ECOLOGICAL AND EVOLUTIONARY IMPLICATIONS FOR THE CONSERVATION OF PANAMANIAN GOLDEN FROGS

by

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Nature's first green is gold
Her hardest hue to hold.
Her early leaf's a flower;
But only so an hour.
Then leaf subsides to leaf.
So Eden sank to grief,
So dawn goes down to day.
Nothing gold can stay.

- Robert Frost

To my Mom and Dad, who have always encouraged me following my dreams

and to Geoff who is always game for an adventure.

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ABSTRACT

The conservation of endangered species is best achieved by studies that consider both ecological and evolutionary aspects of population viability. Integrative research of this type is especially important for amphibian species as they have been plagued by rapid, global declines and extinctions in recent decades. The aims of this dissertation were to contribute to the conservation of two such declining amphibians, the Panamanian golden frogs, *Atelopus varius* and *A. zeteki*, as well as demonstrate the combined utilities of morphological, ecological, and genetic analyses in providing an integrative framework for informing wildlife conservation initiatives. The results of this research engender several important considerations for the conservation of these critically endangered and culturally important amphibians. In my first study, multivariate phenotypic and mitochondrial DNA analyses were used to verify that previously defined management units are supported by concordant patterns of variation among populations as well as to identify phenotypic traits that may be associated with adaptive divergence. The second study examined the contributions of alternative landscape factors to patterns of genetic variation among golden frog populations, highlighting the importance of maintaining the integrity of low slope areas, such as riparian habitat corridors and mountain ridges, for gene flow. The third and final study demonstrated the importance of genetic and phenotypic variability in allowing golden frog populations to adapt to environmental

change by examining the relationship between thermoregulation behavior and susceptibility to a fungal epidemic. Here I demonstrated that the behavioral fever response mounted by one golden frog population is effective in reducing the odds of infection during a chytridiomycosis epidemic. I concluded by summarizing the implications of this work for the management of Panamanian golden frogs and considering the applicability of these findings to the conservation of other endangered species of *Atelopus*.

CHAPTER I

INTRODUCTION

Conservation biology is truly a multidisciplinary science in that it focuses the knowledge and tools of all biological disciplines, from molecular biology to population biology, on the conservation of biodiversity (Soulé & Wilcox 1980). However, to be effective, there must be a high level of integration among the separate fields as each offers unique insight into the processes affecting viability (Clarke 2000). Decisions based on just one or few lines of inquiry run the risk of overlooking factors critical to effective management, especially when faced with high levels of intraspecific variation.

The need for such integrative studies in the conservation of amphibian species is especially poignant as amphibian populations have undergone rapid, global declines and extinctions in recent decades, and many of the most threatened species are also little known (Pounds 2001; Stuart *et al*. 2004; Wake 1991; Young 2001; Young *et al.* 2004). Over 30% of all amphibian species are now considered threatened, and as many as 122 species may have become extinct (Stuart et al. 2004). In many cases, this can be attributed to threats amphibians share with other taxa, including habitat destruction and fragmentation, introduction of predators and competitors, pesticides, acid precipitation, increased ultraviolet irradiation, exploitation by humans, and global climate change

(Berger 1998; Lips 1998; Pounds & Crump 1994; Young 2001). However, the declines and extinctions of as many as 200 frog species across the globe appear to have been caused by a newly-emerged, fungal pathogen called *Batrachochytrium dendrobatidis* (Skerratt *et al.* 2007). This fungus infects the keratinized layers of an amphibian's skin, causing a potentially fatal disease called chytridiomycosis (Longcore *et al*. 1999). In Central America, a north-to-south epidemic wave of this disease has been linked to declines and mass die-offs, resulting in dramatic losses of amphibian biodiversity (Lips *et al.* 2006).

