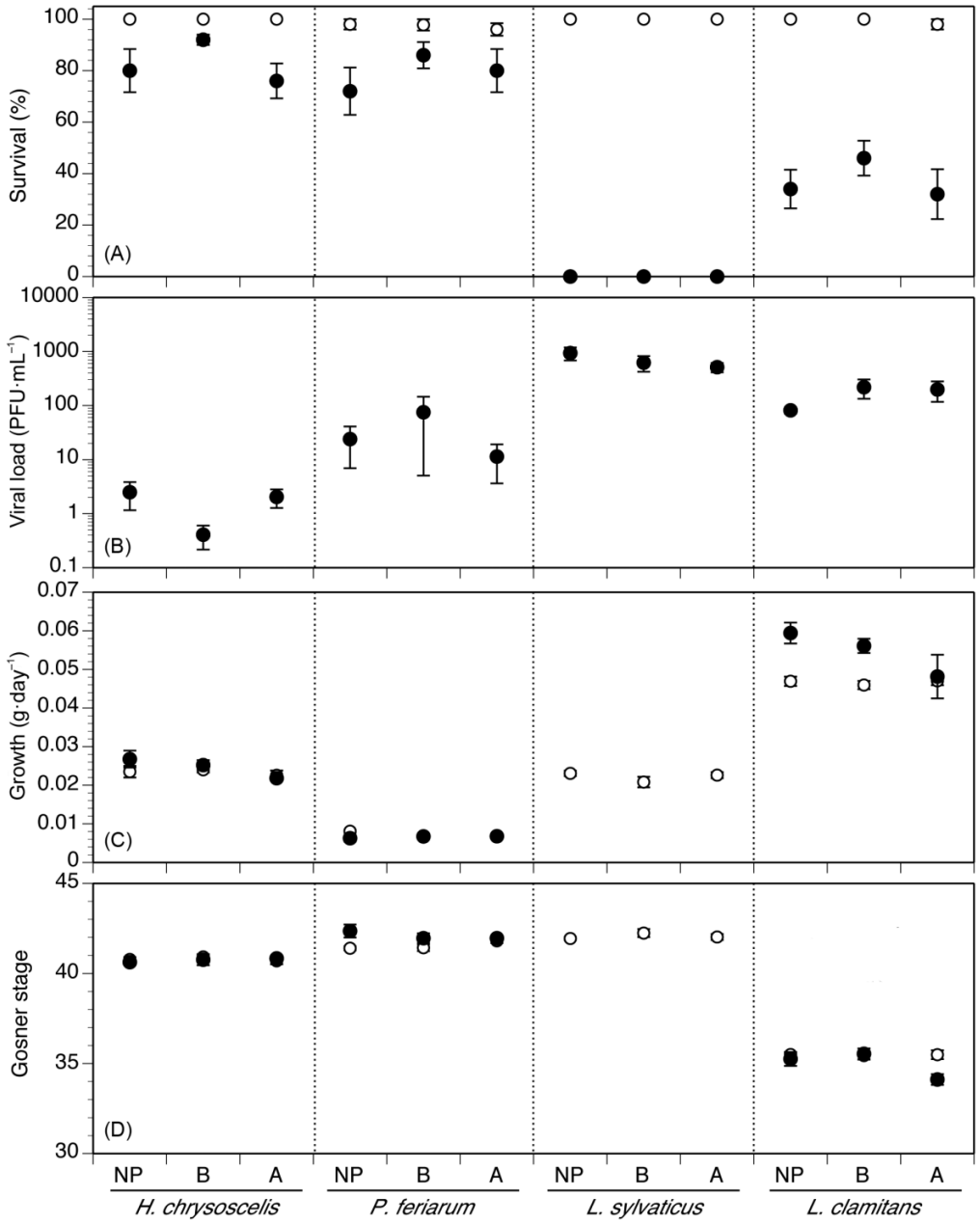


Fig. 2. The effects of predator cues and virus addition on the survival (A), viral load (B), growth (C), and developmental stage (D) of tadpoles of Cope's Gray Treefrog (*Hyla chrysoscelis*), Upland Chorus Frog (*Pseudacris feriarum*), Wood Frog (*Lithobates* (= *Rana*) *sylvaticus*), and Green Frog (*Lithobates* (= *Rana*) *clamitans*). Predator-cue treatments are no predator (NP), giant water bug (*Belostoma flumineum*) (B), and dragonfly *Anax* sp. (A). Open circles represent the no-virus treatment and closed circles represent the virus treatment. Data are means ± 1 SE.



changes could be simple by-products of infection (i.e., host pathology); severe edema associated with infection could reduce tadpole mobility (Gray et al. 2009). Alternatively, Parris et al. (2004) reported that ranavirus-infected *A. tigrinum* had greater activity levels than uninfected individuals. They surmised that the increased activity might be pathogen-induced and help facilitate transmission by increasing the likelihood of contact between hosts (Parris et al. 2004). Ultimately, studies that relate changes in the behavior of amphibian hosts to pathogen transmission within natural communities are necessary to determine whether these behavioral responses are simply by-products of infection or adaptive responses induced by the pathogen to facilitate transmission.

There are very few studies that have examined the role of natural stressors in driving the susceptibility (infection and mortality) of amphibians to pathogens. In other animal taxa, natural stressors, including the threat of predation, have been shown to negatively affect immune parameters and, in some cases, increase susceptibility to diseases (Griffin 1989; Boonstra et al. 1998; Rigby and Jokela 2000). These findings have led to generalizations that natural stressors affect taxa similarly and contribute to host–pathogen dynamics (Carey et al.

1999). Although it appears that the threat of predation may increase susceptibility of amphibians to certain pathogens (Thiemann and Wassersug 2000; Kerby et al. 2011), this effect is far from consistent among species or pathogens (Parris and Beaudoin 2004; Raffel et al. 2010). Our study did not support the hypothesis that the threat of predation increases susceptibility to ranaviruses; however, more studies are needed to verify if this trend holds true across additional amphibian taxa and viral types.

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