FROG DECLINES: EXPLORING CONNECTIONS AMONG CLIMATE CHANGE, IMMUNITY AND DISEASE.

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To the Gang.

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CLIMATE CHANGE, IMMUNITY AND DISEASE.

Frog and toad populations have been declining drastically all over the world. For the past 20 years, marked declines have been recorded for 40% of the frog and toad species in the Monteverde Cloud Forest Preserve (MCFP), Costa Rica, where climate change and disease have been implicated. Our study explored possible connections between climate change, immunity and disease susceptibility, examining in particular how climate-induced changes in pond water levels may influence amphibian immune systems. We examined immune development and function (thymus growth, antibody production and skin graft rejection) in Meadow treefrogs (*Hyla pseudopuma*), a declining species in the MCFP, when tadpoles developed under various water regimes. During the wet seasons of 2001-2004, laboratory experiments were conducted in the MCFP by subjecting tadpoles to constant or declining water levels. Field studies also took place in man-made and natural forest ponds that experienced different rainfall and pond water retention patterns. Our studies suggested that development under shallow or highly variable water levels led to weaker immunity. Under these conditions, both tadpoles and young froglets were less able to reject foreign tissue, an important immune response involved in initially fighting off infection. These data support the hypothesis that climate-induced changes in pond water can influence amphibian immune development as well as function. Collectively, these results may have important implications concerning climate change effects on amphibian populations, as well as the possible role of immunosuppression and increased disease susceptibility in species declines.

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Chapter 1. Introduction: Frog declines, climate change and disease: searching for a connection.

Summary

Anuran population declines in undisturbed, protected areas have spurred researchers worldwide to try and determine cause and effect. In the Monteverde Cloud Forest Preserve, Costa Rica, synchronous amphibian crashes in 1987-1988 resulted in extinctions and declines of 40% of its anuran species. This event and other declines over the past two decades have correlated with periodically drier climatic conditions. Pathogenesis has also been observed in anuran populations, with various pathogens implicated around the globe. One hypothesis to explain this apparent increase in pathogenesis is stress-induced immunosusceptibility, with climate change and possible resulting decreases in pond water levels acting as a stressor. If pond desiccation causes stressful and rapid development, one possible physiological trade-off may include an underdeveloped and/or low functioning immune system. To tie these ideas together, I focus on the effects of low water availability on metamorphosis and immunological development of *Hyla pseudopuma* (Meadow treefrog), a declining species in Costa Rica.

Introduction and justification

Frog populations have been experiencing unexplained declines and extinctions in undisturbed, protected areas, on all 6 continents that they inhabit (Weygoldt 1989; LaMarca & Reinthaler 1991; Carey 1993; Kagarise Sherman & Morton 1993; Pounds & Crump 1994; Drost & Fellers 1996; Laurance et al. 1996, 1997; Pounds et al. 1997, 1999; Lips 1998, 1999; Pounds 2000). Massive declines were first reported in temperate zones in the early 1970's and joined by the tropical zones the following decade (Carey et al. 1999). Declines have been geographically widespread, occurring often at higher altitudes or cooler regions, and resulting in massive mortalities for each impacted species. Tadpole-bearing species in tropical montane habitats have experienced some of the worst declines and extirpations. Researchers have brought forth numerous hypotheses (e.g., habitat fragmentation, pesticide contamination, introduced predators and pathogens, ozone depletion and UV-B exposure, and climate change) to explain the declining populations. However, the exact causes and consequences for these declines remain unknown.

Areas of concern involve the role of amphibians in the ecosystem. In many habitats, these abundant vertebrates are a major source of efficient energy transfer up the food chain (Stebbins & Cohen 1995). They play a substantial role in primary production conversion, nutrient flux, and invertebrate biomass regulation (Seale 1980; Stebbins & Cohen 1995). Amphibians also may serve as environmental indicators for humans and wildlife. They undergo metamorphosis in exposed conditions, experience both aquatic and terrestrial habitats, and have permeable skin, making them susceptible to environmental perturbation (Stebbins & Cohen 1995). Amphibian population declines, therefore, may be indicative of environmental degradation and future extinctions of other organisms (Parsons 1989).

In Monteverde, a premontane region of Costa Rica, Pounds et al. (1999) suggest that amphibians, as well as reptiles and birds, are experiencing community alterations due to climate change. There is evidence that increased sea surface temperatures over time have led to rising orographic cloud bases, limiting moisture to communities in the cloud forest. Mist frequency, which is negatively correlated with sea surface temperatures in the equatorial pacific, has been declining dramatically since the mid-1970's. Pounds et al. (1999) show an association between the frequency of dry-season mist and reptile, bird and anuran community changes.

In the Monteverde Cloud Forest Preserve (MCFP), synchronous amphibian crashes in 1987-1988 resulted in extinctions and declines of 40% of its anuran species (Pounds & Crump 1994; Pounds et al. 1997, 1999; Pounds 2000). This event and other amphibian declines in Monteverde since 1987 have coincided with climatic change and increasingly-frequent El Nino years. Pounds et al. (1997, 1999) documented a strong correlation between the species declines and climatic warming and drying.

Infectious disease has been implicated in amphibian declines around the globe (Carey et al. 1999). While the chytrid fungus *Batrachochytrium dendrobatidis* in Australia and Central America has received much attention, other fungal, bacterial, viral and parasitic pathogens have been observed. *Basidiobolus ranarum* has been isolated from extinct Wyoming toad populations (Taylor et al. 1999). Another fungus, *Saprolegnia ferax*, has been linked to embryo deaths of 3 anuran species in the Pacific Northwest, USA (Kiesecker & Blaustein 1997). In Britain, iridiovirus infection with secondary bacterial infection was determined to be the cause of unusual mortalities of the common frog (*Rana temporaria*, Cunningham et al. 1996). While pathogenesis clearly has played a role in amphibian declines, it may not provide the foundation for explaining the declines. Rather, disease might be a symptom or consequence of an underlying reason. The wide array of pathogens implicated suggests that some environmental perturbation, such as global climate change, may be causing anuran susceptibility to disease.

The scientific community is finally in general agreement that global amphibian declines are occurring. Some scientists are searching for a single global reason; others attribute the declines to multiple environmental perturbations across the globe; and yet others, most recently, are starting to look for connections in climate change, pollutants, epidemiology, and immuity. The lack of clear evidence for any one explanation, and the convincing evidence of a global phenomenon, points to complex causation. With widespread pathogenicity across all 6 continents where amphibians reside, increased susceptibility may be the key -- but how? Is climate change influencing community structure and altering host-pathogen interactions? Is climate change inducing temperature and moisture stress, decreasing immune function?

My research focuses on the question of whether climate change can indirectly lead to immunosuppression, and therefore increased susceptibility to infectious disease. Specifically, I test the hypothesis that reduced or variable pond water levels increases the rate of metamorphosis and negatively affects immune development and function, which would likely lead to disease susceptibility (Table 1). I studied both tadpoles and froglets to better understand the effects of water level on immune development and function at different life stages, and ran experiments in a laboratory-controlled setting as well in the field to explore more natural ecological responses.

Study area and organism

The Meadow Treefrog (*Hyla pseudopuma*) was the study organism in all studies. This species is endemic to montane regions of Panama and Costa Rica (Pounds et al. 1999), and is one of the frog species that declined in Monteverde and has not rebounded to pre-decline proportions (Pounds et al. 1999). This species nevertheless has remained sufficiently abundant for study, and its relatively opportunistic breeding behavior and high fecundity make field and laboratory research feasible (Crump & Townsend 1990; Crump 1991a). In designing my experiments, I used previous observations and research to gain information on *H. pseudopuma* breeding sites, habitat use, relative abundance through time, and tadpole and adult biology (Crump 1984, 1989, 1991a, 1991b; Crump & Townsend 1990; Pounds 2000; pers. obs.).

In the Monteverde region of the Cordillera de Tilarán mountain chain, *H. pseudopuma* has been recorded above 1200m on the Pacific slope and above 900m on the Atlantic slope, straddling the Continental Divide at 1680m (Pounds, unpubl. data; Fig. 1). Within this 8km range a climatic gradient exists, with a drier Pacific slope; increased mist, precipitation and cloud cover at the Continental Divide; and a more gradual decrease in mist, precipitation and cloud cover on the Atlantic slope. Conditions during the breeding seasons of 2000-2004 included unexpectedly low precipitation and frequent wind, breeding ponds that remained dry throughout the breeding season, and unusually rapid and frequent desiccation of rain-filled ponds.

Pond desiccation, plastic metamorphosis and immunity

Initial studies were solely carried out to better understand the ability of *H. pseudopuma* to develop plastically (Chapter 2). In this experiment, we examined the plastic development of *H. pseudopuma* tadpoles natal to two different breeding areas along the climatic gradient in Monteverde, Costa Rica (located on the Pacific slope and Continental Divide). By exposing individuals from both breeding sites to different water level treatments in the lab, we were able to study both environmental and genetic effects on developmental rate, as well as genetic differentiation between sites. At various stages of development, we exposed these tadpoles to either a constant high water level or a sudden decline in water level, simulating what might occur when rainfall is not adequate for maintaining pond water levels. While individuals from both the Pacific slope and Continental Divide responded plastically to lowered water levels, individuals natal to the Pacific slope metamorphosed earlier than the Continental Divide under both high and low water environments, but those from the Continental Divide showed consistent responses to water decline across clutches. These differences suggest genetic differentiation between the Pacific slope and Continental Divide populations.

Various other anuran species have been shown to exhibit plastic metamorphosis in response to pond water loss by increasing their developmental rate and metamorphosing earlier (*Bufo americanus*, Wilbur 1987; *Ambystoma talpoideum*, Semlitsch & Wilbur 1988; *Scaphiopus couchii*, Newman 1989; review by Newman 1992; *Rana temporaria*, Laurila 1997; *Scaphiopus hammondii*, Denver 1997a, 1997b, Denver et al. 1998). This response occurs without chemical cues, conspecific interactions, or temperature changes (Crump 1989; Denver et al. 1998), and may be due to perception of decreasing water volume (Denver et al. 1998) or crowding ((Denver 1997b; Kikuyama et al. 1993; Hayes & Wu 1995). A similar response to pond desiccation was previously found in *H. pseudopuma* by Crump (1989), although it remained undetermined whether this species had responded to crowding, perception of lowered water level, or a synergy of both stimuli.

Research on the desert species *S. hammondii* (Western spadefoot toad) demonstrated that pond drying correlates with activation of the hypothalamic-pituitary-interrenal (HPI) axis, ultimately leading to the production of the stress hormone corticosterone (Denver 1997b, 1998). HPI actuation also triggers production of thyroid hormones (TH: thyroxine (T3) and triidothyronine (T4)) that prompt metamorphosis (Denver 1997b, 1998). The release of corticosterone and thyroid hormones under stressful conditions is synergistic in action, leading to not only earlier but also faster metamorphosis (Kikuyama et al. 1993; Denver 1995, 1997a, 1997b; Hayes 1997; Denver et al. 2002; Boorse & Denver 2004).

If pond desiccation triggers a stress response, increasing developmental rate and earlier metamorphosis, it would follow that developmental or physiological consequences might occur. Body size at metamorphosis has repeatedly been shown to decrease under drying conditions, incurring possible fitness consequences in the form of both survival and reproduction (Crump 1989; Semlitsch et al. 1988; Denver et al. 1998; Newman 1989, 1992). Beyond body size, however, few studies have been performed with the purpose of examining differences in systemic development. Metamorphosis has been exogenously prompted in the laboratory to determine the course of endocrine pathways (Denver 1997b, 1998; Denver et al. 2002; Boorse & Denver 2004), and the conversion from tadpole to adult immunity (Flajnik et al. 1985; Rollins-Smith & Blair 1993; Rollins-Smith & Cohen 1996; Barker et al. 1997; review in Rollins-Smith et al. 1997; Rollins-Smith et al. 1988), but few immunological developmental comparisons have been made between normal and rushed metamorphosis (such as Rollins-Smith et al. 1988). It is reasonable to hypothesize that an increase in developmental rate or stress may lead to a greater chance for developmental errors, or less investment in development, maintenance or repair (Sibly & Calow 1986), and that growth, differentiation rate, and immune development and function may be functionally integrated (Stearns 1998; Newman 1992). One consequence of rapid and/or stressful development could be an underdeveloped or lower functioning immune system (Fig. 2).

To explore how pond water conditions might influence tadpole and froglet immunity, we tested the impact of water declines in the laboratory (Chapter 3), and natural pond water regimes in the field (Chapter 4), on skin graft rejection. Skin allograft rejection is a classical immune model that has been used to study in-vivo foreign tissue destruction for over 60 years (Rollins-Smith 2001; review in Rosenberg & Singer 1992). This type of tissue rejection primarily involves a cellular immune response, and is largely induced by helper and cytotoxic T cells and their related cytokines (reviews in Brown et al. 1975, DuPasquier et al. 1986, Du Pasquier et al. 1989, Edwards & Ruben 1982, and Flajnik et al. 1987; Rosenberg & Singer 1992; Rau et al. 2001; Rollins-Smith 2001).

In our graft rejection experiments, we established two related predictions: 1) If stressful pond water conditions suppress amphibian immune function, tadpoles will be less competent in rejecting their skin grafts. 2) If stressful pond water conditions negatively impact immune development, froglets may also be less competent in rejecting their skin grafts.

In response to greater pond water variation and desiccation in the field, both tadpoles and froglets were slower in rejecting skin allografts. These results suggested that water level variation and decline during tadpole development negatively impacts the development and function of tadpole and froglet immune systems. Collectively, these data support the hypothesis that climate-induced changes in pond water have the potential to influence immune development and function, possibly contributing to disease susceptibility and frog declines.

Additional studies

Our first attempt to explore possible immunological consequences was examining thymus growth in young *H. pseudopuma* froglets after they had undergone a sharp decline in water level in the laboratory during tadpole development. Although thymocyte numbers may not be indicative of an actual immune response, such measures can provide a rough estimate of immune development and function (Rollins-Smith et al. 1988, 1997). For this study I dissected the thymus glands (an immune system organ) one month post-metamorphosis and counted the lymphocytes. Although no differences were apparent between control and sudden low water treatment individuals, the variation in the data was large, and it was unclear whether the results were representative of the water treatments.

We also attempted to study the ability of tadpoles to produce antibodies against foreign antigens, giving a direct measure of humoral immunity. Tadpoles developing in control and low water levels in the laboratory and the field were challenged with injections of sheep red blood cells (Sigma Chemical Co., St. Louis, MO), and their intraperitoneal fluid was collected one week later. Although hemagglutination and ELISA assays were run to quantify antibody production, results were unsatisfactory.

As part of a separate collaborative effort, we also tested the ability of froglets that developed under the various laboratory water treatments and field pond regimes to produce antimicrobial peptides (L. Rollins-Smith, Vanderbilt University). The measure of antimicrobial peptides provides a direct method for studying innate (non-specific) immunity. The peptides are also being challenged in a laboratory assay against the chytrid fungus (*Batrachochytrium dendrobatidis*). This assay will allow us to explore the effectiveness of antimicrobial peptides produced by *H. pseudopuma* against the chytrid fungus, which is a pathogen that has been attributed to species declines in Central America and Australia (Lips 1998, 1999; Berger et al. 1998). This project is on-going, and is not included in this present dissertation. A schematic showing all of the research studies and foci is in Fig. 3.

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Table 1. Break-down of dissertation research questions.

Research focus		Relevance
Effects of pond water limitation	→	Global climate change, dry conditions in Monteverde. (Pounds et al. 1997, 1999).
on differentiation rate and	→	Basic questions regarding plasticity of differentiation and metamorphosis.
immunological development	→	Immunological trade-offs of rapid development? Could this lead to greater disease susceptibility?
of *Hyla pseudopuma*	→	*H. pseudopuma* declines correlate with climatic drying (Pounds et al.1999); *H. pseudopuma* exhibits plastic development (Crump 1989).
in Monteverde, Costa Rica,	→	Historical information on climate and amphibian population changes; unexplained declines (Pounds & Crump 1994; Pounds et al. 1997, 1999; Pounds 2000).

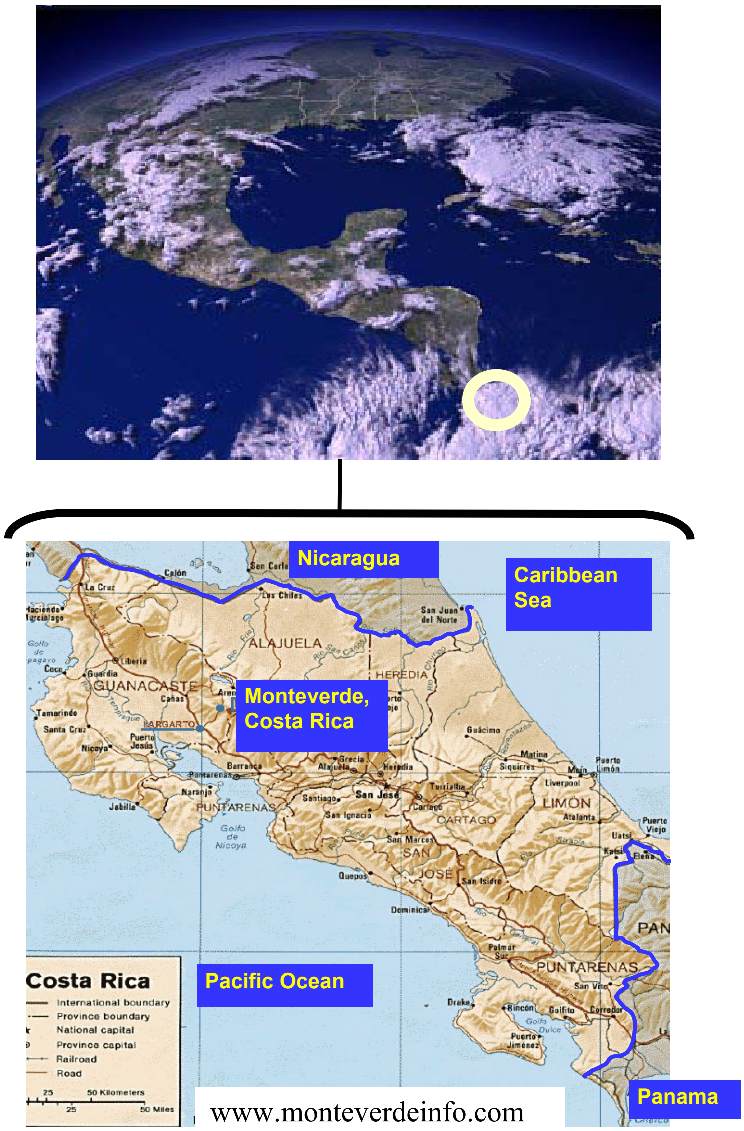

Figure 1. In the Monteverde cloud forest, *H. pseudopuma* is present above 1200m altitude on the Pacific slope and 900m on the Atlantic slope, with the continental divide at 1680m. Within this 8km range a climatic gradient exists, with a drier Pacific slope; increased mist, precipitation and cloud cover at the continental divide; and a more gradual decrease in mist, precipitation and cloud cover on the Atlantic slope.

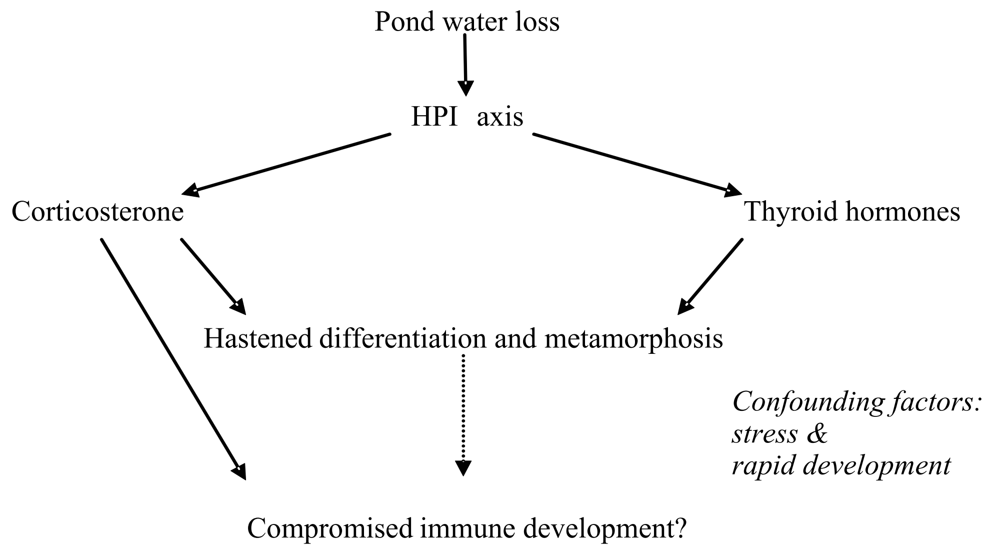

Figure 2. Sequence of hormonal events occurring during plastic development under low water conditions, and their potential implications for immune system development.

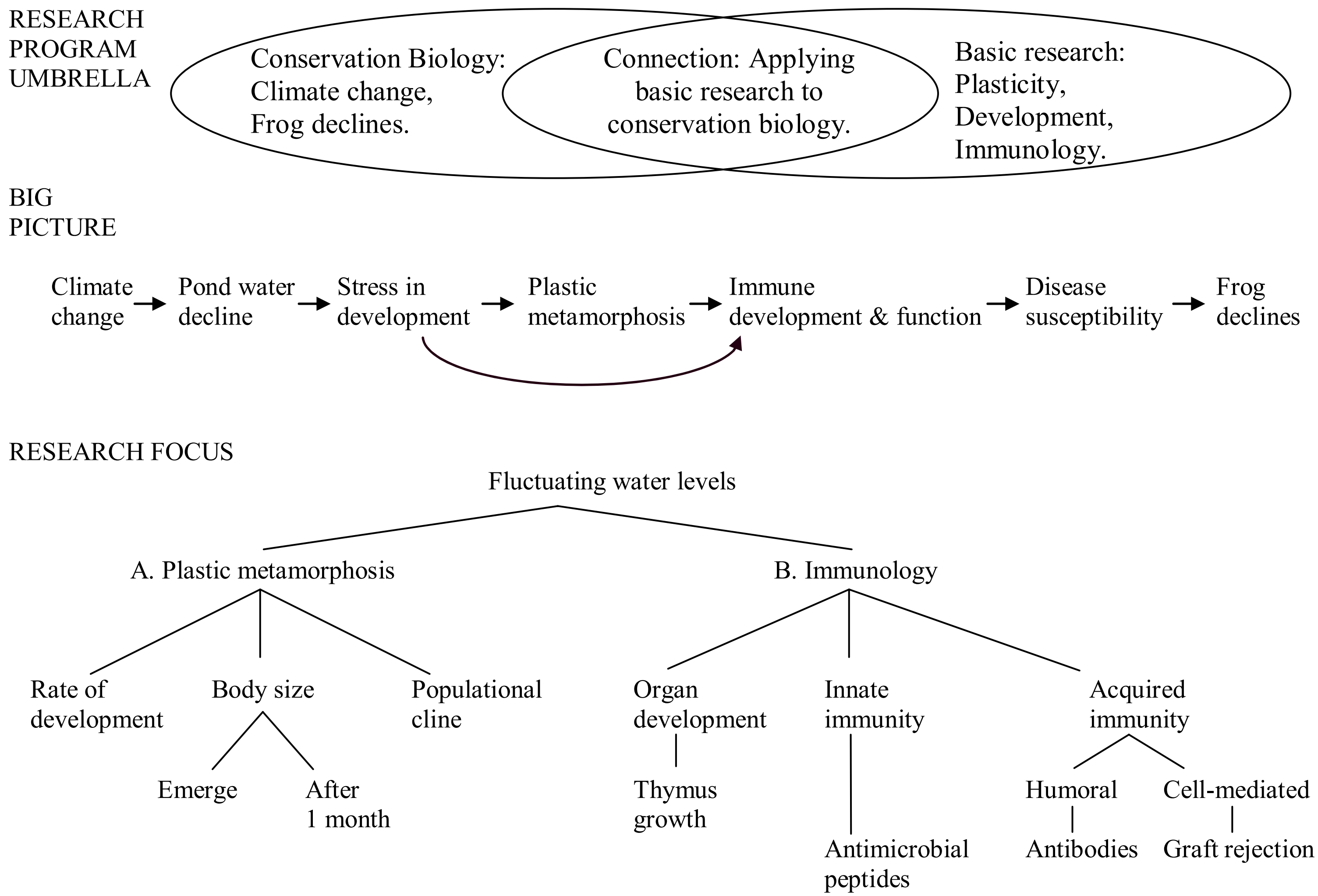

Figure 3. Schematic of Ph.D. research program.