Arguably the most imperiled of all amphibian taxa is the Neotropical genus *Atelopus*. Thus far, 62 of 77 described species (81%) have been classified by the IUCN as extinct or critically endangered (La Marca *et al.* 2005, Pounds *et al.* 2006; Lötters 2007) with declines and extinctions having been attributed to the frogs' extreme sensitivity to environmental perturbations (Lötters 1996), chytridiomycosis, and global warming (Pounds *et al.* 2006). In Panama, two species of golden frogs (*Atelopus varius* and *A. zeteki*) are revered as national icons, but unfortunately are also in critical danger of extinction due to these same threats in addition to over-collection for the illegal pet trade (Zippel 2002; IUCN 2004). While *A. varius* was historically found throughout much of montane Costa Rica and western Panama (Savage 1972), it has disappeared from most of its range (Lips 1998; Pounds and Crump 1994; Zippel *et al.* 2006). *Atelopus zeteki* has also gone missing from parts of its range, which included the area in and around an extinct volcanic crater at El Valle de Anton, Panama (Dunn 1933; Zippel *et al.* 2006). My goals in undertaking this dissertation were to contribute to the conservation of Panamanian golden

frogs (*Atelopus varius* and *Atelopus zeteki*) as well as demonstrate the combined utilities of morphological, ecological, and genetic analyses in providing an integrative framework for informing wildlife conservation initiatives.

At the inception of this dissertation, golden frogs from three Panamanian populations were already being maintained in captivity in U. S. zoos as a precautionary measure against extinction as the chytridiomycosis front grew ever closer (Zippel 2002). However, the amazing morphological and ecological variation among *Atelopus* populations in Panama and the lack of a phylogeny for the group left the taxonomy of these and other populations of golden frogs poorly understood. In addition, little was known about the potential range of the pathogenic fungus, *B. dendrobatidis,* and how the outcome of a chytridiomycosis epidemic might differ across ecologically diverse golden frog populations.

Members of Project Golden Frog, a conservation group whose goal is to prevent the extinction of these culturally significant and endangered amphibians, chose the three golden frog populations for captive management because among them they represented a wide range of phenotypic variation. They also divided the extant golden frog populations into five evolutionarily significant management units (ESUs) based on qualitative descriptors of phenotypic variation (Zippel *et al.* 2006). However, little was known about the genetic relationships among these captive populations and ESUs. In Chapter II, I test the extent to which these ESUs exhibit genetic variation concordant with their phenotypic variation using information from mitochondrial DNA sequences and multivariate

phenotypic analyses. It is my hope that this information will aid in prioritizing management efforts so as to maximize the ability of golden frogs to survive and adapt to their ever changing natural environment.

In Chapter III, I continue to examine the interplay between ecological and evolutionary factors in shaping golden frog diversity by quantifying the extent to which the genetic variation among populations is shaped by several alternative landscape factors. Specifically, the relative influences of (1) riparian habitat corridors, (2) changes in elevation, and (3) climatic suitability on patterns of genetic structure were compared using a causal modeling framework. One of the major goals of this study was to identify specific barriers and/or corridors for gene flow and allow for informed predictions of the effects of alternative management strategies on population connectivity. This information will also be important to consider should the decision some day be made to re-introduce captively bred golden frogs to the wild.

In chapter IV, I shift my focus from understanding and maintaining the functionality of processes generating variation among golden populations to an in-depth look at the role of individual behavior in shaping susceptibility to *B. dendrobatidis* infection during an epidemic. Using a combination of mark-recapture and diagnostic assays for *B. dendrobatidis*, I investigate whether and how the progression of a chytridiomycosis epidemic can be affected by interactions between the genotypes of both the pathogen and its amphibian hosts and the environment in which they are found.

And finally, in chapter five I conclude by summarizing the implications of this dissertation for the conservation of Panamanian golden frogs and consider the applicability of these results to other endangered species of *Atelopus*. I then outline some important areas for future research in the endeavor to prevent the loss of this amazingly diverse and beautiful amphibian lineage.

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CHAPTER II

TESTS OF PHENOTYPIC AND GENETIC CONCORDANCE AND THEIR APPLICATION TO THE CONSERVATION OF PANAMANIAN GOLDEN FROGS

Abstract

Evolutionarily significant units (ESUs) differ in the extent to which they capture, or even consider, adaptive variation, and most such designations are based solely on neutral genetic differences that may not capture variation relevant to species' adaptabilities to changing environmental conditions. While concordant patterns of divergence among datasets (i.e., neutral and potentially non-neutral characters) can strengthen ESU designations, determining whether such criteria are met for highly variable taxa is especially challenging. In this study, I test whether previously defined ESUs for endangered Panamanian golden frogs (*Atelopus varius* and *A. zeteki*) exhibit concordant variation among multiple phenotypic traits and mitochondrial DNA sequences, and the extent to which such divergence corresponds to environmental differences. Multivariate analyses identify phenotypic and genetic differentiation consistent with proposed ESUs and support the status of *A. varius* and *A. zeteki* as separate species. Moreover, the significant association detected between ESU co-membership and genetic similarity, which remained strong after removing the effect of geographic distance, also indicates that genetic differences are not simply due to isolation by distance. Two phenotypic characters (body size and the extent of dorsal black patterning) that differ among ESUs

also covary with environmental differences, suggesting that to the extent that these phenotypic differences are heritable, variation may be associated with adaptive divergence. Lastly, discriminant function analyses show that the frogs can be correctly assigned to ESUs based on simultaneous analysis of multiple characters. The study confirms the merit of conserving the previously proposed golden frog ESUs as well as demonstrates the utility and feasibility of combined analyses of ecological, morphological and genetic variation in evaluating ESUs, especially for highly variable taxa.

Introduction

In the endeavor to protect biological diversity, the field of conservation biology also contends with the challenge of preserving the functionality of evolutionary processes necessary for sustaining it in the face of anthropogenic changes (Moritz 2002; Monsen & Blouin 2003). Defining evolutionarily significant units (ESUs) – the intraspecific units meriting separate conservation (Ryder 1986) – is central to achieving these goals. However, the identification of ESUs is far from straightforward and has been the subject of much debate (Avise 1989; Moritz 1994; Grant 1995; Bowen 1999; Dimmick et al. 1999; Paetkau 1999; Taylor and Dizon 1999; Crandall et al. 2000; Green 2005). Consequently, the extent to which such units consider adaptive variation (Waples 1991; Bowen 1999; Crandall et al. 2000) in addition to patterns of neutral-genetic variation (Avise 1989; Moritz 1994; Dimmick et al. 1999) differs substantially.

Independent characters, such as neutral genetic haplotypes and heritable phenotypes (e.g., Rising and Avise 1993; Ryan and Bloomer 1999; Friesen et al. 2006; Gharrett et al. 2006; Gompert et al. 2006) each provide a separate historical record of population divergence and are expected to show concordant variation if they share a history of divergence (Ryder 1986; Avise 1989; Grady & Quattro 1999). Analysis of such concordant variation provides an evolutionary framework that affords insight into the processes shaping variation and is therefore instrumental in developing conservation strategies to not only sustain viable populations but also maintain the context for selection in the face of a changing environment (Ryder 1986; Dizon et al. 1992; Vogler et al. 1993; Avise 1989; Paetkau 1999; Crandall et al. 2000; Moritz 2002). Irrespective of whether preserving adaptive variation is a significant consideration for a particular conservation initiative, ESU-designations based on multiple characters reduce the risk of being inaccurate. For example, ESUs diagnosed using the single criterion of genetic distinctiveness (which is often based on analyses of mitochondrial DNA) may not be accurate, as recent divergence (Lorenzen et al. 2006), large effective population sizes (Paetkau 1999), or introgression (Gompert et al. 2006) can slow the formation of genetic structure and therefore bias conservation assessments. Likewise, evolutionary relationships and divergence arising from historical processes may not be represented if ESUs are diagnosed solely from phenotypic characters subject to intense selection or that exhibit non-heritable variation (Avise 1989; Grady & Quattro 1999). Additionally, multivariate analyses that reveal concordance among multiple character sets reduce the potential for taxonomic ambiguities, which arise more often when decisions are based on single characters (Avise 1989; Grady & Quattro 1999; Paetkau 1999).