Chapter 2. Plastic metamorphosis along a climatic gradient in Monteverde, Costa Rica.

Introduction

Frog populations have been experiencing unexplained declines and extinctions in undisturbed, protected areas, on all 6 continents that they inhabit (Weygoldt 1989; LaMarca & Reinthaler 1991; Carey 1993; Kagarise Sherman & Morton 1993; Pounds & Crump 1994; Drost & Fellers 1996; Laurance et al. 1996, 1997; Pounds et al. 1997, 1999; Lips 1998, 1999; Pounds 2000). In the Monteverde Cloud Forest Preserve (MCFP), synchronous amphibian crashes in 1987-1988 resulted in extinction or decline of 40% of its anuran species (Pounds & Crump 1994; Pounds et al. 1997, 1999; Pounds 2000). The Meadow Treefrog (*Hyla pseudopuma*), endemic to lower montane areas of Panama and Costa Rica, is a frog species that declined in Monteverde, Costa Rica and has not rebounded to pre-decline proportions (Pounds et al. 1999). In the early 1980s, *H. pseudopuma* could be found by the hundreds during explosive breeding episodes (Crump & Townsend 1990), but after the 1987-1988 frog declines much lower numbers were documented at the same site (1990-1994: maximum at any one census = 12; Pounds et al. 1997). During our field research spanning the wet seasons of 2000-2004, sightings of breeding adults were rare, and breeding congregations were relatively small, even when conditions appeared ideal for reproduction.

The 1987-88 crash and other amphibian declines in Monteverde since 1987 have coincided with climatic change and increasingly-frequent El Nino years (Pounds et al. 1997, 1999). There is evidence that increased sea surface temperatures have led to rising orographic cloud bases, limiting moisture to communities in the cloud forest. Mist frequency, which is negatively correlated with sea surface temperatures in the equatorial pacific, has been declining dramatically since the mid-1970's. An association between the frequency of dry-season mist and anuran abundances over time has been shown, with a strong correlation documented between the anuran declines and climatic warming and drying (Pounds et al. 1997, 1999).

For animals that have evolved to exist in the cool, wet cloud forest environment, increasing temperatures or reduced precipitation resulting from climate change could have far-reaching consequences for reproductive fitness and survival (Pounds & Crump 1994). Montane regions such as Monteverde that are strongly influenced by the tropical Pacific are thought to be especially influenced by atmospheric disturbances and climate change (Pounds & Crump 1994;

Pounds 2001). The El Niño effect has been shown to cause declines in dry season precipitation (mostly in the form of reduced mist), and at times alter wet season rainfall by influencing northeasterly trade winds (Pounds & Crump 1994; Pounds et al. 1999; Pounds 2001). It is logical that these climatic changes might impact anuran breeding ponds, increasing the frequency and duration of low pond water levels and pond desiccation.

In response to pond desiccation, various anuran species exhibit plastic metamorphosis by increasing their developmental rate and metamorphosing earlier (*Bufo americanus*, Wilbur 1987; *Ambystoma talpoideum*, Semlitsch & Wilbur 1988; *H. pseudopuma* Crump 1989; *Scaphiopus couchii*, Newman 1989; review by Newman 1992; *Rana temporaria*, Laurila 1997; *Scaphiopus hammondii*, Denver 1997a, 1997b, Denver et al. 1998). While a similar response to pond desiccation was previously found in *H. pseudopuma* (Crump 1989), it remained undetermined whether this species responded to crowding, perception of lowered water level, or a synergy of both stimuli. This type of developmental plasticity is characteristic of species that have evolved in the unpredictable environment of temporary ponds (Denver 1997a, 1998; Denver et al. 1998; Newman 1989, 1992), and is thought to be an adaptive trait (Wilbur and Collins 1973; Wilbur 1987; Newman 1989, 1992; Denver 1997b).

The goal of the present study was to examine the development plasticity of *H. pseudopuma* tadpoles natal to two different breeding areas along a climatic gradient in Monteverde, Costa Rica. In the laboratory we exposed tadpoles at various stages of development to either a constant high water level or a sudden decline in water level, simulating what might occur when precipitation is not adequate for maintaining pond water levels. Questions regarding genetic differentiation between the field sites, clinal variation, plasticity reaction norms and trade-offs are broached. The data are interpreted from a basic science perspective and within the context of climate change and conservation biology.

Study area and organism

In the Monteverde region of the Cordillera de Tilarán mountain chain, *H. pseudopuma* has been recorded above 1200m on the Pacific slope and above 900m on the Atlantic slope, straddling the Continental Divide at 1680m (Pounds, unpubl. data). Within this 8km range a climatic gradient exists, with a drier Pacific slope; increased mist, precipitation and cloud cover at the Continental Divide; and a more gradual decrease in mist, precipitation and cloud cover on the Atlantic slope.

One of the two breeding sites used for this study is in the Monteverde Cloud Forest Preserve along the Continental Divide (MCFP, Sendero Pantanoso, 1680m elevation). This area is mostly primary cloud forest and is in the Lower Montane Rainforest Life Zone (Holdridge 1982, Pounds et al. 1997). The second breeding site is in Pacific slope cloud forest less than 1km from the MCFP entrance (Golden Toad Laboratory for Conservation, GTLC outdoor man-made ponds,1640m). This site is surrounded by a mosaic of secondary and primary cloud forest and pasture at the lower edge of the Lower Montane Rainforest Life Zone (Holdridge 1982, Pounds et al. 1997).

A climatic gradient exists between the Continental Divide and Pacific slope sites. During the dry season, the Continental Divide is wetter than the Pacific slope because of moisture-laden trade winds that meet the mountain range and dump their moisture, oftentimes in the form of clouds and mist (Pounds & Crump 1994). Climatic differences are less pronounced during the wet season, however, with the heavier conventional storms arriving from the Pacific lowlands as well as advective precipitation coming from the Caribbean (Atlantic) basin (Crump et al. 1992; Pounds & Crump 1994). In addition to basic alee effects, the Pacific slope breeding site is also near a noticeable transition from the Lower Montane Holdridge Life Zone to the warmer and drier Premontane Holdridge Life Zone, which tends to get less rain and mist and more sun (Holdridge 1982). Although the Continental Divide and Pacific slope breeding sites do not tend to differ in timing of first breeding, differing rain patterns during the wet season have the potential to lead to distinctive egg laying and tadpole duration patterns.

As *H. pseudopuma* is an opportunistic temporary-pond breeder with relatively large egg clutches, risk of egg and tadpole mortality may be considered a natural part of their ecology. Indeed, for any species that has evolved developmental plasticity allowing them to breed in temporary ponds, we would expect to see natural occurrences of egg and tadpole mortality due to pond desiccation. However, it is notable that in the MCFP primary cloud forest, tadpoles that are developing in more “ideal” breeding ponds are still suffering from frequent and intense drying episodes.

In 2000-2004, the first heavy rains came between mid-May to mid-June, prompting the first *H. pseudopuma* cohorts to be laid. One to two dry spells occurred in July; and heavier rains came again in early August. The forest breeding ponds were often stagnant and uninhabitable for *H. pseudopuma* by late August, however, due to microorganism and insect predator build-up. This was most noticeable when ponds underwent frequent and intense dry periods throughout the season. Cohorts laid in August usually did not survive far into egg or tadpole development, effectively shortening *H. pseudopuma's* potential breeding season. Earlier in the season, periods of pond desiccation led to the emergence of small and weak individuals struggling to metamorphose and survive.

Methods

Over a 5-day period in June 2001, eggs were collected from a large natural breeding pond historically and currently used by *Hyla pseudopuma* on the Continental Divide (MCFP), and from outdoor man-made ponds used by a natural breeding population on the Pacific slope in Monteverde, Costa Rica (GTLC). At each site, 80 eggs were taken from each of 5 clutches and transferred to the MCFP laboratory. Once the hatchling tadpoles were free-swimming, they were separated into 800 individual plastic 16oz drinking cups to avoid conspecific interactions (water level at 8cm = 255ml volume; 7.5cm diameter at water surface and 5.5cm diameter at bottom). The cups were placed in a stratified design interspersing clutches and treatments on and among shelves to control for environmental variation caused by cup location. Decomposing leaf parts from the forest floor were added to the cups ad-libitum for food, with occasional supplementation with ground rabbit food. Water was changed every 3-4 days until the date of water level change, after which cups with low water levels received a water change every 2 days.

The main treatment effect was water level. High water controls experienced an 8 cm water level throughout their entire tadpole phase (water level at 8cm = 255ml volume; 7.5cm diameter at water surface and 5.5cm diameter at bottom), while sudden low water treatments experienced an initial 8 cm water level followed by a sudden decrease to 2 cm (55ml volume; 6.0cm diameter at water surface and 5.5cm diameter at bottom) (methods modified from Denver 1998). We imparted the sudden decline in water level in order to simulate what may occur in natural ponds when precipitation is inadequate. Because declining water levels tend to cause a developmental delay in early tadpole stages, but hasten development in later tadpole stages (Denver 1997a, 1997b & 1998), water level treatments were initiated only after hindlimb bud emergence (tadpole age = 43-45 days). To account for developmental variation at the time of water decline, the developmental stage of each tadpole was determined using a 10x hand lens (Gosner 1960). After staging, water level treatment designations (high or low) for a small number of cups were switched to ensure even distribution of gosner stages (GS) across treatments. Water level treatment was initiated immediately after staging by refilling the water to 2cm for treatments, or to 8cm for controls. Once tadpoles neared emergence, all cups were checked for emerging individuals every 1-2 days. The duration from water level treatment initiation to emergence served as our measure of developmental rate.

Statistical Analysis

Time to emergence from the date of water level change was analyzed with a MIXED model ANOVA (time to emergence = water level treatment + site + clutch (nested within site) + water level*site + gosner stage as a covariate) (type III F test, $\alpha = 0.05$; SAS 2005). Because the sudden change in water level affected tadpoles at early hindlimb development (GS 27-33) differently than those at later hindlimb development (GS 34-40), statistical analyses were run separately on these two groups. Water level and site were fixed effects, and clutch was designated as random. Because tadpole developmental rate varied before the start of the water level treatment, gosner stage at the time of water treatment initiation provided a significant covariate in the models. Clutch nested within site was also significant, helping to explain variation in the model. Reaction norms were compared visually by looking at the slope of the line for each population, or statistically with the water level*site interaction term (Fig. 2; Via et al. 1995). Plasticity was further examined by analyzing time to emergence separately for the Continental Divide and Pacific slope clutches (MIXED model by site: time to emergence = water level + water level*clutch + gosner stage). Genetic variation in plasticity among clutches was compared visually by looking at the slopes of the various lines within each population (Fig. 3; Via et al. 1995), and statistically by running the above model in a GLM ANOVA and determining the type III mean square (MS) for the water level*clutch (GxE) interaction term (Newman 1992; SAS 2005).

Results

In response to experimental declines in water level, *Hyla pseudopuma* tadpoles of different developmental stages, and from different sites, showed specific patterns of change in their developmental rate. Tadpoles first exposed to a decrease in water level during early hindlimb development (GS 27-33) did not differ significantly in their time to emergence compared to controls ($p = 0.5$, $n = 36$-48 for each site-treatment combination; Early hindlimb development, Fig. 1). Moreover, in the early hindlimb development group the Pacific slope population did not differ in their time to emergence compared with the Continental Divide population under control or lowered water level conditions (site p = 0.5, water level*site p = 0.3).

Tadpoles first exposed to low water level during later hindlimb development (GS 34-40) increased their developmental rate and emerged earlier than controls from both sites (water level treatment p = 0.0001, water level*site p = 0.5; n = 136-149 for each site-treatment combination; Late hindlimb development, Fig. 1). The mean difference in developmental time between constant high water and lowered water level treatments was 1.16 days (5.7%) for the Pacific slope and 2.04d (8.5%) for the Continental Divide. For both treatment and control individuals, mean time to emergence differed between populations (site p = 0.006), with individuals natal to the Pacific slope finishing their development 14% faster under control water conditions than those from the Continental Divide, and 12% faster under low water level compared to their Continental Divide counterparts [mean time to emergence from date of lowered water: Pacific slope: constant high water = 20.6 days (n = 136), lowered water = 19.4d (n = 139); Continental Divide: constant high water = 24.0d (n = 149), lowered water = 22.0d (n = 147)].

At the time of water level treatment initiation (tadpole age = 43-45 days), mean developmental stage did not differ between the populations (mean GS = 34 for both populations). Twenty-nine percent of the Pacific slope tadpoles (n = 112) and 24% of the Continental Divide tadpoles (94) had reached early hindlimb development (GS 27-33) at this point, with both of these groups reaching the same mean developmental stage (GS = 31). Seventy-one percent of the Pacific slope tadpoles (268) and 76% of the Continental Divide tadpoles (295) had reached later hindlimb development (GS 34-40), again reaching the same mean developmental stage (GS = 35). Differences in developmental rate between sites, therefore, only became apparent in the faster developers in the time span between later hindlimb development and emergence, and never occurred in the most slowly developing tadpoles (the 24-29% who had not reached GS 34 by the time of water treatment initiation at day 43-45).

Figure 2 depicts the developmental plasticity of the later hindlimb development group in the form of reaction norms. One can explore the reaction norm at the population level by graphically and statistically comparing the mean phenotypes that are produced by different environments (Via 1993). Reaction norms can be compared visually by looking at the slope of the line for each population, or statistically with the water level*site interaction term (Fig. 2; Via et al. 1995). The lack of significance in the water level*site interaction (p = 0.5) signifies that the slopes of the reaction norms, or mean sensitivity to the water level decrease, are not statistically different for the two populations. However, the Pacific slope population shows 134-157% more variation around the mean, indicating that the mean may not be as representative of the individual clutches (Pacific slope: constant high water mean variance (σ^2) = 29.2, lowered water σ^2 = 37.8; Continental Divide: constant high water σ^2 = 21.8, lowered water σ^2 = 24.1).

To explore further the populational differences in phenotypic plasticity, Figure 3 breaks down the data by depicting the reaction norms of the five individual clutches within each site (n = 18-36 for each site-clutch-treatment factorial). The Pacific slope clutches responded in various ways to the sudden decrease in water level. Their mean time to emergence ranged over 5.3 days compared to a 2.6d range by their Continental Divide counterparts, and had larger within-clutch variances (Pacific slope: constant high water variance (σ^2) range = 11.8-54.3, lowered water σ^2 = 11.7-87.1; Continental Divide: constant high water σ^2 = 8.6-34.9, lowered water σ^2 = 16.6-31.4). Because sample sizes were relatively large (n = 18-36 for each water level treatment) and no major outliers were detected, the greater variation and flatter reaction norms can be considered representative of the population, although it is noted that only 5 clutches from the population were studied.

Genetic variation in plasticity within the populations can be compared visually by looking at the slopes of the various lines for each population (Fig. 3; Via et al. 1995) or statistically with the water level*clutch (GxE) interaction term (Newman 1992). A significant water level*clutch interaction ($p = 0.0001$) for the Pacific slope population signifies that the slopes of the reaction norms differ among clutches. This suggests ample genetic variation in developmental plasticity among the various genotypes in the population. Conversely, non-significance of the water level*clutch interaction term ($p = 0.75$) for the Continental Divide population suggests low genetic variation in the reaction norms of the population. Genetic variation in plasticity can be estimated with the variance of the water level*clutch interaction, measured by the Type III MS for the interaction term alone (Newman 1989, Via 1993). The type III MS of water level*clutch is 67.9 for the Pacific slope, but only 6.3 for the Continental Divide.

Discussion

Developmental plasticity and clinal variation

Hyla. pseudopuma individuals natal to two different breeding sites (Pacific slope and Continental

Divide) along an elevational and climatic gradient in Monteverde, Costa Rica, showed differences in tadpole developmental time and plasticity. By exposing individuals from each of the breeding sites to different water level treatments in the lab (control high water and sudden water level decline), we were able to study genetic effects on developmental rate, as well as genetic differentiation between sites. [For the purposes of this discussion, "genetic" refers to both base DNA and epigenetic effects.] Individuals from both the Pacific slope and Continental Divide breeding sites had the ability to respond plastically to lowered water levels. However, individuals natal to the Pacific slope metamorphosed earlier than the Continental Divide under both high and low water environments, suggesting genetic differentiation.

While most clinal studies examine populations that are altitudinally or latitudinally wide-spread (Berven et al. 1979; Berven 1982; Harris 1984; Blanckenhorn & Fairbairn 1995; de Jong & Brakefield 1998 Trussell 2000; Laugen et al. 2003), this study shows populational differences over a distance of 2-3 km and elevation change of 40m. This relatively short distance is significant because of the climatic gradient that exists between the Continental Divide and the Pacific slope sites. The Pacific slope is the alee side of the mountain and is drier than the Continental Divide. The breeding pond on the Pacific slope site was near a noticeable transition moving from the Lower Montane Holdridge Life Zone into the warmer and drier Premontane Holdridge Life Zone, which tends to get less rain and mist and more sun (Holdridge 1982). Because different rain and sun intensities may lead to greater rates of breeding pond desiccation, it makes sense that the Pacific slope population would evolve to develop quicker.

When tadpoles from both sites were exposed to low water levels during later hindlimb development (GS 34-40), they increased their developmental rate and emerged earlier than controls (Fig. 1). Altered developmental rates were not apparent when younger tadpoles (< GS 34) were exposed to low water levels, however, which was probably due to a developmental delay that was made up for once they reached GS 34. These results are in close agreement with previous research which tested the ephemeral pond breeder *Scaphiopus hammondii* (Western spadefoot toad) under similar conditions (Denver 1998). Although these studies focused specifically on the effects of water decline, desiccation of natural breeding ponds usually leads to increased crowding and competition, which can also cause or intensify precocious development (Crump 1989; Denver 1997b; Kikuyama et al. 1993; Hayes & Wu 1995).

Altered amphibian developmental rate under a stressful environment is not considered a simple proximate consequence, but rather an adaptive trait that allows tne organism to deal with risk of desiccation and death (Wilbur and Collins 1973; Wilbur 1987; Newman 1989, 1992; Denver 1997b). Tadpole perception of a changing environment is thought to involve CRH (corticotropin-releasing hormone) neurons, or "neuroendocrine stress centers" (Denver 1995). CRH has been found to play a similar developmental regulatory role across phylogenetic lines, signaling fetal preterm stress and prompting parturition in humans and other mammals (review in Denver 1997b). Because the role of CRH in stress perception and early metamorphosis and parturition appears to be conserved, it has been suggested that CRH is a "phylogenetically-ancient developmental regulator" (Denver 1997b). In tadpoles, activation of this neuroendocrine axis leads to hormonal and genetic cascades that synergize to alter developmental rate (Kikuyama et al. 1993; Denver 1995, 1997a, 1997b; Hayes 1997; Denver et al. 2002; Boorse & Denver 2004).

Knowledge of the neuroendocrine axis and its role in tadpole development allows us to infer that developmental plasticity, as well as normal larval duration, is subject to natural selection. Via et al. (1995) identified two types of genetic influences involved in plasticity: 1) "allelic sensitivity," or the expression of certain alleles in certain environments, and 2) "gene regulation," or regulatory genes that turn other genes on or off in certain environments (which in turn can influence allelic sensitivity). Plasticity for a trait, as well as the various character states (mean phenotypes under each environment), are thought to be determined by the same loci (Via et al. 1995). Both of these ideas have been supported in research on amphibian metamorphosis, as selection on both normal larval duration and developmental plasticity has been suggested to target endocrine-related genes (Denver et al. 2002; Boorse & Denver 2004). During normal metamorphosis, various genes in gene-regulated cascades are turned on to cause tissue-specific apoptosis, proliferation, differentiation and migration, resulting in an orchestrated system of tissue- and time-sensitive metamorphosis (Denver et al. 2002). Under stressful conditions, neuroendocrine activation leads to related hormonal and genetic cascades that result in the synergism of corticosteroid and thyroid hormones (Denver 1995, 1997a, 1997b; Denver et al. 2002; Boorse & Denver 2004). Ultimately, it is the concentrations of and sensitivities to thyroid hormone that determine metamorphic timing and rate (Denver et al. 2002). Natural selection on endocrine-related genes involved in tadpole development could lead to evolutionary changes in developmental rates under different water conditions (character states). In most cases, it is the evolutionary change in character states that most likely leads to evolution of reaction norms - i.e., a change in sensitivity to different environments (Via et al. 1995).

Selection on rates of precocious development appears to act on later (mid-to-late prometamorphic) tadpole life stages. If tadpoles in earlier developmental stages are physiologically constrained from faster development, or undergo different genetic and hormonal cascades that result in delayed development, there would be little or no phenotypic variation within the realm of precocious development for selection to act on. In addition to developmental plasticity, selection for faster overall tadpole development (as seen in the Pacific slope population, Figs. 1-3) also appears to act on later tadpole life stages. If selection for faster overall development under non-stressful environments predominated at earlier developmental stages, we would have expected to se a difference in developmental rate between the two populations before day 43-35 (the start of water level treatments). This did not occur, however, as genetic differentiation in developmental rate between the sites only became apparent between later hindlimb development and emergence.