Tests of concordance across multiple independent characters are especially important for highly variable species with poorly understood taxonomy (e.g., long-billed larks: Ryan & Bloomer 1999, ciscoes: Turgeon & Bernatchez 2004, and Galapagos petrels: Friesen et al. 2006). When faced with this situation for endangered species, the additional urgency emphasizes the importance of designating ESUs that capture variation essential to population persistence as species have to contend with a variety of challenges, ranging from habitat loss and fragmentation to climate change and disease. In this study, I use multivariate analyses of character concordance to evaluate the adequacy of ESUs previously proposed for conserving the Panamanian golden frogs (*Atelopus varius* and *A. zeteki*) (Zippel 2002). These frogs show a great deal of morphological, ecological, genetic, and demographic variation among geographically proximate populations in western Panama. The species are also critically endangered (IUCN et al. 2004) due to habitat loss and fragmentation, illegal collection for the pet trade, and chytridiomycosis, a fungal epizootic. Patterns of phenotypic and genetic differences among golden frog populations are examined in a quantitative framework, using both single and multivariate analyses, to assess the level of concordance among characters as well as the level of support for five previously proposed ESUs (Zippel et al. 2006) based on qualitative descriptors of phenotypic variation (Fig. 2.1).

In contrast to examples in which species are phenotypically homogeneous but genetically differentiated (jellyfish: Holland et al. 2004, king weakfish: Santos et al. 2006), or phenotypically diverse yet genetically undifferentiated (poison dart frogs: Summers et al.

2004, Sticklebacks: Taylor & McPhail 1999, hamlets: McCartney et al. 2003), the genetic and phenotypic divergence among Panamanian golden frogs is concordant, with populations exhibiting significant differentiation in both. However, the magnitude of divergence differs among phenotypic traits and with the amount of genetic differentiation. This study highlights the profound effect that taxonomy, character choice and the choice of a single versus multiple character approach can have on the interpretation of patterns of variation and their implications for developing conservation priorities. In addition to contributing to our understanding of the processes underlying variation in golden frogs relevant to their conservation, this study demonstrates the general applicability of analysis of molecular variance (AMOVAs) and Mantel tests in (a) identifying ESUs based on the level of concordance among genetic and phenotypic variation and (b) assessing support for alternative evolutionary and ecological mechanisms in the generation of phenotypic variation.

Materials and Methods

Toe-clip tissue samples, photographs, and snout-vent lengths (SVLs) were collected for *A. varius* and *A. zeteki* from populations throughout their extant ranges in Panama (Fig. 2.1); this sampling included 2 to 4 populations per ESU. All animals were captured by hand and released at the point of capture within five minutes of data collection. While *A. varius* was historically found throughout much of montane Costa Rica and Panama, by the time of this study its extant range had been reduced to the area sampled here.

Genetic data

Tissues were preserved in a salt-saturated DMSO and EDTA solution. A total of 141 individuals were sequenced including frogs from 15 populations (Fig. 2.1). Four outgroup specimens were also used: three *A. varius* from Monteverde, Costa Rica (MVZ Herp 149729 and 164816-7) and one *A. senex* from near Volcan Barba, Costa Rica (MVZ Herp 149735). Due to the huge morphological variation seen among *Atelopus* populations in Central America and the lack of a phylogeny for the group, the taxonomy of these species is not well understood. The *A. varius* samples from Monteverde were included in the outgroup as they were 5-6% divergent from any of the ingroup haplotypes and are likely to represent a distinct species. This population is as divergent from Panamanian *A. varius* as are populations of *A. senex* from Costa Rica, and in fact, the Monteverde *A. varius* samples are only 0.5 – 1.2% divergent from *A. senex*, indicating taxonomic confusion for these records. Genomic DNA was isolated using a QIAGEN DNeasy kit according to the standard protocol for animal tissue and 10µL of extracted DNA was added to 20µL of GeneReleaser® (BioVentures) according to the general protocol to remove PCR inhibitors. A 755 base pair fragment of CytB and 630 base pairs of COI was sequenced from the mitochondrial genome using primers COIfXenL and COIfXenH (see Palumbi et al. 1991) for COI and MVZ25-L (see Moritz et al. 1992) and ControlW-H (see Goebel et al. 1999) for CytB. Twenty-five µL PCR reactions with an initial denaturing at 94°C for two minutes (min) followed by 35 cycles of 30 seconds (sec) denaturing at 94°C, 45 sec annealing at either 60°C for CytB or 45°C for COI, and 30 sec extension at 72°C were used. PCR-products were cleaned with a QIAquick kit prior to automated sequencing.