Differential strength of selection (intensity and duration) operating along an environmental gradient can create a cline (Trussell 2000). In the case of our Pacific slope and Continental Divide populations, the strength of selection on plasticity appears to have differed in the recent or long-ago past. Although there was no apparent difference in the slopes of the population reaction norms between sites, genetic differentiation in plasticity was detected in the form of differences in variance. Compared to the Continental Divide, the Pacific slope clutches had a greater range of time to emergence, 134-157% larger within-clutch variance in time to emergence, and greater genetic variation across reaction norms (Fig. 3). In fact, two of the five Pacific slope clutches showed virtually no plasticity in development under low water conditions.

The greater variation in reaction norms within the Pacific slope population might suggest that developmental plasticity is a less-used life strategy in this population. The more predictable drying of ponds that would come from a drier and warmer environment would likely create strong selection for faster developmental time overall. Faster overall developmental rate, then, may be more strongly selected for and play a more important role. While it is possible that the Pacific slope population from which we sampled has experienced relaxed selection in recent history (the man-made ponds retain water throughout the wet season and the tadpoles do not suffer from water declines), two arguments exist against the hypothesis that relaxed selection is mainly responsible for the variation observed. First, the man-made ponds had been in place for only 5 years (1996-2001), and *H. pseudopuma* require a few years to reach sexual maturity. Second, the individuals breeding in the man-made ponds likely were part of a bigger breeding population using natural ponds in the nearby vicinity (approximate distance between the man-made and other natural ponds: 500-1000m). The nearby ponds had experienced complete desiccation at various times during the 2000-2004 wet seasons. It is additionally possible that constraints are acting on developmental rate in the Pacific slope population, and that the individuals have reached some lower threshold in developmental time.

The consistency in plasticity across the Continental Divide clutches suggests a stronger dependence on developmental plasticity as a life strategy. The low variation in character states - and especially in reaction norms - suggests strong selection pressure in recent history. Strong selection pressure for plasticity would have involved greater survival of individuals that were more sensitive and responsive to pond water changes, which would have resulted in lower genetic variation within the population. Strong selection pressure would also indicate that pond drying is an important factor in this population's survival and fitness. The fact that makes this situation especially noteworthy is that the Continental Divide population exists in a supposedly very wet region of the cloud forest.

If climate change leads to further warming and drying in the Monteverde region, we might expect selection in *H. pseudopuma* for shorter overall developmental time, given freedom from constraints and time for evolution to occur. At the same time, we would expect both the Pacific slope and Continental Divide populations to continue to be developmentally plastic, because plasticity allows individuals to escape a drying pond and survive, as well as extend their larval duration and grow larger and stronger when ponds remain wet (Wilbur & Collins 1973; Berven et al. 1979; Berven 1982; Smith 1987; Wilbur 1987; Semlitsch et al. 1988; Crump 1989; Newman 1989; Newman 1992; Newman 1994). There does not appear any substantial cost of developmental plasticity (cost endured by plastic developers under non-stressful environments) that would override these benefits. The question remains how the future evolution of developmental rates in these populations will unfold.

<u>Consequences of developmental plasticity</u>

To understand how natural selection acts on a plastic trait, it is helpful to know how character states expressed earlier in the life cycle might affect the organism's biology later in life (Via et al. 1995). When *H. pseudopuma* tadpoles are subjected to a stressful environment (water decline and/or crowding) and precocious development ensues, they metamorphose at a smaller size (present study; Crump 1989; Beecher et al. 2005b,c). Small metamorphic size as a result of precocious development has been demonstrated in other amphibian species (Wilbur & Collins 1973; Berven et al. 1979; Berven 1982; Smith 1987; Wilbur 1987; Semlitsch et al. 1988; Newman 1989; Newman 1992; Newman 1994), and has the potential to incur long-term fitness consequences (Smith 1987; Semlitsch et al. 1988; Berven 1990. In the Chorus Frog *Pseudocris triseriata* (sister genus to *Hyla* in the Hylidae family), metamorphic body size was found to positively correlate with body size at 1 and 2 years post-metamorphosis, and negatively correlate with male age at reproduction (Smith 1987). In the salamander *Ambystoma talpoideum,* metamorphic size correlated positively with adult size and negatively with female age at reproduction (Semlitsch et al. 1988). Lastly, in the Wood Frog *Rana sylvatica* size at metamorphosis correlated negatively with age at first reproduction and positively with juvenile survival and adult size, with larger adult males having a greater probability of reproduction and larger females producing more eggs (Berven 1990).

Along with decreased reproductive opportunity and output, small metamorphic body size can also affect the ability of a froglet to survive their first dry season. It has been shown, for example, that froglets need a minimum body length/volume ratio and adequate energy reserves to survive harsh climatic conditions in the dry season (Geise 1987). Given the increased frequency of consecutive dry days in Monteverde (Pounds et al. 1999), especially in the context of high juvenile and adult mortality that probably played a role in the documented frog declines (Pounds & Crump 1994), smaller metamorphic size as a result of precocious development could have played a role in *H. pseudopuma* survivorship.

Plasticity trade-offs involve not only body size, but also immune development and function. In various experiments conducted in natural forest ponds and the laboratory, we found that *H. pseudopuma* tadpoles subjected to water declines were less able to reject foreign tissue during both their tadpole and young froglet life stages, indicating a blunted immune response (Beecher et al. 2005b,c). The fact that the froglets suffered lower immunocompetence one month after being released from the stress indicates that the immune system was negatively affected not only in its function, but also in its development. What makes these findings even more interesting is the fact that when two of our Continental Divide ponds experienced frequent and intense desiccation, all experimental tadpoles maintained in our pond enclosures died from disease (Beecher et al. 2005c).

Conclusion

In this study, we examined the plastic development of *H. pseudopuma* tadpoles natal to two different breeding areas along the climatic gradient in Monteverde, Costa Rica (located on the Pacific slope and Continental Divide). By exposing individuals from both breeding sites to different water level treatments in the lab, we were able to study both environmental and genetic effects on developmental rate, as well as genetic differentiation between sites. At various stages of development, we exposed the tadpoles to either a constant high water level or a sudden decline in water level, simulating what might occur when precipitation is not adequate for maintaining pond water levels. While individuals from both the Pacific slope and Continental Divide responded plastically to lowered water levels, individuals natal to the Pacific slope metamorphosed earlier than the Continental Divide under both high and low water environments, but those from the Continental Divide showed consistent responses to water decline across clutches. These differences suggest genetic differentiation between the Pacific slope and Continental Divide populations.

Although the current relative abundance of *H. pseudopuma* in disturbed areas lowers conservation priority for this species, population numbers in the MCFP remain low. While opportunistic breeding behavior appears to be common in *H. pseudopuma* in fringe or disturbed habitats (Savage 2002), breeding behavior does not appear nearly as opportunistic in the intact forest. We would expect individuals within intact forest to have greater choice of suitable breeding ponds, leading to less selective pressure for opportunistic breeding behavior. We therefore must ask whether the genetic differences that exist between the MCFP Continental Divide population and the more disturbed Pacific slope population might extend beyond developmental rate to breeding behavior and ecology. The impacts of climate change on the future evolution of their developmental rate and plasticity, and the effects of stress and developmental trade-offs on disease susceptibility during these times of amphibian declines, may thus be quite different for *H. pseudopuma* in intact forest populations.

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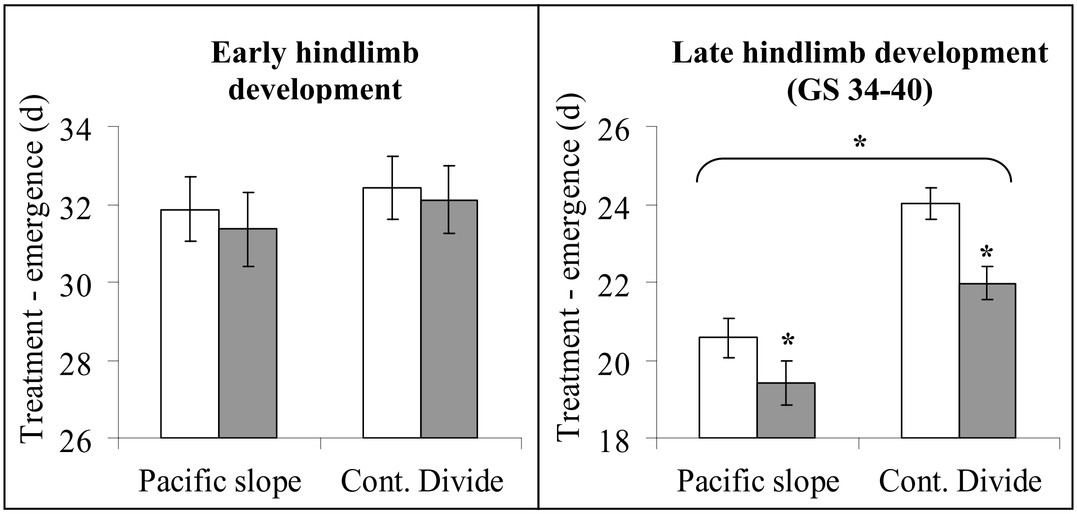

Figure 1. Mean time to emergence of *H. pseudopuma* tadpoles (eggs were collected from two breeding sites along a climatic gradient in Monteverde, Costa Rica, 2002). Tadpoles were exposed to either constant high (8cm, shaded bars) or sudden low water levels (8cm dropped to 2cm at GS 35-36, open bars), with the decline in water level imposed on tadpoles during early (GS 27-33) or late (GS 34-40) hindlimb development. Developmental time was calculated from the initial change in water level until emergence. **Early hindlimb development**: water level treatment, site, and water level*site were all non-significant ($p \geq 0.3$; n = 36-48 for each site-treatment combination). **Late hindlimb development**: water level treatment $p = 0.0001$, site $p = 0.006$, water level*site $p = 0.5$ (n = 136-149 for each site-treatment combination). Asterisk denotes significance at $p \leq 0.05$.

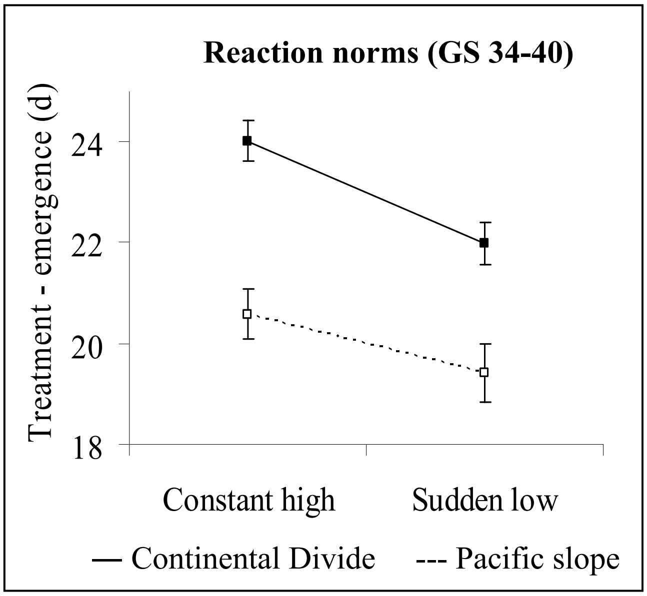

Figure 2. Comparison of *H. pseudopuma* developmental reaction norms by site (research design as in Fig. 1). The lack of significance in the interaction term water level*site (p = 0.5) signifies that the slopes of the reaction norms were not different. However, the Pacific slope population shows more variation around the mean, suggesting that the mean is not necessarily representative of the individual clutches (Fig 4).

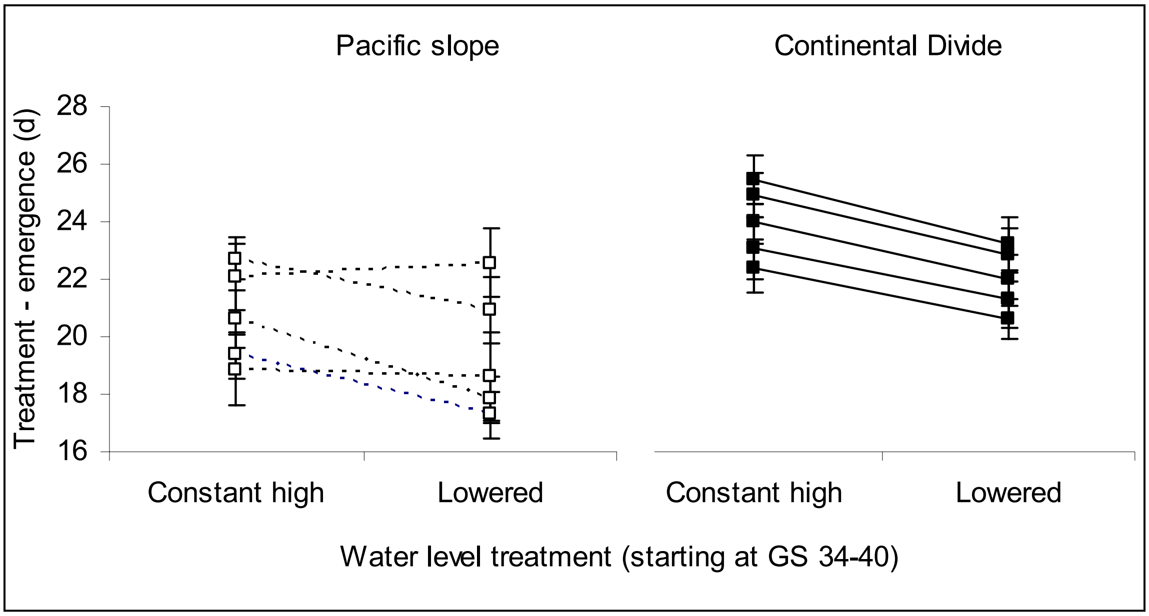

Figure 3. Comparison of *H. pseudopuma* developmental reaction norms for clutches within each site (Monteverde, Costa Rica, 2002). Each site was analyzed separately (research design as in Fig. 1; n = 18-36 for each site-clutch-treatment factorial). The Pacific slope population has a (1) greater range of character states among clutches, (2) larger variance of the mean character states within each clutch, and (3) greater variation in reaction norms among clutches. [**Pacific slope**: water level treatment p = 0.0005, water level*clutch interaction p = 0.0001. **Continental Divide**: water level treatment p = 0.0001, water level*clutch interaction p = 0.75].

Chapter 3. Reduction in water level during tadpole development impairs froglet skin graft rejection.

Introduction

The topic of amphibian declines has been prominent in the scientific literature for the past decade, with a petition especially of late for interdisciplinary research that simultaneously addresses the topics of environmental stress (e.g., climate change), immunosuppression, and disease as potential causes of species declines. The urgent need to understand the role of human-induced environmental stressors on amphibian immunosusceptibility and population declines has been proclaimed by long-term researchers in the field (e.g., Rollins-Smith 2001), a sentiment that has been echoed throughout the amphibian literature. There have been calls for well-planned observational and experimental studies that target interacting environmental factors contributing to amphibian declines (Alford & Richards 1999); interdisciplinary studies that examine the relationship of climate change and disease (Storfer 2003); and studies to help us recognize possible environmental factors and synergisms leading to disease susceptibility (Kiesecker et al. 2004). While it has been hypothesized that environmental stress and immunocompromise may be behind the well-documented increases in pathogenesis and population declines (Kiesecker & Blaustein 1995; Halliday 1998; Carey et al. 1999; Rollins-Smith 2001), much remains to be learned about environmental influences on the immunity of declining species (Gantress et al. 2003).

The purpose of our study was two-fold. One of our goals was to work within the contexts of the amphibian declines in Costa Rica where climate change and disease both appear to be important factors (Pounds et al. 1997, 1999; Lips 1998, 1999; Pounds 2000). In the Monteverde Cloud Forest Preserve (MCFP), Costa Rica, wide-spread species declines have been documented multiple times in the past 20 years, including a crash in 1987-88 that ended in extinction or decline of 40% of the anuran species (Pounds & Crump 1994; Pounds et al. 1997, 1999; Pounds 2000). Climatic changes and increasingly-frequent El Nino years were strongly correlated with these declines, a particularly relevant finding given that montane regions strongly influenced by the tropical Pacific are especially affected by atmospheric and climatic disturbances (Pounds & Crump 1994; Pounds et al. 1997, 1999; Pounds 2000). Amphibian disease has also been a concern, as the prevalence and influence of specific anuran pathogens (e.g., chytrid fungus *Batrachochytrium dendrobatidis*) has increased markedly throughout Central America (Lips

1998, 1999). To simultaneously address the issue of climate change and disease susceptibility, we designed a study that would examine the effects of pond desiccation on frog immunocompetence.

Our second goal was to place our findings within the context of existing knowledge of endocrine and immune responses of tadpoles and froglets. By comparing our results with hormone and immune profiles from other amphibian development studies, we aimed to better understand the mechanisms that underlie immunosusceptiblity and that are possibly intertwined with climate change, disease and species declines. In particular, we took into account published reports of the stress hormone corticosterone (CORT) and thyroid hormone (TH) levels during stressed and unstressed tadpole development (Denver 1998), and the effects of CORT and TH on immune system function and development (Rollins-Smith et al. 1988).

It has been well established that water decline during tadpole development can prompt and increase the production of CORT and TH (Denver 1997a, 1997b; Denver et al. 1998). These hormonal changes have been shown in a number of species to alter development rate (Wilbur 1987; Semlitsch & Wilbur 1988; Crump 1989; Newman 1989, 1992; Laurila 1997; Denver 1997a, 1997b; Denver et al. 1998; Beecher et al. 2005a). It has also been determined that increased levels of CORT and TH during tadpole development can lower lymphocyte numbers and depress skin graft rejection. Both of these events serve as indices of immunosuppression (Rollins-Smith et al. 1988).

In order to address the role of climate-induced stress on amphibian immunity, we studied the developmental and immune effects of water decline on *Hyla. pseudopuma* (Meadow treefrog) tadpoles and froglets. *H. pseudopuma* is a species endemic to montane regions of Panama and Costa Rica (Pounds et al. 1999). It is also one of the frog species that has suffered declines in the MCFP and, like many of the species there, it is has not rebounded (Pounds et al. 1997, 1999; Pounds 2000). It is logical that moisture limitations apparently contributing to these declines might impact amphibian breeding ponds, and that this would most likely manifest in increased frequency and duration of pond water desiccation. In fact, observations during the wet seasons of 2000-2004 included inconsistent precipitation, historic frog breeding ponds that remained dry throughout the breeding season, and unusually rapid and frequent desiccation of rain-filled ponds where *H. pseudopuma* tadpoles were developing.

In the MCFP laboratory, *H. pseudopuma* tadpoles were subjected to one of two manipulations: a constant water level throughout their larval development, or a sudden decline in water level approximately half-way through their larval development. Our methods of water decline were modified from Denver (1998) so that we could best use his neuroendocrine profiles of tadpole development. After the tadpoles metamorphosed, we examined the froglets' ability to reject skin allografts to determine whether development under stressful water conditions had immune consequences. Skin graft rejection was also used in studies that examined TH effects on amphibian immunity, wherein lymphocyte profiles were generated (Rollins-Smith et al. 1988).

Skin allograft rejection is a classical immune model that has been used to study in-vivo foreign tissue destruction for over 60 years (Rollins-Smith 2001; review in Rosenberg & Singer 1992). This type of tissue rejection primarily involves a cellular immune response, and is largely induced by helper and cytotoxic T cells and their related cytokines (reviews in Brown et al. 1975, DuPasquier et al. 1986, Du Pasquier et al. 1989, Edwards & Ruben 1982, and Flajnik et al. 1987; Rosenberg & Singer 1992; Rau et al. 2001; Rollins-Smith 2001). An effective immune response against foreign skin is similar to that against pathogens, making skin graft rejection a proxy for disease resistance. In our experiment, we established two related expectations: 1) If stressful pond water conditions suppress amphibian immune function, tadpoles will be less competent in rejecting their skin grafts. 2) If stressful pond water conditions negatively impact immune development, froglets may also be less competent in rejecting their skin grafts.

Methods

In June 2002, *H. pseudopuma* eggs of one clutch were collected from a natural breeding population on the Pacific slope of Monteverde, Costa Rica (outdoor man-made pond, Golden Toad Laboratory for Conservation), and transferred to the MCFP laboratory. Once the hatchling tadpoles were free-swimming, they were separated into individual plastic 16oz drinking cups to avoid conspecific interactions. The cups were placed in a stratified design interspersing treatments so that we could control for environmental variation caused by cup location. Degrading leaves from the forest floor were ground in a blender with MCFP spring tap water to create a leaf puree, and one-third of a teaspoon of the puree was added to each cup every 3 days. Powder from ground Spirulina vegetarian tablets was added to the cups at the same time to ensure proper nutrition.

The main treatment effect was water level. High water controls experienced an 8 cm water level throughout their entire tadpole phase (water level at 8cm = 255ml volume; 7.5cm diameter at water surface and 5.5cm diameter at bottom), while sudden low water treatments experienced an initial 8 cm water level followed by a sudden decrease to 2 cm (methods modified from Denver 1998). Because developmental stage influences amphibian neuroendocrine and developmental responses to water level changes [tadpoles younger than Gosner Stage 34 (GS34) tend to delay development, while more advanced tadpoles tend to hasten development (Denver 1997a, 1997b & 1998; Beecher et al. 2005a)], water level treatments were initiated only after partial hindlimb development at GS35-36 (NF54-55) (Gosner 1960; Nieuwkoop & Faber 1956). Prior to treatment initiation, therefore, we monitored tadpole development by periodically staging them with a 10x hand lens or stereoscope (Gosner 1960). When all twenty research tadpoles (10 control, 10 low water) reached GS 35-36 (Day 27), treatment was initiated by refilling the water to 2cm for the low water group, and changing the 8cm of water for controls. Once tadpoles were nearing emergence, cups were covered with mesh and checked for emerging individuals daily. The duration from water level treatment initiation to emergence served as our measure of developmental rate. Food was provided for post-metamorphic froglets in the MCFP laboratory by leaving the window open and the light on for approx. 30 minutes every evening, creating a swarm of insects.

Efforts were made to control or account for variables known to influence graft rejection when designing our skin graft experiments. Temperature, age, developmental stage, skin graft size, and genetic differences between recipient and donor have all been shown to influence rate and likelihood of rejection (reviews in Edwards & Ruben 1982 and Flajnik et al. 1987). Temperature was held relatively constant in the laboratory by stratifying cups across treatments; age and developmental stage were recorded and accounted for throughout the experiment, and developmental time was considered in the statistical analyses; skin grafts were cut to a size that was small relative to body size, and froglet weight was considered in the analyses; donor and recipients were all collected from the same clutch, decreasing the influences of genetic variation; and the two sibling skin donors [which had been housed under control conditions] were stratified across treatments to decrease the chances of greater genetic similarities within a particular treatment.

Froglets received skin grafts on Day 70, approx. 3-3.5 weeks following emergence ($\overline{x}$ = 23d post-emergence or 14d post-metamorphosis) using methods modified from Horton (1969) and Du Pasquier & Bernard (1980). After euthanizing the sibling skin donors with tricaine methane sulphonate (MS 222), the dorsal skin was removed and carefully flattened out in amphibian phosphate buffered saline (APBS, insert Carolina Biological Supply cat #) on an upside-down glass petri dish. Using a clear piece of plastic marked with a 1.5mm^2 square as a guide, the donor skin was cut into squares with a fine scalpel under a dissecting scope. Care was taken to make swift clean cuts and avoid pinching the skin with tweezers, preventing damage to the graft that might cause tissue necrosis unrelated to our study.

Prior to implanting the skin grafts, we recorded froglet wet weight (mg) and anesthetized the recipients by immersion in MS-222. Using fine surgical scissors under a dissecting scope, a slit was made in the recipient froglet's dorsal skin, and the donor skin was placed at the opening with the edges slipped underneath (Du Pasquier & Bernard 1980). This technique ensured that injury to the recipient would be minimized, and that skin would cover the entire dorsal area at all times. Once the skin graft was in place, an antibiotic solution (mercurochrome) was applied externally to the graft region to help prevent infection, the bottom of the recipient's plate was re-moistened, and the recipient placed in a 20C degree refrigerator for 30 minutes to allow graft adhesion while the froglet remained anesthetized. After removal from the refrigerator, most froglets were active within 20 minutes. Three days following implantation, the recipient skin was trimmed under anesthesia to expose the entire area of donor skin, and the antibiotic solution was again externally applied to the graft region to help prevent infection.

Grafts were followed for signs of rejection from Day 8 to Day 28 post-graft implantation. Every other day, froglets were examined under a dissecting scope to note changes in tissue appearance. Percent of graft area with complete loss of pigmentation was used as criteria for rejection, and the proportion of the graft rejected or healed was recorded over time. The froglets were maintained in the laboratory until their graft sites completely healed. All healthy froglets were released back to their natal breeding site in accordance with the MCFP permit.

Statistical Analysis

Graft rejection over time was analyzed using a repeated-measures ANOVA ($\alpha = 0.05$). Each individual was a subject, and the within-subject repeated variable was observation, each of which represented a span of 2 days. The between-subject analysis determined if a difference existed in percent graft rejection between water level treatments (control high vs. sudden low). The independent variables in the model were water level treatment and observation, and time to emergence and weight at grafting were explored as covariates. Froglet weight and emergence date were also analyzed as dependent variables in two-tailed GLM univariate ANOVAs, with water level treatment as the independent variable (type III F test, $\alpha = 0.05$) (SAS 2005).

In the repeated measures analysis, as our dependent variable "percent rejection" was a proportion with a large skew to one or both tails, it violated assumptions of normality and was not amenable to transformation. Repeated measures therefore were run with the process PROC GENMOD, which allows the user to choose an alternative response probability distribution for non-normal data (Orelien 2001; Pedan 2001; Moser 2004; Simpson et al. 2004; Johnston & Stokes 1997; GENMOD procedure, SAS 2005). We tested models using various response probability distributions and error covariance structures (working correlation matrix used to model the correlation structure between observations within each subject) for the best fit. Any models that did not have a deviance score (value/df) close to 1 in The Criteria For Assessing Goodness of Fit output were considered inadequate, and the model with the deviance score closet to 1 was considered to have the best fit (Pedan 2001; Simpson et al. 2004). The final GENMOD model used a compound symmetry (cs) working correlation matrix with a gamma distribution [default non-linear link function: power (-1)]. A gamma distribution with an inverse power function best describes continuous response data that has one tall peak at or close to zero, rapidly declining thereafter with a long and fat tail (Simpson et al. 2004; Clark et al. 2005). Such a distribution was often seen in our data, with the exception of a second peak at 100% rejection for some observations (Fig. 2). To be able to use the gamma distribution, all rejection data points of 0% had to be changed to 1%. For greater in-depth discussion on GENMOD model building, refer to Beecher et al. (2005c).

We also analyzed the raw data with the more conventional MIXED repeated measures procedure (SAS 2005). Like GENMOD, MIXED can handle missing observations by employing a likelihood-based estimation method (in this case restricted maximum likelihood or REML), and allows the user to choose an appropriate within-subject covariance matrix (Wolfinger & Chang 1995; Hamer & Simpson 2000; Moser 2004; MIXED procedure, SAS 2005). In testing various model distributions, the model with the largest negative Akaike's Information Criterion (AIC) value in the Model Fitting Information output was considered to provide the best fit (Wolfinger & Chang 1995; Wolfinger 1996). The final MIXED model employed a variance components (VC) correlation matrix. We tested the main effects with a type III F test. Because both PROC GENMOD and PROC MIXED gave similar results, we report the p values from PROC MIXED because it is a more widely-used statistical measure, and to coincide with the statistical reporting in Beecher et al. (2005c). [GENMOD results available from the first author].

Results

Tadpoles exposed to a sudden water decline took significantly longer as froglets to reject their skin grafts (n = 191: *water level treatment* p = 0.0241; *observation* p = 0.0001. Fig. 1). A difference in mean percent rejection rate between water level treatments was first observed 20 days following graft surgery, and was maintained through the end of our experiment (d 28). At Day 20, the mean percent rejection for controls jumped 25% (from 18% to 43%), but not until Day 28 did the mean percent rejection for sudden low water treatments jump a corresponding amount (33%, from 21% to 54%). The general patterns of rejection were similar, however, as can be seen in the almost parallel slopes and line trajectories. This similar yet delayed pattern suggests that the sudden low water treatment individuals suffered a lag time in immune response.

Figure 2 shows the distributions of individual froglet responses for each treatment over time. By Day 20 and through Day 26, the constant high water group took on a bimodal distribution with frequency peaks at 0% and 100% rejection, and with a smaller number of individuals showing intermediate rejection levels. For the sudden low water treatment, very few individuals exceeded 0% rejection until Day 24, with a skew toward 0% rejection lasting through Day 26. By the end of the experiment (Day 28), the control group showed a skew toward 100% rejection, while the sudden low water treatment group still displayed a bimodal distribution. At the time of the first major jump in mean percent rejection for each treatment (control: Days 18-20, low water: d 26-28), the distributions of individual responses underlying those means were similar between trratments (Fig. 2).

Tadpoles exposed to a drop in water level also showed a trend toward quicker development (from the time water treatments began until emergence), although this difference was not significant (constant high = 21.4 days (n = 10), sudden low = 20.4 days (n = 8); p = 0.2024. Fig. 3). As an explanatory variable in the graft rejection repeated measures analysis, time to emergence was not significant by itself, but the interaction of water treatment*time to emergence was significant (p = 0.0001) (Fig. 4). Froglet weight at the time of grafting also differed between water level treatments, with sudden low treatment individuals averaging 15% lower body weight compared to controls (mean weight: constant high = 312mg (n = 10), sudden low = 267 (n = 8); p = 0.0154. Fig. 3). In the graft rejection repeated measures analysis, froglet weight was not a significant explanatory variable by itself, but the interaction of froglet weight*water level was significant (p = 0.0001) (Fig. 4).

To elucidate the impact of froglet weight on graft rejection, we deleted the extremes from the data set (2 low water individuals <250mg and 3 high water individuals >325mg), and ran analyses on the remaining individuals where the two groups overlapped in weight [constant high $\bar{x}$ = 289mg, range = 268-310 (n = 7), sudden low $\bar{x}$ = 279, range = 264-300 (n = 6)]. Using this new data subset, weight was no longer significant as a dependent variable in the univariate anova (n = 13: p = 0.25), and neither weight nor its interaction with water level provided significant covariates in the rejection repeated measures analyses ($p \geq 0.2$). Nonetheless, compared to controls, low water treatments still lagged in their graft rejection (n = 143: p = 0.0084).

Discussion

Subjecting tadpoles to sudden water decline compromised their ability to reject foreign tissue as young froglets. The most apparent difference between control and sudden low water treatment groups was the timing of graft rejection, with the first major jump in mean percent rejection occurring over a week later for the low water treatment. As no froglets actually accepted their skin grafts (which was confirmed within one week after the last recorded observation), this was not a case of active tolerance (Flajnik et al. 1987), but rather immunosuppression. Because the onset of rejection lagged for the low water level treatment while other general patterns of rejection did not differ (seen in the almost parallel slopes and line trajectories, Fig. 1), it appears that the immune responses once fully underway were basically comparable, but that the initial stages of rejection were compromised.

Individuals exposed to a drop in water level developed more quickly and weighed less at the time of grafting. Faster development time was apparent as a trend, with lack of significance most likely due to small sample size; a similar and larger laboratory study conducted in 2001 showed significance for comparable developmental patterns (Beecher et al. 2005a). When tadpoles increase their developmental rate, they are oftentimes smaller at metamorphosis. This consequence of precocious metamorphosis has been noted in various species (Wilbur & Collins

1973; Berven et al. 1979; Berven 1982; Smith 1987; Wilbur 1987; Semlitsch et al. 1988; Crump 1989; Newman 1989, 1992, 1994), and has the potential to incur long-term fitness consequences (Smith 1987; Semlitsch et al. 1988; Berven 1990; review in Beecher et al. 2005a). Even given these differences, time to emergence and froglet weight alone did not explain the decreased immune response to foreign tissue.

We considered the possibility that we were seeing differences in rejection rate because some froglets had extra time to develop their immune systems. Although we had held constant the number of days from egg laying until froglet grafting (all eggs came from one clutch, and all grafts were performed the same day), this method did not allow us to control for the number of days from metamorphic climax to grafting. Because developmental time differed among individuals, those individuals that metamorphosed quicker would have had additional time to develop their adult-type immune systems. This concern was alleviated by the fact that the low water group had slightly more time on average to recover immunologically, and yet they rejected their grafts slower.

We also considered the possibility that we were seeing differences in rejection rate due to skin grafts composing different percentages of the froglets' bodies, a pertinent concern given that the low water froglets were smaller. To address this issue, we ran a graft rejection analysis on a subset of the data, including only those individuals that overlapped at intermediate weights. Although weight did not differ between treatment groups in this data subset, the low water treatment individuals still lagged in their graft rejection. Lower body weight therefore cannot fully explain the decreased ability of individuals exposed to sudden low water levels to reject their skin grafts.

The interactions of time to emergence-by-water level, and froglet weight-by-water level, with regards to graft rejection were produced by two main events. In the constant high water treatment, the three largest individuals (two of whom took relatively long times to emerge) rejected their grafts quickly (Fig. 4; data is shown for Day 24 post-grafting). The influence of these data points created a weak correlation between rejection and development time, and a stronger correlation between rejection and weight. In the sudden low water treatment, however, many individuals showed little or no rejection until Day 28 across development times and weights. While the relatively quick graft rejection by one low water treatment individual influenced the trendlines in Fig. 4, if we exclude that individual no patterns exist. For the data subset where we excluded the smallest and largest froglets, the correlations and interactions disappear, illustrating again that neither time to emergence nor froglet weight can fully explain differences in rejection rate (Fig. 4).

To elucidate what most likely occurred in our tadpoles and froglets, we can make use of valuable endocrine and immune profiles from other amphibian development studies. Profiles of CORT and TH levels during tadpole development, as well as thymocyte numbers during tadpole and froglet development, are summarized in Fig. 5. Specifically, tadpoles were exposed to a gradual water decline (Fig. 5a) or to exogenous TH (Fig. 5b) during development, both of which caused earlier metamorphosis and smaller body size (Rollins-Smith et al. 1988; Denver 1998). Although species, treatments and time frames differed among our studies, making it unrealistic to extrapolate exact timing and magnitude of responses, it is reasonable to infer similar hormonal and immune trajectories.

Based on previously published data, the age and developmental stage at which we first subjected *H. pseudopuma* tadpoles to sudden water decline most likely occurred when CORT and TH levels were relatively low (front of gray-shaded area in Fig. 5a; Jolivet-Jaudet & Leloup Hatey 1984; Denver 1998; Glennemeier & Denver 2002a). Although amphibian species have been shown to vary in their exact patterns of corticosteroid plasma levels during development, it appears that relatively low levels during most of prometamorphosis (GS35-40) are characteristic across species (review in Jolivet-Jaudet & Leloup Hatey 1984; Glennemeier & Denver 2002a). Our initial water decline also most likely occurred when thymocyte and splenocyte numbers were climbing to reach full expression (front of gray-shaded area in Fig. 5b; Flajnik et al. 1987; Rollins-Smith et al. 1988). Under normal conditions the thymus reaches its peak maturity during late prometamorphosis (GS40-41), before the time when CORT and TH levels rise together (mid-climax, GS43-44) (reviewed in Du Pasquier et al. 1986, 1989).

The response of *H. pseudopuma* tadpoles to water decline most likely paralleled that of the temporary pond breeder *S. hammondii*, with sharp rises in whole-body CORT and TH (Fig. 5a; Denver 1998). Timing and magnitude of these hormonal surges under stress are earlier and greater than under control conditions. This finding is underlined by the unstressed *R. pipiens* CORT profile in Fig. 5a, which corresponds with the unstressed *S. hammondii* profile but extends to later developmental stages (Glennemeier & Denver 2002b). Other stressors related to water decline, such as chronic crowding and food limitation, have also been shown to very rapidly increase corticosteroid levels in tadpoles (Hayes 1997 - Steroids as...; Glennemeier & Denver 2002b; review in Belden & Kiesecker 2005). These sharp CORT and TH hormonal surges are known to be part of a neuroendocrine response to stress in prometamorphic tadpoles in which the hormones synergize to prompt early metamorphosis (Denver 1995, 1997a, 1997b; Denver et al. 2002; Boorse & Denver 2004). This stress response is even stronger when water declines are sudden and severe, with whole-body CORT and TH increasing more markedly. Although not measured in the present study, the thyroid hormone thyroxine (T4) in particular can rise as much as 37-fold (Denver 1998).

With respect to pond water desiccation, the research connecting water stress to CORT and TH levels provides striking relevance to findings connecting CORT and TH to immunity. Figure 5b shows a study in which tadpoles were exposed to a low-dose regimen of T4 that was seemingly within biological limits (Rollins-Smith et al. 1988), and possibly characteristic of T4 levels during pond water desiccation. In response to T4 exposure, *X. laevis* tadpoles experienced a dampening of lymphocyte proliferation, reaching only 60% normal thymocyte and 35% normal splenocyte numbers at their peak (Rollins-Smith et al. 1988). While the subsequent loss of lymphocytes at metamorphic climax is normal and necessary to reorganize the immune system and prevent autoimmune problems (Rollins-Smith et al. 1992, 1997), tadpoles exposed to T4 went on to destroy a dramatically greater percentage of lymphocytes at metamorphosis, losing 90% of their thymocytes (vs.a normal 38%) and 79% of their splenocytes (vs. 43%) (Rollins-Smith et al. 1988).

The fate of lymphocytic re-expansion after metamorphosis, during the time when our froglets were rejecting their grafts (gray-shaded area in Fig. 5b), is unclear. Although the study shown in Fig. 5b documented a return to normal lymphocyte number by one month post-metamorphosis, these data were offset by a second experiment that showed a continued dampened splenocyte number 5 months post-metamorphosis suggesting longer-term immune consequences (Rollins-Smith et al. 1988). It seems unlikely that precociously developing individuals (as in our sudden low water treatment) could rebound to normal lymphocyte numbers within only one month, especially as it takes 4-8 months under normal circumstances for froglets to reach immune maturity (review in Rollins-Smith 1998).

While TH may indirectly affect lymphocyte function and abundance, it appears that it is CORT that can directly lower lymphocyte numbers (Rollins-Smith & Blair 1993; Barker et al. 1997;

Rollins-Smith et al. 1997; review in Rollins-Smith 1998). CORT is the most prevelant corticosteroid found in frog plasma (review in Krain & Denver 2004), and at biologically-relevant concentrations in-vitro and in-vivo it has been shown to inhibit proliferation and increase apoptosis of splenocytes and thymocytes (Rollins-Smith & Blair 1993; Barker et al. 1997). During times of high unbound plasma levels (as seen during metamorphosis), CORT can induce T cell apoptosis directly, as well as indirectly via promoting cell death pathways (review in McEwen et al. 1997; Rollins-Smith et al. 1997). It appears, therefore, that a significantly greater rise in CORT during water stress can directly lead to a less-developed lymphocyte population during tadpole development and following metamorphosis.

Beyond reducing lymphocyte number, glucocorticoids during chronic stress can influence the immune system in various ways. Because immature T cells in particular are highly susceptible to glucocorticoid-induced apoptosis, raised CORT levels tend to influence T cell selection by decreasing the number of T-Helper 1 cells (Th1, cell-mediated immunity) in favor of T-Helper 2 cells (Th2, antibody-mediated immunity) (review in McEwen et al. 1997). Glucocorticoids also have been shown to alter immune cell trafficking and cytokine production; dampen T cell differentiation, MHC expression, proinflammatory gene expression, and leukocyte infiltration into sites of inflammation; and interfere with antigen presentation (Chrousos 1998; reviews in McEwen et al. 1997, Petrovsky 2001 and Wadhwa et al. 2001; Marques-Deak et al. 2005). In general, therefore, glucocorticoids are thought to inhibit the cellular immune response.

Because most prior studies on CORT have not extend beyond the period of metamorphosis (e.g., Fig. 5a), it is unclear what the CORT levels and corticosteroid-induced immune effects were probably like at the time of grafting our froglets 3-3.5 weeks post-metamorphosis. It is reasonable to reflect that immune effects such as decreased lymphocyte number, altered Th1/Th2 ratios, and dampened T cell differentiation and MHC expression might carry over at least for the first part of a froglet's life. Lingering stress effects on the hypothalamic-pituitary-interrenal (HPI) axis and related neuroendocrine-immune interactions are also possible, as suggested in studies where prenatally-stressed rats and pigs showed lower in-vitro lymphocyte proliferation 1-2 months after birth (reviews in Kay et al. 1998 and Tuchscherer et al. 2002). Because we are interested in water stress-induced immunosuppression in tadpoles as well as froglets, and have documented depressed skin graft rejection during both life stages (Beecher et al. 2005b), it is helpful to examine both direct and resultant effects of CORT on foreign tissue rejection.

Skin allograft rejection is largely induced by thymus-derived T cells, requiring both helper (Th) and cytotoxic (Tc) cells (reviews in Brown et al. 1975, DuPasquier et al. 1986, Du Pasquier et al. 1989, Edwards & Ruben 1982, and Flajnik et al. 1987; Rosenberg & Singer 1992; Rau et al. 2001; Rollins-Smith 2001). For graft rejection to initiate, antigen-presenting dendritic (Langerhans) cells of the grafted donor skin must emigrate into the surrounding skin and lymph system, activating the recipient's Th cells that recognize foreign histocompatibility antigens (Rosenberg & Singer 1992). Following this activation, the resulting cellular responses involve both non-specific and antigen-specific reactions. Destruction of the graft epidermis, oftentimes the first sign of rejection, is a process of non-specific inflammation caused by inflammatory cells and cytokines (produced by inflammatory and T cells). To initiate dermal destruction, the activated Th cells secrete lymphokines that in-turn activate lymphokine-dependent Tc cells, leading to Tc cell proliferation and cytotoxicity (Rosenberg & Singer 1992). Tc cells can then respond in a non-specific as well as antigen-specific manner (Rosenberg & Singer 1992; Rau et al. 2001), and serve as the main effector cells (immune cells responsible for cell-mediated cytotoxicity) mediating graft rejection (Rosenberg & Singer 1992). Once the immune response is well underway, Tc cells do not appear as essential, however, suggesting the role of additional immune effector cells at this point (Rau et al. 2001).

In various studies, corticosteroids have been shown to interfere with each of the above mechanisms. In addition to decreasing T cell numbers, raised corticosteroid levels can depress the activity of antigen-presenting cells and Th cell activation (Highet & Ruben 1987). They can also dampen inflammation by down-regulating MHC class II expression on macrophages (required for antigen presentation to T cells) (Celada et al. 1993), and curbing immigration of macrophages, eosinophils and neutrophils into areas of inflammation (review in McEwen et al. 1997). Finally, raised corticosteroids levels can cause deficient Th lymphokine (IL-2) production and Tc lymphokine receptor expression (Highet & Ruben 1987), thereby down-regulating Tc cell proliferation, differentiation and cytotoxicity. In fact, depressing Tc cell populations experimentally has been shown to result in a lag in rejection rate (Rau et al. 2001), which was the main consequence of water decline in our study.

The lag in graft rejection seen in our water-stressed individuals could have also been due to disparities in the timing of froglet immune maturation. It has been suggested that precocious developers may suffer developmental asynchrony between froglet lymphocyte re-expansion (which is stage-dependent), and optimal MHC class I antigen expression (which is age-dependent) (Rollins-Smith et al. 1988). In effect, individuals that metamorphose earlier would begin their second lymphocyte proliferation at an earlier age, perhaps before MHC class I expression is fully upregulated (Rollins-Smith et al. 1988). As lymphocytes are selected and learn to recognize self by coming into contact with MHC-expressing thymic cells (Flajnik et al. 1985), MHC class I expression that is not yet fully upregulated at the time of froglet lymphocyte re-expansion could temporarily create a more larval-like T cell population (Rollins-Smith & Cohen 1996; Rollins-Smith et al. 1988). Because rejection rate partially depends on disparate MHC histocompatibility between donor and recipient (DiMarzo & Cohen 1982 - "an in vivo study..."), a larval-like T cell population would cause a froglet to be less competent in rejecting foreign tissue.

Conclusion

The impacts of developmental stress on froglet immunity are relevant to the diseases and declines occurring in frog populations in Monteverde, as well as globally. In our study, stressful effects of water decline during tadpole development, which in certain areas seems to be occurring as a result of climate change (Pounds & Crump 1994; Stewart 1995; Pounds et al. 1997, 1999; Kiesecker et al. 2001; Pounds 2001; Burrowes et al. 2004), depressed the ability of young froglets to reject skin allografts. Because skin graft rejection primarily involves cellular immunity, this experimental measure can serve as a proxy for mounting a response against certain pathogens.

The fact that stress-induced rises in CORT appear intimately tied to depressed cellular immunity can be applied to situations of disease in the wild. For example, it is known that glucocorticoids tend to down-regulate particular cytokines (IL-2 and IFN-γ) involved in cell-mediated immune responses against parasites and intracellular bacteria (review in McEwen et al. 1997). Because cellular immunity provides the primary defense against pathogens, a diminished response may make individuals initially more susceptibile to infection (Chrousos 1998; review in Wadhwa et al. 2001). In this manner, there may indeed by an immunosuppressant link among climate change, disease and frog declines.