Phenotypic and environmental data

Digital photographs of the dorsal and ventral surfaces, as well as body size (SVL) measurements were taken in the field for 213 adult male frogs from the same 15 populations mentioned above (Fig. 2.1). Only adult males were measured because body size is a sexually dimorphic trait in this species. To quantify individual color variation, the color and contrast values among digital photographs were standardized by adjusting midtones to a target value using the "auto color" command in Adobe© Photoshop© CS version 8.0. Yellow and black colors were characterized with Image-Pro Discovery version 4.5 (Media Cybernetics©) by averaging the RGB (red, green, blue) color values from three random points on the frog's dorsal surface. Individual variation was summarized with a principal components analysis (PCA). PCAs of averaged R, G, and B values extracted single significant components and for both yellow and black colors and the R, G, and B values all contributed significantly to the Z-scores (z-yellow = 0.744R + 0.896G + 0.807B and z-black = 0.890R + 0.983G + 0.881B). The z-scores for the significant components of yellow and black dorsal colors and two other phenotypic traits - the percentage of black patterning on the dorsal surface of each frog (as quantified with Image-Pro Discovery) and the SVL (as measured in the field with dial calipers) – were analyzed in both univariate and multivariate frameworks (see below). Color and pattern variables as well as body size were chosen for analysis because they are (1) highly variable among populations and (2) potentially adaptive. Because golden frogs are highly toxic, their black and yellow coloration is likely aposematic and an adaptation to avoid predation. Body size differences may also represent adaptations to variation in climate throughout their range.

Elevation and locality data for each population was measured using a hand-held GPS unit. Climate data were extracted for each locality with the program DIVA-GIS (version 5.2: www.diva-gis.org), using values from WorldClim interpolated climate layers (apx. 1-km spatial resolution). These layers were developed from monthly climate measurements compiled from weather stations around the globe for the years 1950-2000 (Hijmans et al. 2005). We used the program to extract the climate values for each locality given its geographic coordinates. Nineteen bioclimatic variables reflecting annual trends in temperature and precipitation, seasonality, and extreme environmental factors (e.g. temperature of the coldest month) were used (see www.worldclim.org/bioclim.htm for detailed descriptions of individual variables). Variation in the bioclimatic variables and elevation among populations was summarized using PCA, resulting in three composite climate variables (hereafter referred to as bioclim 1-3, Table 2.1).

Statistical analyses

Because of taxonomic uncertainty (see below), all statistical analyses were conducted treating the putative species, *A. varius* and *A. zeteki*, separately, as well as considering them as a single taxon. Pronounced variation within *A. zeteki* and *A. varius* and problems with the specimens and characters used historically to diagnose the two species raise uncertainty about their taxonomic status. The original description of *A. zeteki* as a subspecies of *A. varius* was based on differences in coloration, color patterning, and body size of frogs from El Valle, Panama (Dunn 1933). However, this designation was not based on a comparison with Panamanian *A. varius*, but instead *A. cruciger* from

Venezuela and a specimen of unknown taxonomy from Colombia (Lötters 1996). Differences in skin toxins (Villa et al. 1988) and male calls (Cocroft et al. 1990) were later used to elevate *A. zeteki* from a subspecies to a species. However, subsequent studies have shown that the toxin which was supposedly unique to *A. zeteki* is also found in *A. varius* (Yotsu-Yamashita et al. 2004). Furthermore, the bioacoustic differences of Cocroft et al. (1990) actually represent intraspecific differences in *A. varius* calls as their "*A. zeteki*" specimen was in fact from a population of *A. varius* (Zippel et al. 2006). Further studies are needed to determine whether the two nominal species can be differentiated by call.