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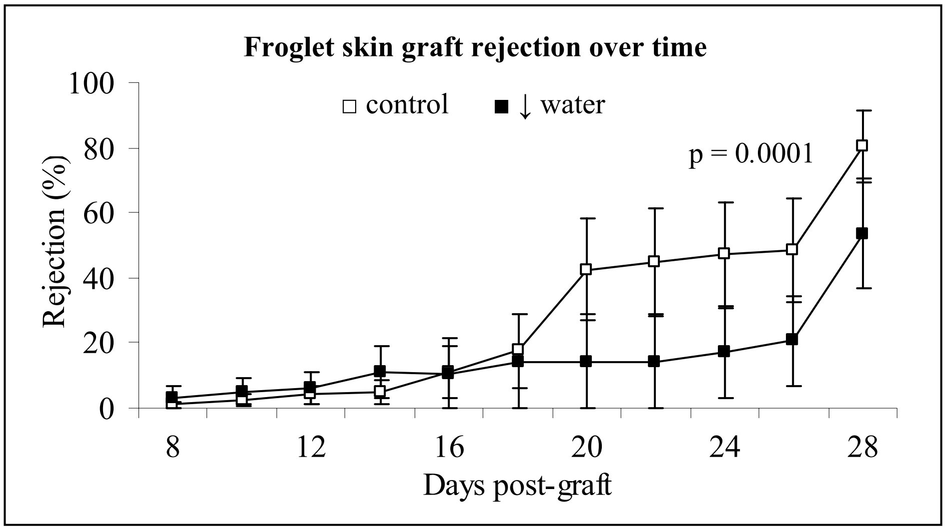

Figure 1. Mean percent graft rejection (skin pigmentation loss) over time (*H. pseudopuma*, Monteverde, Costa Rica, 2002). Tadpoles were exposed to constant high (8cm) or sudden low water levels (8cm dropped to 2cm at GS 35-36). Froglets were grafted 2.5-3 wks post-metamorphosis and percent rejection recorded every 2 days. Number of froglets = 18 (constant high: 10, sudden low: 8); total number of repeated measures observations: n = 191 (constant high: 110, sudden low: 81).

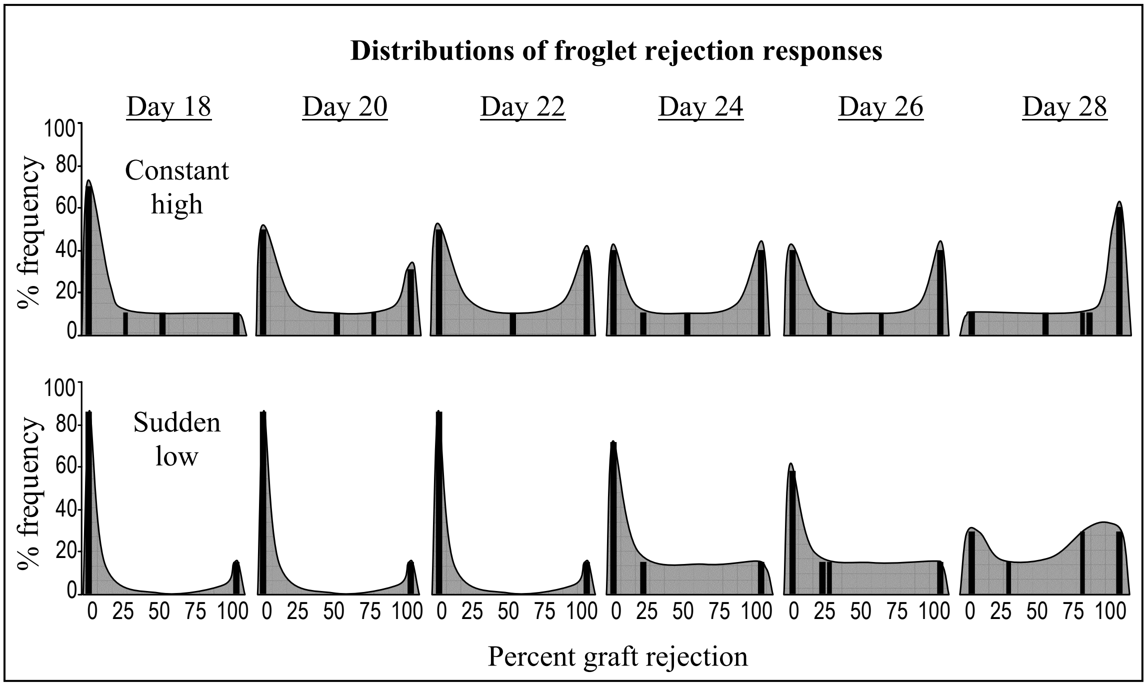

Figure 2. Distributions of percent rejection among individual froglets for each water treatment over time (constant high: 8cm, sudden low: 8cm dropped to 2cm at GS 35-36; *H. pseudopuma*, Monteverde, Costa Rica, 2002). Number of froglets/treatment/day: constant high n = 10, sudden low n = 7 (1 low-water individual died on d16).

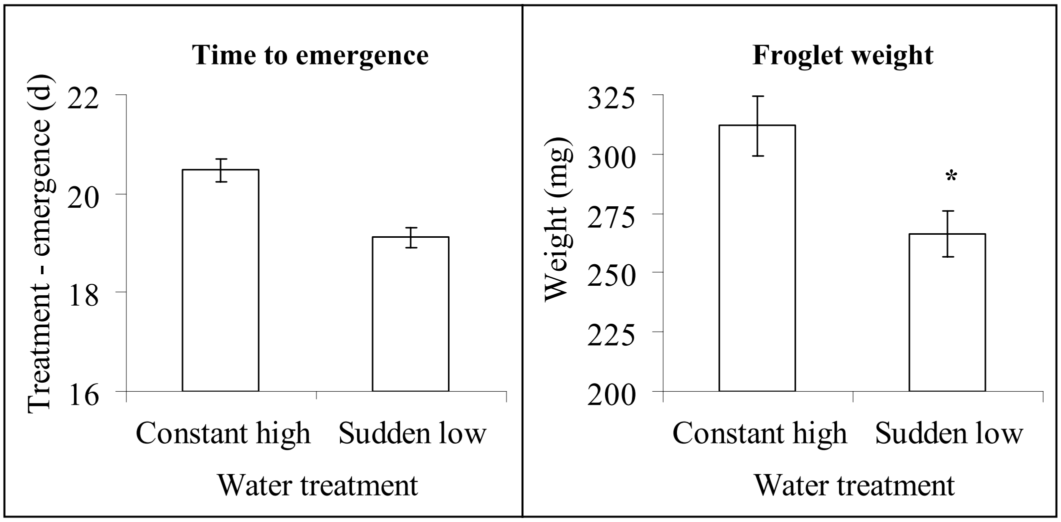

Figure 3. Mean time to emergence and mean froglet weight (*H. pseudopuma*, Monteverde, Costa Rica). Tadpoles were exposed to constant high (8cm) or sudden low water levels (8cm dropped to 2cm at GS 35-36). Asterisk denotes significance at $p \leq 0.05$. Developmental time was calculated from the initial change in water level until emergence, and froglet weight was recorded at the time of graft surgery 2 weeks post-metamorphosis (constant high n =10, sudden low n= 8).

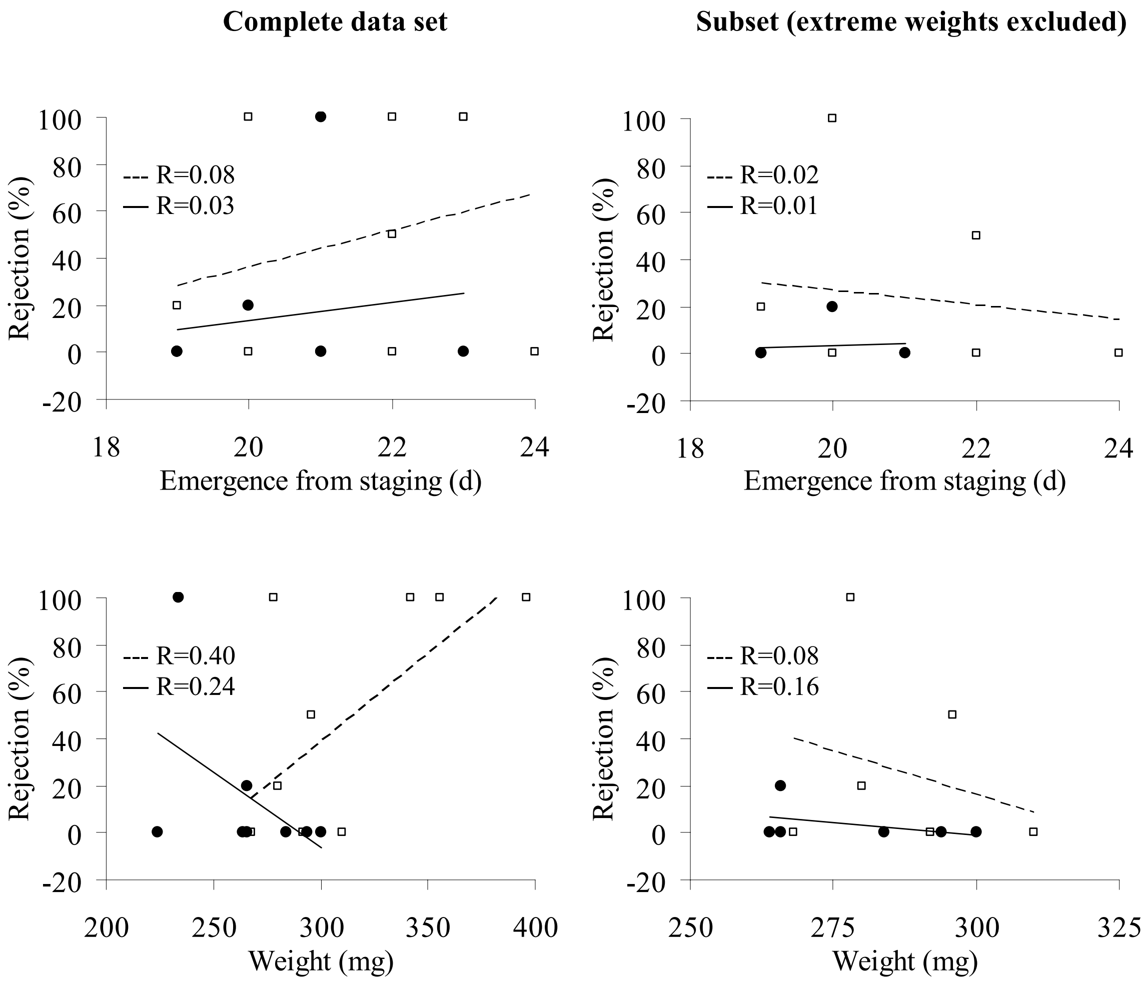

Figure 4. Interactions of froglet weight-by-water level, and time to emergence-by-water level, in regards to percent graft rejection. Time to emergence was calculated from the initial change in water level until emergence, and froglet weight was recorded at the time of graft surgery 3-3.5 weeks post-emergence. Data is shown for Day 24 post-grafting [constant high n = 10, sudden low n = 7 (1 low-water individual had died on d16)]. The data set subset excluded the two smallest low water individuals (<250mg) and the three largest high water individuals (>325mg) (constant high n = 7, sudden low n = 6; some data points represent more than one individual). Square symbols represent individual data points, and lines represent linear regression trendlines. □ --- constant high: 8cm; ■ —sudden low: 8cm dropped to 2cm at GS 35-36.

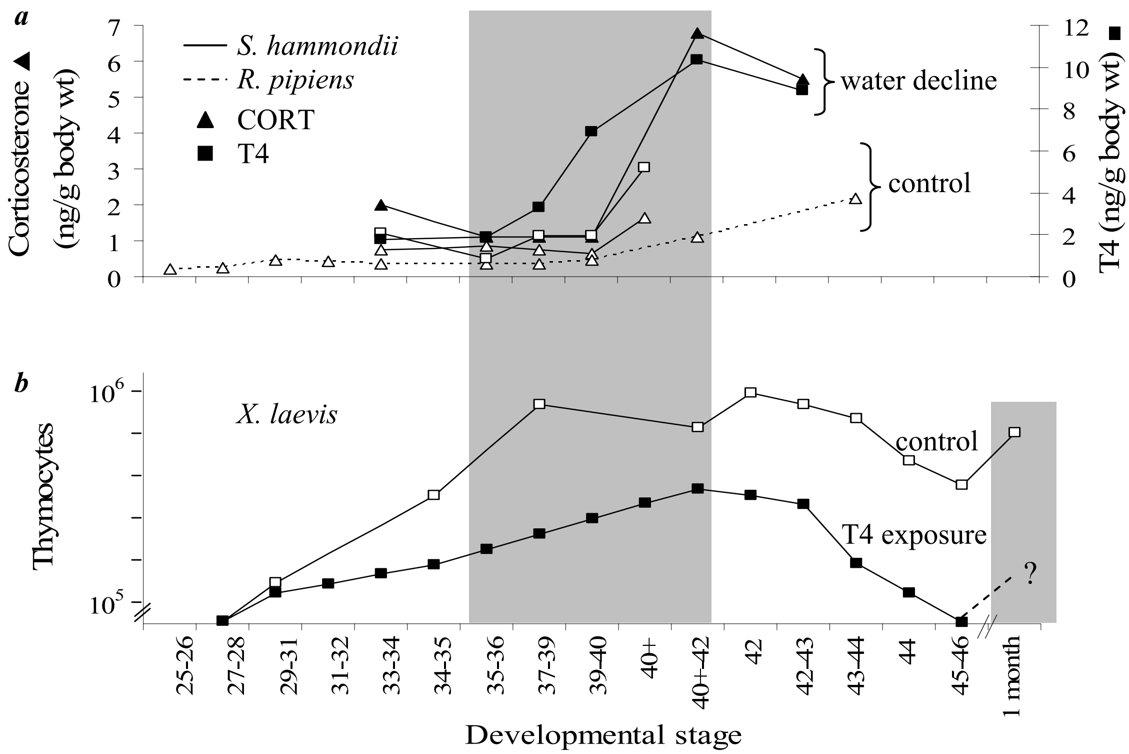

Figure 5. Neuroendocrine and immune profiles in anurans. The large gray area represents the time period of water decline in the present study, and the small gray area represents the time period of froglet graft rejection. **(a)** Whole-body CORT and T4 levels during unstressed and stressed tadpole development [modified from Denver (1998) and Glennemeier & Denver (2002a)]. *S. hammondii* tadpoles were exposed to a gradual water decline starting at GS32/NF52 (Denver 1998). **(b)** Tadpole and froglet whole-organ thymocyte numbers during and after normal and T4-induced development [modified from Rollins-Smith et al. (1988)]. Thyroidectomized *X. laevis* tadpoles were exposed to a low-dose regime of T4 starting at GS27/NF49; similar differences were seen in splenocytes.

Chapter 4. Tadpole and froglet graft rejection during and after exposure to pond water regimes in the field.

Introduction

According to The World Conservation Union Global Amphibian Assessment, more than 2400 amphibian species world-wide (43%) are thought to be experiencing population declines (Stuart et al. 2004). Over 400 of these species are considered rapidly declining, although almost half of them exist in areas with no obvious environmental degradation. Of these “enigmatic” declines, climate change and disease are the most commonly cited causal factors in the research literature (Stuart et al. 2004). While these two events are oftentimes documented separately, studies have begun to surface connecting climate change with disease as contributing to the frog declines.

One phenomenon that has been extensively investigated in relation to both climate change and frog declines is the El Niño - Southern Oscillation effect (ENSO) (review in Carey & Alexander 2003). The Southern Oscillation is a reversal in surface air pressure across the tropical Pacific Ocean, which results in changes in sea surface temperatures (Trenberth & Hoar 1996). It is in the equatorial Pacific where changes in the Southern Oscillation are most strongly linked to El Niño, or anomalous oceanic warming events (Trenberth & Hoar 1996). In 1976 much of the eastern tropical Pacific Ocean experienced a warming of sea surface temperatures (the “1976 Pacific climate shift”), and since then ENSO events have increased in frequency, duration and intensity (Guilderson & Schrag 1998).

Warmer sea surface temperatures during El Niño years in the equatorial Pacific have been shown to affect climatic patterns over land, and in the process impact frog populations (Pounds & Crump 1994; Pounds et al. 1999; Pounds 2001; review in Carey & Alexander 2003). Montane regions that are strongly influenced by the tropical Pacific are thought to be especially influenced by these atmospheric and oceanic disturbances (Pounds & Crump 1994; Pounds 2001). In Monteverde, Costa Rica, the El Niño effect has been shown to cause declines in dry season precipitation (mostly in the form of reduced mist), and at times alter wet season rainfall by influencing northeasterly trade winds (Pounds & Crump 1994; Pounds et al. 1999; Pounds 2001). At this same site an association was found between the frequency of dry-season mist and anuran abundances over time, documenting a strong correlation between climatic warming and drying and anuran declines (Pounds et al. 1997, 1999). Although no diseases were documented in conjunction with these declines, serious amphibian diseases and declines were seen in other parts of Central America (Lips 1998, 1999).

Recently, ENSO effects have also been discovered to influence frogs in the Oregon Cascade Mountains (Kiesecker et al. 2001). In the Pacific Northwest, ENSO cycles correlate with precipitation patterns (review in Kiesecker et al. 2001), and in various years reduced winter precipitation resulted in shallower montane breeding ponds (Kiesecker et al. 2001). The shallowness of the pond waters allowed greater UV-B penetration into *Bufo boreas* frog embryos, causing higher fungal-related mortalities (Kiesecker & Blaustein 1995; Kiesecker et al. 2001). This finding changed the focus of UV-B influences on frog declines from simply stratospheric ozone depletion to broader climatic changes. Elevated sea-surfaces temperatures in the tropical Pacific, a causation factor discussed with regard to the Monteverde frog declines (Pounds et al. 1997, 1999), was thus not only linked to declines in other montane regions, but also to disease outbreaks.

To understand how climate changes may impact breeding pond conditions, we can look at how climate and weather patterns affect soil hydrology and water retention patterns. Climate and weather patterns can influence pond breeding waters both during and outside of the wet season, a factor noted in both the Monteverde and Oregon Cascade Mountain frog declines (Pounds & Crump 1994; Kiesecker et al. 2001). During the dry season, mist deposition, temperature and wind patterns impact soil evaporation (Pounds & Crump 1994). During the wet season, rainfall fills and maintains ponds and recharges ground water. During dry years the depth of the water table appears to be especially important for temporary ponds, as it can provide water seepages into the soil (Pounds & Crump 1994). A simulation study of climate change effects on wetland hydrology has predicted that higher temperatures and periods of less precipitation concurrent with climate change patterns will cause higher evaporation rates and lower soil moisture levels (Osterkamp et al. 2001). It is logical that such climatic changes might similarly impact anuran breeding ponds, increasing the extent and frequency of pond water variation, duration of low pond water levels, and dangerous periods of pond desiccation. Our questions center on whether such changes in breeding ponds might impact amphibian immunity and make tadpoles and froglets more susceptible to disease.

Therefore, within the greater context of climate change, disease and frog declines, we studied the impact of pond water variation and depth on tadpole and froglet immune development and function. Specifically, we studied the effects of water depth and variation by exposing tadpoles to four different pond water regimes in the field (steady high, steady low, low variation, high variation). To determine whether amphibians developing under the various water regimes experienced immune consequences, we examined the abilities of tadpole and froglet to reject skin allografts.

Skin graft rejection is a classical immune model that has been used over 60 years for studying in-vivo foreign tissue destruction (Rollins-Smith 2001; review in Rosenberg & Singer 1992). An effective immune response to foreign skin requires the same three basic phases that an effective immune response to pathogens does: 1) the detection of foreign bodies and antigenic presentation to T cells, 2) lymphocyte proliferation and mobilization, and 3) attack by cellular and/or humoral immune components (McEwen et al. 1997). We therefore considered skin graft rejection a proxy for mounting an immune response against pathogens, providing resistance to disease. In our experiments, we established two related expectations: 1) If stressful pond water conditions suppress amphibian immune function, tadpoles will be less competent in rejecting their skin grafts; and 2) If stressful pond water conditions suppress immune development, froglets may also be less competent in rejecting their skin grafts.

Study area and organism

The Meadow Treefrog (*Hyla pseudopuma*), endemic to lower montane areas of Panama and Costa Rica, is a frog species that has declined in Monteverde, Costa Rica and has not rebounded to pre-decline proportions (Pounds et al. 1999). In the Monteverde region of the Cordillera de Tilarán mountain chain, *H. pseudopuma* has been recorded above 1200m on the Pacific slope and above 900m on the Atlantic slope, straddling the Continental Divide at 1680m (Pounds, unpubl. data). Our studies take place at sites on the Continental Divide where this species used to be abundant, but where post-decline numbers are still relatively low.

H. pseudopuma tadpoles were exposed to four different pond water regimes: “steady high”, “steady low”, “low variation” and “high variation”. We defined these regime classifications by the means and variations of water depth (Table 1, Fig. 1). For the steady high regime, we employed concrete ponds that maintained relatively high water levels throughout the study. The steady low regime differed from the steady high regime primarily in water depth. While the steady low regime ponds were natural forest ponds and thus encompassed some variation, the amount of variation was minimal. The low variation regime ponds had a mean water level comparable to the steady high ponds, but varied in water depth over time. The high variation regime ponds encompassed greater absolute and/or percent changes in water level/volume over time, as well as greater frequency of changes. At times these ponds experienced extreme desiccation.

Steady high regime ponds were outdoor man-made ponds located at the Golden Toad Laboratory for Conservation (GTLC, Pacific slope, Monteverde, Costa Rica). Low variation, high variation and steady low regime ponds were natural forest ponds located in the MCFP in swampy areas along the Continental Divide (Lower Montane Holdridge life zone; Holdridge 1982). The limited and high variation ponds were scattered along the MCFP's Sendero Pantanoso (Swampy Trail) on a relatively flat area (at a 1km scale) of the Continental Divide (Robert Lawson, unpubl. data). The regimes were separated by approximately 0.5km, and the ponds within each regime were in proximity to one another. The steady low ponds were in the MCFP's Bosque Enano (Dwarf Forest) located in a saddle-shaped depression along the Divide's ridge crest, approximately 3.5km from the Pantanoso site. Both the Pantanoso and Enano sites consisted mostly of primary cloud forest, but the Enano site was dominated by elfin forest due to constant heavy wind exposure. Small high-elevation swampy areas like Pantanoso and Enano occur repeatedly along the crest of the Cordillera de Tilarán (Tilarán Mountains; Robert Lawson, unpubl. data).

The soil and hydrology of Pantanoso and Enano are thought to be quite similar to one another. While water moves relatively easily through the surface and uppermost horizon of the soils that had developed on the Monteverde Formation of the upper Cordillera de Tilarán, it tends to divert sideways (following the topography) when it hits the lower less-permeable horizon (Clark et al. 2000). In level areas this lateral flow is slow or impeded, and locally swampy areas result (Clark et al. 2000). As Enano is located along the ridge crest, it receives more mist and cloud deposition than Pantanoso during the dry season. Specific to our sites, the Enano ponds (steady low regime) were located in more characteristically swampy areas, the pond bottom topography was much less variable across space, and the ponds usually remained wet throughout the entire wet season. Our Pantanoso ponds (low variation and high variation regimes) oftentimes could hold deeper water when full, but were more directly dependent on rainfall to stay wet.