Genetic data. Phylogenetic trees were estimated using maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference with the sequence data from the two mitochondrial genes concatenated into a single haplotype for each individual (GenBank Accession numbers for CytB and COI: EF494908 - EF494999). MP and ML analyses were performed in PAUP*4.0b10 (Swofford 2002) using 500 random-stepwise heuristic searches with tree-bisection-reconnection branch-swapping and branch support was assessed using 1000 and 100 nonparametric bootstrap replicates for MP and ML, respectively. An HKY + Γ model (Hasegawa et al. 1985) was selected as the best fit model (π_A = 0.26842, π_C = 0.24303, π_G = 0.14292, π_T = 0.34564, t-ratio = 6.624, α = 0.2226, rate categories = 5) using DT-ModSel (Minin et al. 2003) and used for ML and Bayesian analyses. Four chains of 10^7 generations with trees saved every 10^3 generations were used for the Bayesian analysis after a burn in of 10^4 generations (MRBAYES 3.0b4:

Huelsenbeck and Ronquist 2001). Net sequence divergence (Nei 1987) was estimated using DnaSP ver. 4.10.

Analyses of molecular variance (AMOVA) with individuals nested by (a) population and (b) the proposed ESUs were performed in ARLEQUIN 2.0 using 1000 permutations, and a Kimura 2-Parameter distance matrix with $\Gamma = 0.2226$. Mantel tests were used to test for correlations between (a) log-transformed genetic (F_{ST}-values, the dependent variable, or DV) and straight-line geographic distances (the independent variable, or IV) (Slatkin 1993), and (b) log-transformed genetic distances (DV) and an indicator matrix (0,1) (IV) that designates populations as members of the same (0), or different (1) ESUs. These tests were performed following the procedure of Legendre and Legendre (1998) in IBD version 1.5 (Bohonak 2002) with 10^4 randomizations. Partial-Mantel tests were used to assess correlations between (a) log-transformed genetic (DV) and geographic distances (IV) while controlling for ESU membership (DV), and (b) log-transformed genetic distance (DV) and ESU membership (DV) while controlling for geographic distance (IV).

Phenotypic variation. To test whether and how individuals from the five proposed ESUs and two putative species differ in phenotype, individual variables encompassing four morphological aspects of phenotype were analyzed separately, as well as jointly. As variances were highly unequal across ESUs and species, Kruskal-Wallis tests (nonparametric alternative to ANOVA) were used to compare means and nonparametric multiple comparison tests (Dunnett's C) were used to determine which groups differed significantly for a particular phenotypic character. Multivariate discriminant function

analyses (DFAs) were used to determine the extent to which individual frogs can be differentiated according to ESU (we did not use DFAs to test for statistical significance of phenotypic differences among ESUs because the covariance matrices were unequal and this could affect *P*-values, see Manly 2005). Cross-validation of ESU classifications was accomplished by classifying each individual by the functions derived from all others (leave-one-out classification) and prior probabilities for each ESU were set proportionally.

To assess the relative support for alternative causes of variation among golden frog populations, pairwise Mantel tests were performed among genetic, geographic, morphological, and environmental variables. Correlations between (a) morphological (DV) and environmental (IV) phenotypic variables and (b) phenotypic variables (DV) and genetic distance (pairwise F_{ST}-values, IV) were investigated using Mantel tests in the Microsoft Excel© 2000 PopTools add-in (Hood 2002). Because pairwise Mantel tests were used to screen for potential correlations rather than test specific hypotheses about the relationships between pairs of variables, significance levels were not adjusted for multiple hypothesis testing (see Nakagawa 2004 and Roback & Askins 2005). Least-square linear regressions (run in SPSS, ver. 14.0) were then used to test for correlations among pairs of variables with significant Mantel tests and generate hypotheses about their causes that could be tested in the future.