Methods

In June 2004, young *H. pseudopuma* tadpoles (1.5 wks of age) were collected from a natural breeding population on the Pacific slope of Monteverde, Costa Rica (outdoor man-made pond, Golden Toad Laboratory for Conservation), and transferred to the MCFP laboratory. Based on the number and size of the tadpoles, their schooling behavior, the size of their natal pond and knowledge of the pond's adult breeding behavior in previous years (2000-2003), the tadpoles probably consisted of one or two clutches at the most. Observations of the tadpoles prior to collection, body size at collection, the time required to reach certain developmental stages in the laboratory, and rainfall data marked the egg-laying date at the very end of May. Because there was a major rain event on May 26 (Pounds, unpubl. data), and heavy breeding likely took place the following day, May 27 was designated as Day 0 for aging purposes.

To house the tadpoles in the laboratory, the same basic methods for maintaining control groups in Beecher et al. (2005a,b) were used. The tadpoles were separated into individual plastic 16oz drinking cups to avoid conspecific interactions (water level at 8cm = 255ml volume; 7.5cm diameter at water surface and 5.5cm diameter at bottom), and cups stratified across designated studies, treatments (pond water regimes) and ponds to control for environmental variation in the room. Degrading leaves from the forest floor were ground in a blender with MCFP spring tap water to create a leaf puree, and a third of a teaspoon was added to each cup every 3 days. Powder from ground spirulina vegetarian tablets was added to the cups once a week to ensure proper nutrition.

Efforts were made to control or account for variables known to influence graft rejection when designing our skin graft experiments. Temperature, age, developmental stage, skin graft size, and genetic differences between recipient and donor have all been shown to influence rate and likelihood of rejection (reviews in Edwards & Ruben 1982 and Flajnik et al. 1987). Temperature was held relatively constant in the laboratory by stratifying cups across treatment (pond water regime) and pond designations, and although pond water temperature was not recorded in the field, 2002 water temperature measurements at the GTLC, Pantanoso and Enano ponds were comparable (mean daylight temperature: 62°C). Age and developmental stage were recorded and accounted for throughout the experiment, and developmental time was considered in the statistical analyses; skin grafts were cut to a size that was small relative to body size (as small as logistically possible), and froglet body weight was considered in the analyses; recipients were collected from 1-2 clutches, decreasing the influences of genetic variation; and skin donors were stratified across treatments and ponds to decrease the chances of greater genetic similarities within particular groups of animals.

All tadpoles were maintained in the laboratory during their remaining early development. Because developmental stage influences amphibian neuroendocrine and developmental responses to water level changes [tadpoles younger than Gosner Stage 34 (GS34) tend to delay development, while more advanced tadpoles tend to hasten development (Denver 1997a, 1997b & 1998; Beecher et al. 2005a)], transfer to the field enclosures were delayed until all tadpoles reached partial hindlimb development at GS34-37 (Gosner 1960; Nieuwkoop & Faber 1956). Tadpole development was monitored in the laboratory by periodically staging random individuals with a 10x hand lens or stereoscope, and when the majority reached GS34-37 every tadpole was weighed (mg) and staged (Day 28-30) (Gosner 1960). Tadpoles that did not fall within GS34-37 at this time were omitted from further study, and, if healthy, they were released back to their natal pond in accordance with the MCFP permit.

To supply non-relative skin donors, tadpoles of similar developmental age were collected from one of our high variation regime ponds (MCFP, Sendero Pantanoso). Located 2-3km away from the graft recipients' natal pond, there is evidence that these two breeding populations are genetically distinct (Beecher et al. 2005a). Genetic differentiation between graft recipients and donors was especially important for the tadpole study, as tadpoles tend to reject grafts only if they differ in major MHC haplotypes (Chardonnens & Du Pasquier 1973; reviews in Flajnik et al. 1987, Rollins-Smith 1998 and Rollins-Smith & Cohen 1996). Skin donors were assigned to either the tadpole or froglet study, and were maintained in the laboratory under control conditions.

Individuals in the tadpole study received skin grafts within four days of final staging. Grafting day was stratified across the four pond water regimes, and each day tadpoles destined for two different ponds were grafted (8 ponds total; grafting on Days 29-33, $\bar{x}$ = d 31). For each day of grafting, surgeries were alternated between the two groups of tadpoles in order to stratify skin donors across ponds, as well as to control for possible researcher effects over time. Sixteen to twenty tadpoles per pond were grafted, with 14-17 tadpoles eventually placed in each pond after omitting those with lost or sub-par grafts.

Tadpoles were grafted using methods modified from Horton (1969) and Du Pasquier & Bernard (1980). After euthanizing skin donors with tricaine methane sulphonate (MS 222), the dorsal skin was removed and carefully flattened out in amphibian phosphate buffered saline (APBS, insert Carolina Biological Supply cat #) on an upside-down glass petri dish. To ensure that the skin grafts would not cover too large of an area which could lead to recipient death or graft acceptance, the dorsal skin was cut into 0.5mm^2 squares (Horton 1969; DiMarzo & Cohen 1982). To succeed in the difficult process of cutting such small pieces of skin, a clear piece of plastic was penned with a 0.5mm^2 square and held above the donor skin while cutting under the stereoscope with a microsurgical blade (sterile miniature-edged with lance tip). Care was taken to make swift clean cuts and avoid pinching the skin with tweezers, preventing damage to the graft that might cause tissue necrosis unrelated to our study.

Recipient tadpoles were anesthetized by immersion in 1:5000 MS 222 and placed on their left side on moist sterile gauze under the stereoscope. Using a microsurgical blade and fine forceps, an approximate 0.5mm square area of dorsal skin was removed and a skin graft was placed on top. Although tadpole skin was difficult to work with, care was taken to limit the amount of extra area exposed that would not be covered by the graft. Immediately after graft implantation, an antibiotic solution (mercurochrome) was externally applied to the graft region to help prevent infection. The gauze tissue was rewetted (keeping the graft site dry), and the tadpoles were placed in a 20C degree refrigerator for 30 minutes to prevent waking to allow for graft adhesion. Most tadpoles regained consciousness within 10 minutes after removal from the refrigerator (discerned by voluntary movement), and actively swam within seconds of their return to their newly-changed water. To allow for initial healing and return to robustness, grafted tadpoles were maintained in the laboratory 1-2 days following their surgery.

Grafted (tadpole study) and ungrafted (froglet study, 25/cage) tadpoles were transferred simultaneously within each pond (transferring on Days 31-35; $\bar{x}$ = d 33). Tadpoles for the two studies were housed in side-by-side cages that were subjectively placed to best capture the pond dynamics. The tops of the cages rose above the highest water level possible to ensure that the tadpoles would experience all vertical water level changes, and to ensure access to the water surface for oxygen (necessary for *H. pseudopuma* survival). Because the tadpoles were unable to forage outside of the cages, at every visit a small amount of decaying leaves from the pond bottom was placed into the cages, as well as ground spirulina algae to ensure proper nutrition.

Cage foundations were constructed out of hard wire mesh molded into the shape of cylinders wide enough to prevent dense crowding horizontally (**insert diameter**). To prevent tadpole escape and damage, an inner soft plastic mesh lining with small holes and a closed bottom was attached. The inner lining extended above the cage, allowing it to be tied off in the field. The inner and outer mesh pieces were fit snugly into and knotted to a plastic potting base. To provide sturdiness and anchoring in the forest ponds, three evenly-spaced and vertically aligned pieces of PVC piping were attached to each cage, and the legs inserted into the pond bottoms. In the man-made (steady high regime) ponds, instead of PVC the cages were secured to the pond perimeters with rope. All cages were placed flush against the pond bottoms. At times of extreme pond water desiccation, water was added up to the rims of the cage bases (3cm high) to help prevent tadpole deaths. At the man-made ponds, cage water depth was recorded once when the ponds were full and again whenever the water lines dipped below the full mark, and at the forest ponds water depth in the cages was recorded every 2-3 days.

Tadpole skin grafts were followed for signs of rejection from Day 7 after graft implantation until healing was complete. Rotating among the different forest areas every 3-4 days [2 GTLC ponds (steady high regime); 4 Pantanoso ponds (low variation and high variation regimes); and 2 Enano ponds (steady low regime)], tadpole grafts were examined in the field. To prevent certain regimes from being observed earlier than others on a continual basis, the order of visitation was occasionally varied. To follow graft rejection, on each visit the tadpoles were carefully removed from their cages, their graft sites were illuminated with a headlamp using halogen and regular bulbs, and the grafts examined with a (**insert magnification, brand & type**) hand lens. Percent of graft area with complete loss of pigmentation was used as criteria for rejection, and the proportion of the graft rejected or healed was recorded over time. Along with rejection data, additional notes taken on graft appearance and tadpole appearance and development aided in identification, allowing us to follow graft rejection through time for each tadpole. Upon the visitations to each site, the tadpoles in the froglet study cages were also periodically checked in order to monitor general health and note any deaths.

As individuals from the tadpole and froglet studies emerged from their cages, they were returned to the MCFP laboratory. It was necessary to wait for the emerging individuals to crawl up the inner cage linings on their own, as those that were removed earlier did not survive the trip back. Because tadpoles that had completely healed their graft site before emergence had been immediately released back into their natal pond, time to emergence was not analyzed for the tadpole study. It was recorded in full for the froglet study, however [developmental rate: mean caging date (d31) to emergence]. Metamorphosing individuals were maintained in the laboratory in16-oz mesh-covered cups, and food was provided for the fully metamorphosed froglets by leaving the window open and the light on for 30 minutes every evening, creating a swarm of insects. To help ensure adequate nutrition, additional insects were periodically caught with nets from surrounding tall grasses, laced with amphibian nutrient powder and dropped into the cups.

Monitoring of tadpole graft rejection was continued every three days in the laboratory. After 100% rejection and complete healing of their graft sites, all healthy individuals were released back to their natal site in accordance with the MCFP permit. For the froglet study, development was checked daily for each individual until they reached complete metamorphosis (complete absorption of their tail; mean day of metamorphosis across water regimes = d 63-66 / 2 months, 11d on average following emergence). Froglet graft surgeries were subsequently scheduled for 2-2.5 wks post-metamorphosis ($\bar{x}$ = 16d / range = 14-18d post-metamorphosis, or 25-29d / 3.5-4wks following emergence for each water regime).

Froglet grafting was performed according to the tadpole grafting methods, with a few modifications. The froglet skin donors that had been collected as tadpoles had metamorphosed in the laboratory, and as in the tadpole study they were of the same approximate age and developmental stage as our recipients. Froglet weight (mg) was recorded just prior to grafting. The froglet grafting techniques used in Beecher et al. (2005b) were attempted first, but rejected for this study. In those methods, a slit was cut in the recipient's dorsal skin, the graft was laid on top, and the graft edges were tucked underneath the recipient's skin and later exposed three days later by trimming back the overlaying skin. Skin grafts of this study's small size ($0.5mm^2$) did not survive well using this technique, however, as a large portion of the grafts were covered by overlaying skin and immediately started to die. Therefore, the methods in the tadpole graft study were used for cutting, where an area of approximately $0.5mm^2$ was exposed, and care was taken to limit excess exposed areas. Once the skin graft was in place, an antibiotic solution (mercurochrome) was applied externally to the graft region to help prevent infection, the bottom of the recipient's plate was re-moistened, and the recipient placed in a 20C degree refrigerator for 30 minutes to allow graft adhesion while the froglet remained anesthetized. After removal from the refrigerator, most froglets were active within 20 minutes.

Starting Day 3 post-grafting, froglet skin grafts were followed every 3 days for signs of rejection.

Unlike the tadpole study (above) or our froglet laboratory study where larger skin grafts were used (Beecher et al. 2005b), the small froglet grafts in this study tended to shed their epidermis before showing classical signs of rejection (a response also seen in Horton 1969). Because it was possible to follow graft rejection more closely in the laboratory, not only was percent graft area with complete loss of pigmentation recorded, but so too was percent area covered by epidermis, percent area darkly opaque [due to lymphocyte accumulation under the graft (Horton 1969)], percent area white with mottled pigmentation (in the later stages of rejection), and percent area healed with own skin. The most useful data was percent area with complete pigmentation loss (including healed areas), just as was reported for the other studies.

Froglet graft rejections were observed until Day 19 post-grafting. At this point, unfortunately some froglets experienced random deaths due to fungal-infested grasses placed in their cups, causing lower sample sizes and skewed results. The cause of death was easily determined by the visible sign of fungal growth, and because a simple change of the grasses in the cups immediately prevented all future deaths. The surviving froglets were maintained in the laboratory until they completely healed their graft site with their own skin, and all healthy individuals were released back to their natal breeding site in accordance with the MCFP permit.

Concurrent with the froglet cage study, graft rejections by froglets that had developed naturally and uncaged in their ponds were observed. These individuals had experienced true pond water conditions during their entire embryonic and tadpole phases. The emerging individuals were collected from one steady high and one high variation pond as they crawled onto our cages and the pond perimeters, and only those whose tails showed absorption not exceeding the first few days following emergence were selected. Development of each individual was checked daily in the laboratory until they completed metamorphosis (100% tail absorption), and graft surgeries were subsequently scheduled.

The same grafting methods were used for the froglet uncaged study as in the froglet cage study, except that skin donors for the steady high regime recipients (natal to the Pacific slope) were collected from one of our Continental Divide ponds, and donors for the high variation regime recipients (natal to the Continental Divide) were collected from one of our Pacific slope ponds. As in the froglet cage study, all skin donors had been collected as tadpoles and maintained them in the laboratory through metamorphosis. Time between metamorphosis and grafting differed by 5-6 days between the regimes, with froglets from the steady high regime grafted at d19-20 (2.5-

3wks) post-metamorphosis, and those from the high variation regime grafted at d25 (3.5wks) post-metamorphosis. As the graft observations ran concurrent to the froglet cage study, the same problem of random deaths and decreased sample sizes was encountered due to the bad grasses that placed in their cups. Graft observations were therefore ended on Day 13 for the steady high regime, and on Day 19 for the high variation regime.

Statistical Methods

Graft rejection for the tadpole and froglet cage studies was analyzed over time using a repeated-measures ANOVA ($\alpha = 0.05$), which accounts for correlations among observations for each individual (Wolfinger & Chang 1995). Each individual was a subject, and the within-subject repeated variable was observation, each of which represented a span of 3-4 (tadpole study) or 3 (froglet study) days. The between-subject analysis determined if a difference existed in percent graft rejection among pond water regimes (steady high, steady low, low variation and high variation). The independent variables in the model were water regime and pond-nested-in-regime. Although we were unable to explore time to emergence or weight at grafting as covariates because of power issues, we did examine them as dependent variables in two-tailed GLM univariate ANOVAs, with independent variables water regime and pond-nested-in-regime (type III F test, $\alpha = 0.05$) (SAS 2005).

In the repeated measures analysis, as our dependent variable "percent rejection" was a proportion with a large skew to one or both tails, it violated assumptions of normality and was not amenable to transformation. Two steps were taken to remedy this situation. First, early and late observations which encompassed mostly 0 or 100% rejection were deleted from the data sets (the tadpole study data set thus included observations from d 10-32, and the froglet study data set included observations from d 13-19). Second, repeated measures were run with the GENMOD procedure in SAS which fits alternative models for non-normal data, allowing the user to choose an alternative response probability distribution and proceed with repeated measures analyses (Orelien 2001; Pedan 2001; Moser 2004; Simpson et al. 2004; Johnston & Stokes 1997; GENMOD procedure, SAS 2005). To estimate parameters in the repeated measures analysis, GENMOD uses generalized estimating equations (GEE) (Hu et al. 1999; Orelien 2001; GENMOD procedure, SAS 2005). GEE methods employ a quasi-maximum likelihood approach (Hu et al. 1999; Simpson et al. 2004; GENMOD procedure, SAS 2005) that allows subjects to be retained in the analysis regardless of missing data (Orelien 2001). We tested the main effects using a type III contrast, which is akin to a GLM type III sums of squares analysis except that it uses likelihood ratios instead of sums of squares (Simpson et al. 2004; GENMOD procedure, SAS 2005). To compare water regimes, we constructed separate contrast statements.

When designing a GENMOD repeated measures model, the user can specify the response probability distribution that best fits their data (GENMOD procedure, SAS 2005). The probability distributions available in GENMOD are from the exponential family, and include binomial, Poisson, normal, gamma, and inverse Gaussian (GENMOD procedure, SAS 2005). GENMOD is considered an extension of traditional linear models because it employs a non-linear link function that relates the response means to linear predictors (Johnston & Stokes 1997; GENMOD procedure, SAS 2005). The error covariance structure - i.e., the working correlation matrix used to model the correlation structure between observations within each subject - can also be specified in GENMOD (Orelien 2001; GENMOD procedure, SAS 2005). While choosing a working correlation close to the true structure increases the chances of parameter convergence, choosing the correct working correlation matrix is not necessary as the matrix is ultimately fitted through an iterative process (Johnston & Stokes 1997). Further discussion on correlation structures and model fitting can be found below.

We also analyzed the raw data with the more conventional MIXED repeated measures procedure (SAS 2005). Like GENMOD, MIXED can handle missing observations by employing a likelihood-based estimation method (in this case restricted maximum likelihood or REML), and allows the user to choose an appropriate within-subject covariance matrix (Wolfinger & Chang 1995; Hamer & Simpson 2000; Moser 2004; MIXED procedure, SAS 2005). We tested the main effects with a type III F test, and the regime differences with a multiple comparison of least squares means (Tukey adjustment). MIXED averages its LSMEAN values across the repeated measures, using standard errors that reflect the chosen covariance structure (Wolfinger & Chang 1995).

Specification of an appropriate covariance matrix is more important in MIXED because it directly influences the tests of the fixed effects (Wolfinger & Chang 1995). Examples of covariance structures that are commonly seen in repeated measures analyses, and that I explored, are: variance components (VC: the default structure, assumes independence among the repeated measures - i.e., observations closer to one another are no more dependent on one another than are those that are far apart); compound symmetry (CS: assumes equal variance among equally correlated repeated measures); Huynh-Feldt (HF: assumes different variances among the repeated measures); and Auto-regressive Lag 1 (AR(1): assumes that the correlations among the repeated variables are proportional to the distance between them - i.e., observations closer to one another are more positively correlated) (Hamer & Simpson 2000).

To identify adequate distributions and correlation structures, and to determine the best-fitting GENMOD and MIXED models, I followed three steps. First, I only considered distributions and covariance structures that were appropriate for repeated measures and for our type of data. Once this was determined, I ran the analyses using different model specifications, discarding any models that did not lead to parameter convergence or that prompted warnings from the SAS log [e.g., the warning "The negative of the Hessian is not positive definite" can indicate that the model is inappropriate (Pedan 2001)]. The last step compared the remaining models using fitness criteria. In GENMOD, I consulted The Criteria For Assessing Goodness of Fit output. Any models that did not have a deviance score (value/df) close to 1 were considered inadequate, and the model with the deviance score closet to 1 was considered to have the best fit (Pedan 2001; Simpson et al. 2004). In MIXED I consulted the Model Fitting Information output, considering the model with the largest negative Akaike's Information Criterion (AIC) value as having the best fit (Wolfinger & Chang 1995; Wolfinger 1996).

The final GENMOD repeated measures analyses for our tadpole and froglet cage studies used a compound symmetry (cs) working correlation matrix and a gamma distribution [default non-linear link function: power (-1)]. A gamma distribution with an inverse power function best describes continuous response data that has one tall peak at or close to zero, rapidly declining thereafter with a long and fat tail (Simpson et al. 2004; Clark et al. 2005). Such a distribution is seen in our data, with the exception of a second peak at 100% rejection for some observations (Fig. 2 in Beecher et al. 2005b). To be able to use the gamma distribution, all rejection data points of 0% had to be changed to 1%. The final MIXED repeated measures analyses for our tadpole and froglet cage studies employed a variance components (VC) correlation matrix. While the VC correlation structure was not available in GENMOD, the VC and CS correlation structures in MIXED yielded similar results. Because both GENMOD and MIXED gave similar results of significance, we report the p values from MIXED because it is a more widely-used statistical measure, and because it allowed us to control for multiple-comparison experimental error rate. [GENMOD results available from the first author].

For the froglet uncaged study, both PROC GENMOD and PROC MIXED were again used to analyze graft rejection (same distributions and covariance structures as above), although not with repeated measures. Because the first three observations revealed no graft rejection for either regime, and only one of the two regimes remained in the study after the fourth observation, we ran an ANOVA on the fourth observation (d 13) alone (two-tailed type III test, $\alpha = 0.05$). The independent variable in the model was water regime (steady high or high variation), and weight at grafting was explored as a covariate. Because both PROC GENMOD and PROC MIXED gave similar results, we again report the p values from PROC MIXED. We also analyzed weight at grafting as a dependent variable in a two-tailed PROC GLM univariate ANOVA (type III F test, $\alpha = 0.05$).

Results

Pond water depth and variation significantly influenced the ability of tadpoles to reject skin grafts (n = 618: *water regime overall effect* p = 0.0001; *pond(regime)* p = 0.0023. Tadpole cage study, Fig. 2). Within 10 days of grafting, tadpoles exposed to steady high water levels exceeded all other pond water regimes by a difference of >20% rejection, continuing to lead in rejection rate by a comparable amount until their graft sites completely healed (*Tukey* p = 0.0001 for all comparisons with other regimes). Exposure to our steady low and low variation water regimes slowed graft rejection to similar extents (steady low vs. low variation *Tukey* p = 0.3119). The low variation regime tadpoles demonstrated a trend toward even slower rejection rate after d16, however, corresponding to a second decline in water level (Fig. 1). The slowest graft rejection was in the high variation regime. After more than two weeks (d 16) these tadpoles averaged only 30% graft rejection, falling more than half way behind the steady high regime (mean rejection $\bar{x}$ = 68%) and at least a third of the way behind the steady low and low variation regimes ($\bar{x}$ = 45-51% rejection) (*Tukey* p = 0.0001 for all comparisons with other regimes).

Exposure to greater pond water variation and lower water depth during tadpole development also lowered the ability of froglets to reject skin grafts (n = 174: *water regime overall effect* p = 0.0105; *pond(regime)* p = 0.1698. Froglet cage study, Fig. 2). Compared to all other regimes, froglets that had been exposed to steady high water levels during their tadpole development rejected their grafts faster (*Tukey* $p \leq .05$ for all comparisons with other regimes). By Day 16 post-grafting, the steady high regime froglets exceeded the other water regimes by a difference of >25% rejection. For those individuals that had been exposed to the steady low and low variation water regimes, froglet graft rejection was slowed to similar extents (steady low vs. low variation *Tukey* p = 0.8955). We were unable to include the high variation regime in the analysis because all of the tadpoles died from disease prior to metamorphosis.

For froglets that had developed uncaged in their natal ponds, high variation in pond water again led to slower graft rejection. Although sample sizes were low in this study because of high mortality upon grafting (mean survival: 28%), we were able to detect differences in rejection ability. While the steady high regime froglets were rejecting their grafts by Day 13 (mean rejection $\bar{x}$ = 8%), the high variation regime showed no signs of rejection at this point (n = 10: p = 0.0053. Froglet uncaged study, Fig. 2). The high variation regime showed only minor signs of initial rejection a week later (Day 19), when mean percent rejection reached only 3%.

In both the froglet cage and uncaged studies, changes in water level during tadpole development influenced metamorphic body size, with the difference still notable within the first month after metamorphosis. In the froglet cage study, the steady high regime individuals (which weighed the most: $\bar{x}$ = 147mg, n = 33) were significantly heavier than the low variation individuals (which weighed the least: $\bar{x}$ = 132mg, n = 27) (*water regime overall effect* p = 0.0175, *Tukey* p = 0.0126. Froglet cage study, Fig. 3). The steady low regime froglets were at an intermediate weight ($\bar{x}$ = 140mg, n = 19), and did not differ significantly from either of the other two regimes (*Tukey* p ≥ .36). In the froglet uncaged study, development in the high variation pond had a drastic effect on froglet weight (Froglet uncaged study, Fig. 3). The steady high regime froglets averaged 98mg, but the high variation regime froglets only averaged a very small 63mg, a 30% difference (p = 0001; rejection analysis covariate p = 0.0020). In fact, froglet weights from the two regimes did not overlap at all [range: steady high = 90-110mg (n = 4), high variation = 58-72 (n = 6)].

Changes in water level altered the time required for the tadpoles to reach emergence. Compared to the steady high regime, the steady low regime averaged 7.9% faster development, while the low variation regime averaged 10.4% slower development [time to emergence from the average caging date (d31), Froglet cage study: steady high $\bar{x}$ = 19.0 days (n = 33), sudden low = 17.5 days (n = 19), low variation = 21.0 days (n = 27). *Water regime overall effect* p = 0.0001, *Tukey* p ≤ .0291.]. Compared to each other, the steady low regime emerged an average 20% faster than the low variation regime.

The number of tadpoles present in the cages changed over time due to emergence or death. The shaded area in Fig. 2 signifies the span of time during which emerging individuals were removed from the cages and brought back to the laboratory. Changes in cage density, as well as status of emergence and metamorphosis, did not appear to alter the mean rejection trajectories. This is best seen in the continually smooth curve of the steady high regime (Tadpole study, Fig. 2). In the high variation regime we stopped observing grafted tadpoles after Day 16 because of the number of accumulated deaths. No enclosed tadpoles from this regime, in either the tadpole or froglet cage study, survived to emergence. Similar deaths occurred in one of the two steady low ponds, but not until a week later (d 22). In all of these cases the deaths appeared to be pathogenic, and samples were preserved in ethanol.

Discussion

Exposure to pond water variation and desiccation during tadpole development compromised the ability of tadpoles and young froglets to reject foreign tissue. Because we did not document any cases of skin graft acceptance in any of our studies, the differences in rejection rate did not appear to be cases of active tolerance (Flajnik et al. 1987) but rather immunosuppression. Immune responses lagged in response to extreme pond desiccation as well as variation in pond water level. Greater absolute and percent changes in water level/volume over time, and greater frequency with which these changes occurred, were associated with more pronounced effects on immunity. Because slower graft rejection under these conditions was observed not only in tadpoles but also in froglets, both immune system function and development appeared to be impaired.

The most apparent difference in allograft rejection among our pond water regimes was in the timing of rejection. Compared to the steady high regime, tadpoles and froglets from the steady low and low variation regimes delayed their graft rejection by 3-4 days, while those from the high variation regime delayed rejection by 1 week. We saw similar results in a controlled laboratory study, where exposure to sudden water decline during tadpole development resulted in delayed froglet sibling skin graft rejection by over 1 week (Beecher et al. 2005b; data shown in Fig. 2). While the timing of the onset of rejection differed in all of these studies, other general patterns of rejection appeared similar. For each study, once rejection was underway we saw almost parallel slopes and line trajectories across water level treatments (Fig. 2). This similar yet delayed rejection pattern suggests a lag time in immune response.

The fact that water regimes differed primarily in the onset of rejection, and that this pattern corresponded across all of our studies, bolstered the incomplete data in our froglet cage and froglet uncaged studies. In the froglet cage study, given that by our last observation (d19) the steady low and low variation regimes were lagging behind the steady high regime by >20% rejection, it is relatively safe to assume that they would have continued to lag in rejection rate until complete healing. Because of the variation in percent rejection between the steady low and low variation regimes, however, we are less confident in assuming that they would have continued to share similar rejection trajectories with each other throughout the entire rejection process. In the froglet uncaged study, even with short observation time periods and low sample sizes, the lag in graft rejection rate by the high variation regime matched that seen in the tadpole study. Because differences in graft rejection were similar among all of our studies, it is reasonable to conclude that the patterns we observed were representative of the different water regimes.

Of the two high variation ponds, the pond that encompassed the greatest absolute amount of variation had the most negative immune impacts on tadpoles, even though at times the water was quite deep (Fig. 1). Not only did extreme pond desiccation influence tadpole immunity (as was seen in both high variation ponds), then, but so did absolute changes in water depth / volume. A similar relationship was suggested by the low variation ponds, where water was relatively deep even when the levels were at their lowest (Fig. 1). Individuals in this case appeared to respond more to the changes in water level than to the actual water depth. We expect that if the low variation ponds were shallower, or if the water level variation were greater (intermediate between our limited and high variation regimes), the tadpoles would have experienced even greater stress impacts on their immune development and function. We were able to observe the impact of additional water stress in the low variation ponds, in fact, when water level declined during the cage studies for a second time (Arrow in Fig. 1; most individuals across regimes were still in their cages). In conjunction with this water decline, the low variation tadpoles appeared to undergo a further dampening of their graft rejection response, lagging behind even the steady low regime from this point on (5-10% rejection lag from d19-32; Tadpole cage study, Fig. 2).

Although our steady low and low variation regime froglets appeared to have comparable rates of graft rejection, they differed in their developmental timing responses. Exposure to the steady low regime caused tadpoles to increase their developmental rate compared to the steady high regime, while exposure to the low variation regime led them to ultimately delay development (Fig. 4). If we treat the steady high regime as our control, the reaction norms for the two other regimes were relatively similar in absolute slope but opposite in direction. The increase in developmental rate by the steady low regime tadpoles was in line with our expectations, as we saw *H. pseudopuma* tadpoles respond this way to sudden water declines in the laboratory (Beecher et al. 2005a,b). Of all of our pond water regimes in the field, in fact, the steady low regime was most like our water decline in the laboratory, in that there was a sudden change in water level/volume that was maintained through emergence. It has been shown that even when tadpoles are housed separately under controlled conditions, decline of water depth or volume can prompt developmental rate changes (Denver et al. 1998; Beecher et al. 2005a). These changes occur without chemical cues or temperature changes (Denver et al. 1998), and can occur in response not only to water decline, but also to the stresses of crowding and food limitation (Denver 1997b; Denver et al. 1998; Kikuyama et al. 1993; Hayes & Wu 1995; Boorse & Denver 2004). During prometamorphosis, these types of stressors tend to accelerate development (Denver 1998; review in Denver et al. 2002).

Tadpoles respond not only to water decline, but also to pond water replenishment. Exposure to water decline followed by water replenishment has been shown to produce a trend toward smaller metamorphic body size, but longer developmental time (Denver et al. 1998). We observed this same response in our low variation regime, which produced froglets that were both small in body size and late in emergence (Froglet cage study, Figs. 3&4). When tadpoles are exposed to longer periods of water decline or drastic desiccation, however, they tend to metamorphose smaller and faster even when water is replenished (Denver et al. 1998). Our uncaged study demonstrated this pattern, with the high variation regime individuals clearly developing quickly in response to drastic water desiccation, and emerging with very small body sizes (Fig. 3). Even with the different responses in metamorphic plasticity observed among our regimes, it is apparent from the body weight and graft rejection data that all three of our alternative water level regimes were stressful, albeit to different extents.

It is possible that weight and percentage of body area covered by the skin grafts influenced rejection rate, although these factors do not appear to adequately explain the observed rejection differences. In the tadpole cage study, for instance, individuals were grafted prior to experiencing their pond water conditions and diverse growth rates. Furthermore, our laboratory study demonstrated that depressed froglet graft rejection following tadpole exposure to water decline can occur without weight serving as a major explanatory factor (Beecher et al. 2005b). The transition into emergence, metamorphosis and post-climax did not appear to alter the mean rejection trajectories, as is best seen in the steady high regime's smooth rejection trajectory over time (Tadpole cage study, Fig. 2). In the froglet uncaged study, populational (genetic) differences could have influenced rejection rates, but as the rejection differences coincided with our other studies it is reasonable to infer that we were seeing mostly environmental effects. We also considered the possibility that rejection differences in the uncaged study resulted from some froglets having extra time to develop their adult-type immune systems, as time from metamorphosis to grafting differed by almost a week between treatment groups. This concern was alleviated by the fact that the high variation regime froglets had 5-6 more days to build their adult immune systems, and still rejected their grafts slower.

While variable food and cage densities may have influenced our results, it is also unlikely that the rejection differences were a unique result of these factors. We provided the caged tadpoles with decaying pond leaves and spirulina algae at every visit to help assuage any differences in food availability within the pond water, and we did not see trends in graft rejection that followed the different food availabilities within the ponds themselves. For example, the steady low ponds appeared to house less leaves, detritus and algae than the low variation or high variation ponds, but graft rejection in this regime was similar or better than these other two regimes. The changes in tadpole density in the cages over time also did not appear to alter mean rejection trajectories. The inadequacy of alternative explanations to account for our results, and the agreement of graft rejection differences across all four of our studies - which included laboratory and field conditions, caged and uncaged housing, and tadpole and froglet life stages - strengthen the conclusion that it was water level/volume variation and desiccation that caused the major differences in tadpole and froglet immunity.

In three of our ponds we observed disease and consequent death. Outward signs of the disease were in the form of a skin malady, with white polyps appearing most often on the sides and ventral surface. While the malady at this stage was most obvious under a stereoscope, disease progression resulted in larger, more numerous white polyps noticeable to the naked eye and covering almost the entire body. Similar symptoms were observed in 1997 among diseased and dying amphibians of the high-elevation preserve of Reserva Forestal Fortuna, Chirique Province, Panama (K. Lips 1997, unpubl. data). The disease in this case was thought to be a Ranavirus infection, but because only a few tadpoles were infected in this way no additional tests were run. We have preserved our samples for analysis.

In both of our high variation ponds, all of the caged individuals suffered disease and death before or during metamorphosis, with none of the individuals living to reach the froglet stage. The two high variation ponds were in close proximity to one another, and probably shared water and moving soil at times of heavy rainfall and flooding. Aside from the obvious transmission probabilities, however, circumstances suggest that exposure to these high variation pond conditions increased the tadpoles' susceptibility to disease and death. During times of drastic water declines and desiccation, the majority of the individuals developing uncaged in the high variation ponds appeared to be in poor condition. Although we did not methodologically sample tadpole abundance or condition outside of the cages, personal observations included decreases in tadpole numbers that greatly exceeded the number of individuals metamorphosing, and early emerging individuals that were very small and weak.

We observed similar disease symptoms in one of our steady low cages, but the pathogenesis did not occur until later and a lower number of individuals were affected. While it is possible that in the course of our field work we transferred the pathogens from the high variation ponds to this steady low pond, the patterns of disease did not follow any particular transmission route. Even though we always observed both steady low ponds on the same day (in different orders of visitation), we never documented any visible signs of disease in the other steady low pond. Furthermore, although we usually observed the low variation ponds on the same day as the high variation ponds (in different orders of visitation), both low variation ponds appeared disease-free throughout the studies.

We considered the possibility that disease itself may have slowed the graft rejections. In this were true, one could argue that disease may have been responsible for the weaker immune responses, and not the other way around. Multiple lines of evidence refute this argument, however: 1) At no time did the tadpoles in the diseased steady low pond lag in rejection rate behind those in the healthy steady low pond. 2) The low variation regime, which showed no signs of disease, lagged behind the steady high regime in both the tadpole and froglet cage studies. 3) The similarities in graft rejection differences observed among all four of our studies, which included a controlled laboratory study, suggest that the depressed immune responses were primarily due to the water level treatments. Collectively, these facts suggest that disease was not responsible for the differences in rejection rate among our regimes. It is reasonable, however, to suggest that perhaps the greater levels of pathogenicity were a result of immunosusceptibility.

It is quite possible that the slower graft rejection responses were at least partially due to depressed T cell number and/or activity (Beecher et al. 2005b). Skin allograft rejection is primarily a cellular immune response, and is largely induced by helper and cytotoxic T cells and their related cytokines (reviews in Brown et al. 1975, DuPasquier et al. 1986, Du Pasquier et al. 1989, Edwards & Ruben 1982, and Flajnik et al. 1987; Rosenberg & Singer 1992; Rau et al. 2001; Rollins-Smith 2001). It has been well established that water decline during prometamorphic development prompts and increases the production of corticosterone and thyroid hormones (Denver 1997a, 1997b; Denver et al. 1998), and that during tadpole development and metamorphosis these hormones can directly and/or indirectly increase lymphocytic apoptosis, decrease lymphocytic proliferation and depress lymphocytic function (Rollins-Smith et al. 1988; Rollins-Smith & Blair 1993; Barker et al. 1997; Rollins-Smith et al. 1997; review in Rollins-Smith 1998). In fact, depressing Tc cell populations experimentally has been shown to result in a lag in rejection rate (Rau et al. 2001), which was the main consequence of water decline in our study

Immunocompromization of tadpoles and young froglets could play a significant role in their disease ecology. Chytridiomycosis and ranaviral disease, both considered emerging diseases and at times coinciding with secondary bacterial infections, have been implicated in various frog declines in different parts of the world (Halliday 1998; Carey et al. 1999; Daszak et al. 1999). Tadpoles are the life stage most at risk from ranaviruses, with 100% mortality often seen in cases of infection (Daszak et al. 1999). Tadpole susceptibility has been seen with Frog Virus 3 (FV3), a major species within the Ranavirus genus (family Iridoviridae) that has infected various amphibian genera and species around the world, and considered a potential major threat to amphibian populations (Gantress et al. 2003). While tadpoles are usually just carriers of the chytrid fungus, metamorphosis into froglets with keratinized skin cells suddenly makes the individuals much more susceptible to the disease (Berger et al. 1998; Rollins-Smith 1998). Young froglets are already at a disadvantage following metamorphosis because their immune system is reorganizing and won't reach maturity for 4-8 months (Rollins-Smith 1998). According to our study, individuals that develop under stressful pond water conditions will be even less immunocompetent, most likely making the already-vulnerable tadpoles and young froglets more susceptible to the pathogens.

While our study examined immune development and function in the face of water decline, we can only make inferences as to the actual susceptibility of the tadpoles and froglets to disease. It has been shown, however, that water decline in temporary ponds can indeed increase disease rates. In a study comparing parasitic trematode infection of the gray tree frog (*Hyla versicolor*), a controlled gradual decline in pond water resulted in higher parasitic loads (Kiesecker & Skelly 2001). Additionally, in the Oregon Cascade Mountains, Southern Oscillations / El Nino effected low winter precipitation, causing shallower montane ponds (Kiesecker et al. 2001). The shallower pond waters caused greater UV-B exposure for *Bufo boreas* embryos, resulting in higher fungal-related mortalities (Kiesecker & Blaustein 1995; Kiesecker et al. 2001).

Conclusion

In the Monteverde frog declines, a strong correlation was established among climate change, precipitation and frog declines (Pounds et al. 1999). Recently, links has also been discovered among climate change, precipitation, amphibian breeding pond conditions and disease susceptibility (Kiesecker & Blaustein 1995; Kiesecker et al. 2001). Our study adds to this body of work by showing how low or variable pond water conditions can negatively impact tadpole and froglet immune development and function.

In response to greater pond water variation and desiccation in the field, both tadpoles and froglets in our study were slower in rejecting foreign skin grafts. Skin graft rejection is primarily a cellular immune, and is largely induced by helper and cytotoxic T cells and their related cytokines (reviews in Brown et al. 1975, DuPasquier et al. 1986, Du Pasquier et al. 1989, Edwards & Ruben 1982, and Flajnik et al. 1987; Rosenberg & Singer 1992; Rau et al. 2001; Rollins-Smith 2001). Cellular immunity oftentimes provides a primary defense against pathogens (Chrousos 1998; review in Wadhwa et al. 2001), and a depressed cellular immune response can make individuals more susceptible to initial infection (Chrousos 1998; review in Wadhwa et al. 2001). Thus, stress experienced by tadpoles during development can play a role in amphibian disease ecology. Collectively, these data support the hypothesis that climate-induced changes in pond water have the potential to influence immune development and function, possibly contributing to disease susceptibility and frog declines.

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Table 1. Pond water regimes: mean pond water depth and mean variation in depth at pond cages (n = 2 ponds/regime, Monteverde, Costa Rica, 2004). Mean depth and variation calculated for 7/1-7/24 when tadpoles were caged.

Pond water regime	Mean depth (cm) [a]	Mean variation	Location
Steady high	**47**	**0.3**	GTLC man-made ponds (Pacific slope)
Steady low	**22**	**17**	Saddle along the Divide ridge crest (Bosque Enano)
Low variation	54	**44**	Flat area along the Divide (Sendero Pantanoso)
High variation	17	**302**	Flat area along the Divide (Sendero Pantanoso)

[a] Mean depth is most meaningful for the steady high and steady low regimes; it is not as representative of the other regimes because of their inherent variation. The above data represents pond depth only at cage locations. The limited and high variation ponds exhibit even greater variation across space and time when entire ponds are taken into consideration.

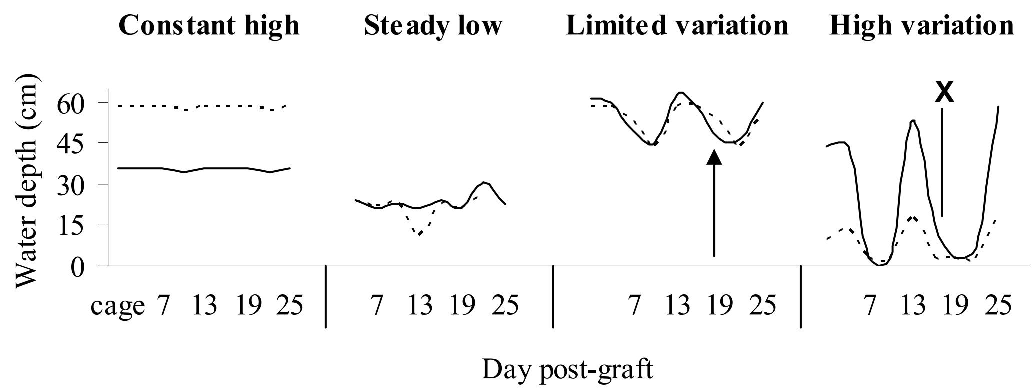


Figure 1. Pond water depth at cages, spanning the time from when all tadpoles were caged until all had emerged (Monteverde, Costa Rica, 7/1-24/2004). Tadpole and froglet field study cages were placed adjacent to one another in each pond (2 cages/pond, 2 ponds/regime). The solid and dashed lines refer to one of the two ponds in each regime. Day post-graft on the x axis refers to the tadpole field study (caged tadpoles in the froglet field study had not received skin grafts at this stage, although they were in the ponds during this time). Arrow: low variation tadpoles appeared to begin to lag behind steady low tadpoles in mean percent graft rejection. X: accumulated deaths halted further tadpole graft observations.

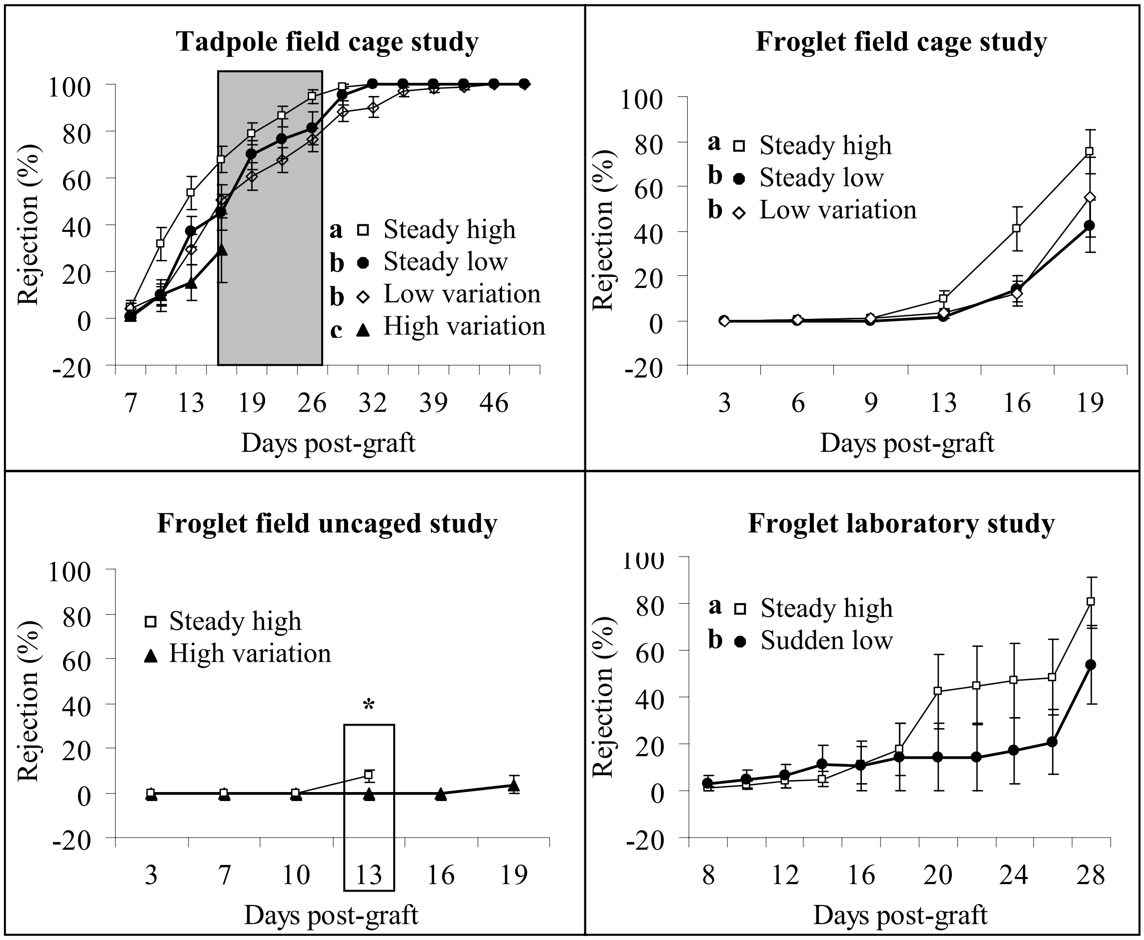


Figure 2. Mean percent graft rejection (skin pigmentation loss) over time (*H. pseudopuma*, Monteverde, Costa Rica). Letters and asterisk denote significance at $p \leq 0.05$; see text for methods. **Tadpole cage study**: The shaded area signifies the span of time during which emerging individuals were removed from the cages and brought back to the laboratory for continued graft observation. Number of repeated measures observations (d 10-32): n = 618 (steady high: 222, steady low: 151, low variation: 206 and high variation: 39). **Froglet cage study**: Number of repeated measures observations (d 13-19): n = 174 (steady high: 71, steady low: 50, low variation: 53). **Froglet uncaged study**: Individuals developed naturally in their natal ponds; n = 10 (steady high: 4, high variation: 6). **Froglet laboratory study**: Data modified from Beecher et al. (2005b). *H. pseudopuma* tadpoles were exposed to steady high (8cm) or sudden low water levels (8cm dropped to 2cm at GS 35-36 and maintained until emergence), and froglets were grafted 2.5-3 wks post-metamorphosis with sibling donor skin (Monteverde, Costa Rica, 2002). Percent rejection was recorded every 2 days. Number of repeated measures observations: n = 191 (steady high: 110, sudden low: 81).

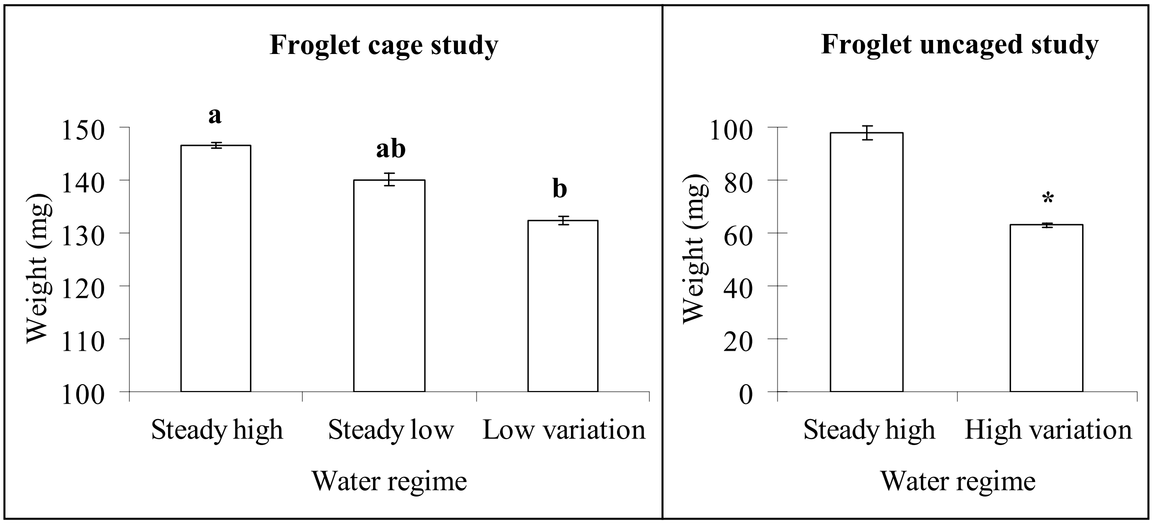


Figure 3. Mean froglet weight at the time of graft surgery (*H. pseudopuma*, Monteverde, Costa Rica, 2004). Letters and asterisk denote significance at $p \leq 0.05$. **Froglet cage study**: Individuals were caged in ponds as tadpoles at GS34-37 (2 ponds/water regime), and were weighed 2-2.5 wks post-metamorphosis (2004). Number of froglets: n = 79 (steady high: 33, steady low: 19, low variation: 27). **Froglet uncaged study**: Individuals developed naturally in their natal ponds (1 pond/water regime) and were weighed 2.5-3 (steady high) and 3-3.5 (high variation) weeks post-metamorphosis (2004). Number of froglets: n =10 (steady high: 4, high variation: 6).

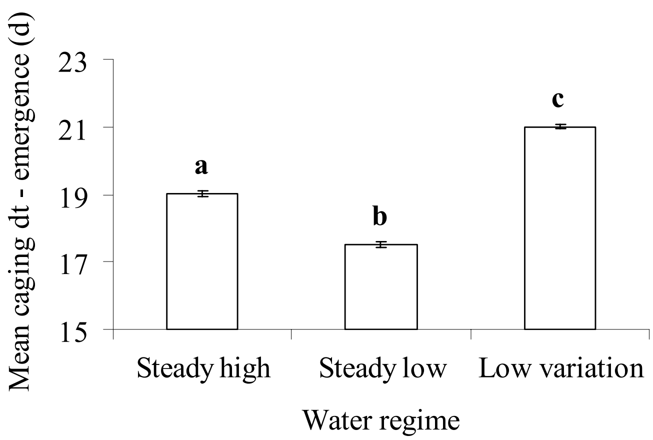
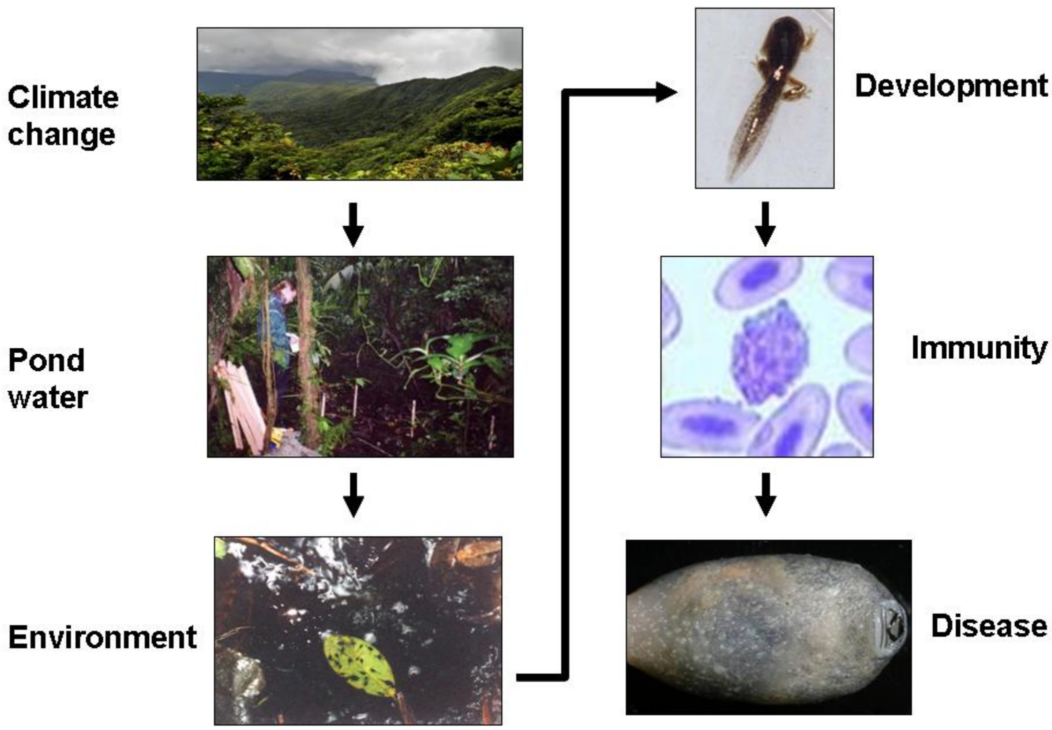
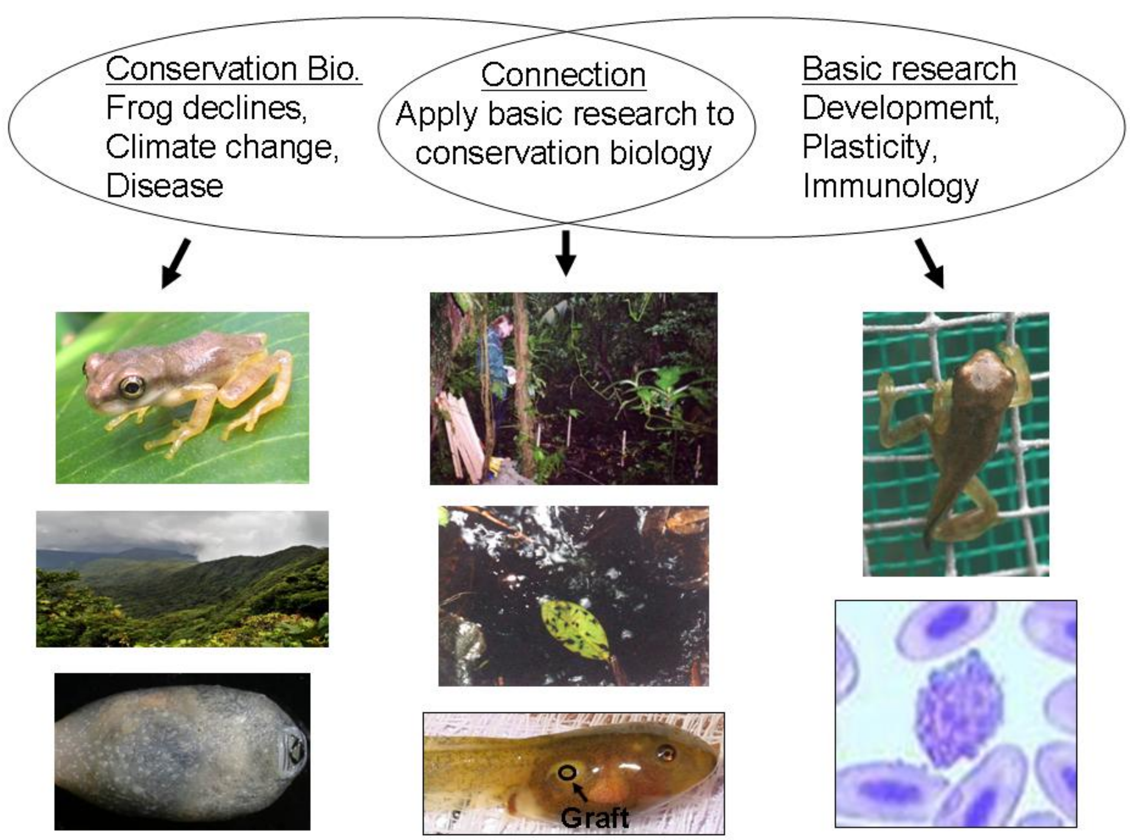
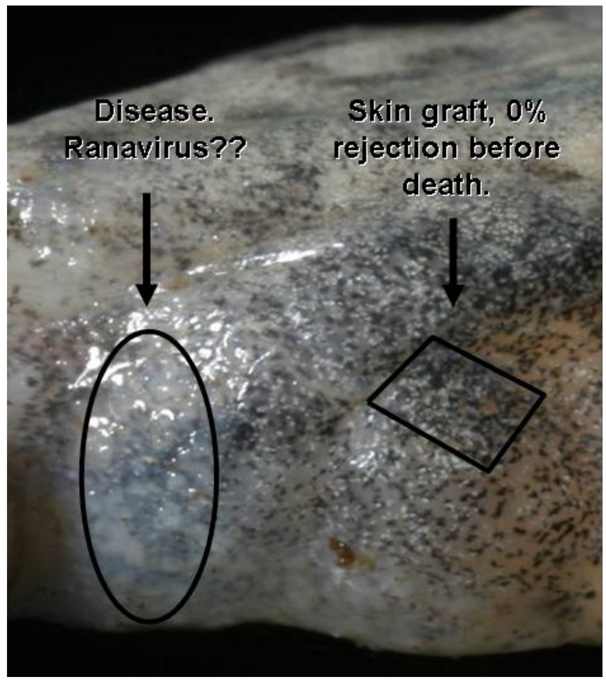


Figure 4. Mean time to emergence for individuals in the froglet cage study (*H. pseudopuma*, Monteverde, Costa Rica, 2004). Letters denote significance at $p \leq 0.05$. Tadpoles were caged in ponds starting at GS34-37 (2 ponds / water regime), and time to emergence was calculated from the mean caging date. Number of individuals: n = 79 (steady high: 33, steady low: 19, low variation: 27).

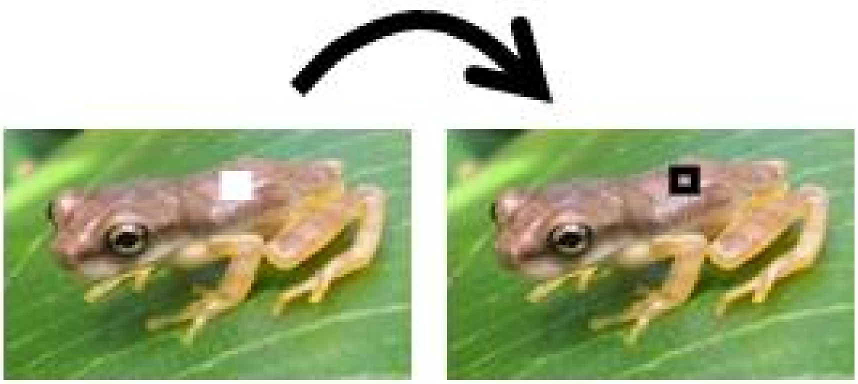
Research model

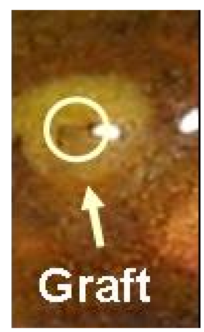


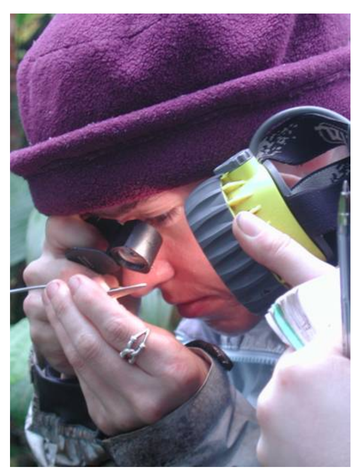



Disease.
Ranavirus??
Skin graft, 0%
rejection before
death.


Hind limb
Graft




Graft



Nancy A. Beecher

♦ ***Ph.D. student, Indiana University*** 5/99 - 11/05

Research: Frog declines, climate change and disease: searching for a connection.

Major: Biology. Minor: Public & Environmental Affairs. GPA 3.9.

Research Objectives: To examine environmental influences on frog metamorphosis and immune system development, for the purposes of enhancing theoretical knowledge and conservation efforts.

Research

- Studied and conducted dissertation research in Monteverde, Costa Rica for 6 years (1999-2004 summers).
- Collaborated with U.S. and Costa Rican researchers, Reserve personnel, and the Monteverde community.
- Developed interdisciplinary research spanning ecology, conservation, evolution, physiology & development.
- Independently raised over $28,000 in funds.
- Independently managed logistics of international research and mentored 5 undergraduates in Costa Rica.

Teaching

- Awarded the IU Biology Dept Outstanding Associate Instructor Award (nominated 3 times).
- Committed to offering high-quality education and critical thinking training to young adults.
- Taught 14 courses from 1999-present, encompassing over 1,200 students.

Evolution and Diversity L111 (3 cr.): Taught 3-5 discussion sections.	Sp05,02,01,00/Fa03,02
Endocrinology Lab Z566 (2 cr.): Co-taught 1 lab.	Spring 2004
Biol Mechan L112 (3 cr.): Supervised 10 undergrad instructors, taught 1 discuss sxn.	Fall 04/Spring 03
Graduate Biometry Seminar: organized seminar, arranged speakers, lead discussion.	Spring 2002
IU Costa Rica undergraduate course (3 cr.): Co-taught, full responsibility.	Winter 2001/2
Evolution L318 (3 cr.): Taught 3 discussion sections.	Fall 2001
Humans and the Biological World (3 cr.): Taught 2 laboratory sections.	Fall 2000
Biology Lab L113 (3 cr.): Full responsibility for lab and discussion.	Fall 1999

- Gave 7 guest lectures encompassing ecology, animal behavior, conservation and quantitative genetics.

♦ ***Biostatistician and Geographical Imaging Analyst, University of Nebraska - Lincoln*** 6/98 - 6/99

- Created ArcView layer for Sandhill Crane migration project in central Nebraska.
- Performed TNTmips extractions, Fragstats analysis, and SAS programming for a project aimed at analyzing landscape use by birds and developing a model to categorize wetlands using satellite imagery.
- Statistical analysis of horticulture and agroforestry projects.

♦ ***M.S., Univ. Nebraska – Lincoln*** Major: Ecology GPA 3.8. Minor: Biometry GPA 4.0. 8/94 - 5/98

M.S. Thesis: Beecher, N. A. 1998. Birds and agroecological relationships in organic and non-organic farmland. M.S. Thesis, University of Nebraska, Lincoln, Nebraska, USA. 158pp.

Research Objectives: To compare bird and arthropod abundance, richness and composition in organic and nonorganic farmland, and to assess relationships between birds and chemical inputs, farming practices, arthropods, and vegetation. To understand agriculture as an ecological system and find ways to enhance wildlife conservation and biological pest suppression on farmland.

Research design and implementation

- Developed and implemented all experimental designs.
- Strengthened avian research by initiating, planning, budgeting and implementing arthropod study.
- Performed field work at 30 agricultural research sites over 2 field seasons:
  - Identified 54 species of birds, surveyed vegetation, and collected 860 arthropod samples.
  - Developed a collaborative relationship, completed questionnaires with 22 private landowners.

Supervision and funding

- Wrote independent and collaborative grants, managed research money for payroll, supplies and travel.
- Hired, trained and supervised 1 undergraduate in the field and 2 undergraduates in the laboratory.

♦ ***Editor/Analyst of Scientific Literature, BIOSIS, Inc. (Biological Abstracts)*** 8/92 - 9/93

Read, analyzed and indexed biological journal abstracts (approximately 50/day) to create library databases. Trained employees and maintained quality control after 8 months of employment.

♦ ***The Pennsylvania State University, B.S. degree*** GPA 3.3. 8/88 - 5/92

Major: Life Science. Volunteered in emergency rooms and hospital wards, observed surgeries.
Minor: Spanish. Studied abroad at the Univ. Salamanca, Spain (read, write and speak Spanish).

REFEREED PUBLICATIONS

Beecher, N. A., R. J. Johnson, J. R. Brandle, R. M. Case, and L. J. Young. 2002. Agroecology of birds in organic and nonorganic farmland. Conservation Biology 16:1620-1631.

Beecher, N. A., G. E. Demas, and C. Nelson. Developmental plasticity and clinal variation in the context of climate change and frog declines. *In preparation*.

Beecher, N. A., G. E. Demas, and C. Nelson. Suppressed foreign tissue rejection by tadpoles and young froglets as a tradeoff of precocious metamorphosis. *In preparation*.

Beecher, N. A., G. E. Demas, and C. Nelson. Suppressed antibody production by tadpoles and young froglets as a tradeoff of precocious metamorphosis. *In preparation*.

Beecher, N. A., G. E. Demas, and C. Nelson. Frog declines, climate change and disease: interpreting the connections. *In preparation*.

PUBLISHED PROCEEDINGS

Johnson, R. J., J. R. Brandle, N. J. Sunderman, R. L. Fitzmaurice, N. A. Beecher, R. M. Case, M. E. Dix, L. J. Young, M. O. Harrell, R. J. Wright, and L. Hodges. 1996. Wildlife as natural enemies of crop pests. Proc. 8th Natl. Extension Wildlife and Fish. Specialists Workshop. (Bellingham, WA. June 26-29).

PUBLISHED ABSTRACTS and PRESENTATIONS

Ph.D.: Frog declines - exploring connections among climate change, immunity and disease

Beecher, N. A., G. E. Demas, and C. Nelson. 2005. Ctr. Integrated Stud. Animal Behavior (CISAB) conference (Bloomington, IN. April 22).

Beecher, N. A., G. E. Demas, and C. Nelson. 2005. Student Conference on Conservation Science (Durham, NC. March 16-18).

Beecher, N. A., G. E. Demas, and C. Nelson. 2005. Society for Integrative & Comparative Biology (SICB) conference (San Diego, CA. April 22).

M.S.: Integrating wildlife and agriculture - birds in organic and nonorganic farmland

Beecher, N. A., R. J. Johnson, R. M. Case, J. R. Brandle, and L. J. Young. 1997. Proc. Midwest Fish and Wildlife Conference 59:141. (Milwaukee, WI. Dec. 7-10).

Beecher, N. A., R. J. Johnson, R. M. Case, and J. R. Brandle. 1996. Proc. Midwest Fish and Wildlife Conference 58:86. (Omaha, NE. Dec. 8-11).

Beecher, N. A., R. J. Johnson, R. M. Case, and J. R. Brandle. 1996. Proc. Annual Meeting, American Society of Agronomy. (Indianapolis, IN. Nov. 3-8). p. 54.

Beecher, N. A., R. J. Johnson, R.M. Case, J.R. Brandle, and L.J. Young. 1996. Proc. NE Acad. Sci. 116:16. (Lincoln, NE. Apr. 26).

GRANTS and HONORS RECEIVED

Ph.D.

Indiana University Biology Department Outstanding AI (nominated 3x)	$500	4/05
Indiana U. Graduate Professional & Student Org. travel grant	$150	2/05
Indiana U. Biology Department travel grant	$250	2/05
Indiana U. College of Arts & Sciences travel grant	$200	11/04
Indiana U. Ctr. Integrated Stud. Animal Behavior (CISAB) travel grant	$500	12/04
Indiana U. College of Arts & Sciences travel grant	$300	11/04
Eloise Gerry / Graduate Women in Science (GWIS) research grant	$2000	7/04
Society for Integrative & Comparative Biology (SICB) travel grant	$2000	5/04
Dona G. Graam / I.U. Biology Dept fellowship for women in science	$2-3,000ea.	5/2000-2004
Declining Amphibian Populations Task Force (DAPTF) research grant	$2020	4/04
Indiana U. Biology Department research award	$250ea.	5/03,3/02,2/01,2/00
Explorers Club research grant	$1200	4/02
Tinker Found. / I.U. Ctr. Latin Amer. & Caribbean Stud. research grant	$1250	5/02
Indiana U. Ctr. Integrated Stud. Animal Behav. (CISAB) research grant	$500,$400	8/01,5/00
Organization for Tropical Studies (Peace Frogs) research grant	$455	7/00
Sigma Xi Grants-in-Aid of Research	$705	6/00
Indiana U. Biology Dept. fellowship for OTS Tropical Ecology course	$2000	5/99
Total Ph.D. funds	$28,630	

M.S.

Center for Great Plains Studies, Research Grant-in-Aid for Graduate Students		$570	11/97
Sigma Xi Science Student Travel Grant		$150	11/97
NE Chapter, The Wildlife Society Graduate Scholarship		$500	12/96
Wildwood Travel Fund Grant		$150	11/96
Univ. Nebraska Research Council Grant-in-Aid		$3000	7/96
USGS Regional Water Resources Competitive Grants Program	(Did not receive)	$64,000	
EPA 1997 STAR Grants for Research Program	(Did not receive)	$355,000	
Lincoln Journal Star and Nebraska Farmer feature articles on M.S. research			1/98, 7/97,12/96

COMMUNITY SERVICE

Presented Ph.D. research in Spanish to the community of Monteverde, Costa Rica.	09/2004
Presented M.S. & Ph.D. research to community groups, local libraries and landowners.	1996-2005
Conducted a community-outreach spider research project for Indiana Brown County State Park.	01-05/2002
Volunteered for project in Mexico studying overwintering grassland birds for genetic conservation.	02/1999
Community outreach with the IU African American Dance Company.	2003-2005