Results

Genetic variation

Two strongly supported clades (Fig. 2.2) corresponding generally to populations of *A. varius* and *A. zeteki* were recovered in each analysis (MP, ML and Bayesian). For the MP analysis, one hundred twenty-six of the 1385 characters were parsimony informative and 304 equally parsimonious trees of 213 steps were found. The two clades are separated by 3.32% net sequence divergence (Nei 1987, equation 10.20). All but 3 populations were restricted to one of the two clades – individuals from one *A. zeteki* population (A) and two *A. varius* populations (G and N) are distributed across both clades, although haplotypes from the El Valle clade were rare - 7 of the 43 individuals from these populations carried haplotypes from the El Valle clade (Figs. 2.1-2.2). Repeated DNA extraction and re-sequencing confirmed that these populations were indeed composed of a mixture of haplotypes from the two clades. Sixty unique mitochondrial haplotypes, 49 of which were population-specific, resulted from the 141 individuals sequenced, excluding outgroups (Table 2.2).

Genetic structure was also revealed by the F_{ST}-analyses, with significant F_{ST}-values observed among the majority of pairwise population comparisons (Table 2.2), including geographically proximate ones. For example, F_{ST}-values were significant between populations F and J despite being separated by only 1.5 km (Fig. 2.1). The AMOVAs showed significant hierarchical structuring of genetic variation as well (Table 2.3). When considered as higher groupings, both ESUs and species (*A. varius* vs. *A. zeteki*) explained a significant partitioning of genetic variance among populations (Table 2.3a, b).

However, the effect of ESU groupings on genetic structure was not consistently significant when the species were analyzed separately – ESU groupings explained a significant amount of genetic variance in *A. varius* (Table 2.3c) but not *A. zeteki* (Table 2.3d).

Genetic and geographic distances were correlated in each species (*A. varius* Mantel: $r = 0.47$, $P = 0.002$; *A. zeteki* Mantel: $r = 0.817$, $P = 0.04$). This pattern of isolation by distance was also significant if the taxa are analyzed as representing a single species (Mantel: $r = 0.56$, $P < 0.005$), even after controlling for correlations associated with ESU membership (partial-Mantel: $r = 0.37$, $P = 0.005$). The general pattern of isolation by distance is also observed in analyses of the separate species after correcting for ESU membership; while the pattern remains significant in *A. zeteki* when considered alone, the relationship is not significant in *A. varius* after controlling for correlations associated with ESU membership (partial-Mantel: $r = 0.79$, $P = 0.043$, and $r = 0.25$, $P = 0.062$, respectively).

Examining the genetic similarity of individuals from the same ESUs, compared to individuals from different ESUs, there is a significant relationship between genetic distance and ESU membership when all five ESUs are considered together (Mantel: $r = 0.52$, $P < 0.005$), even after the effect of geographic distance is removed (partial-Mantel: $r = 0.29$, $P = 0.007$). In analyses of the species separately, the correlation between genetic distance and ESU membership was only significant in *A. varius* (Mantel: $r = 0.57$, $P <$

0.005; partial-Mantel: $r = 0.454$, $P = 0.007$ with the effect of geographic distance removed, whereas in *A. zeteki* the correlation was Mantel: $r = 0.363$, $P = 0.099$).

Phenotypic and environmental variation

Analyses of the five ESUs detected significant differences in the means of each of the four morphological variables (SVL, % black, z-yellow and z-black, Kruskal-Wallis: $\chi^2_4 \geq 17.37$, $P \leq 0.002$). Pairwise comparisons to identify which ESUs differed significantly from each other revealed that the mean phenotypes did not differ among all the ESUs (Dunnett's C, Table 2.4). For example, the extent of black patterning on the dorsum (% black) differed in 9/10 pairwise comparisons and body size (SVL) differed in 8/10 comparisons. Surprisingly, the pairs of ESUs that did not show significant differences in these two phenotypic variables (ESUs 2 & 3 for the black trait, and ESUs 1 & 3 and 1 & 4 for SVL) are found in different mtDNA clades (one from *A. zeteki* clade and the other from *A. varius* clade, see Figs. 2.1- 2.2). These ESUs also exhibit significant differences in the other morphological variables, suggesting that the similarities in the extent of black patterning on the dorsum and body size are likely due to convergence.

Comparisons between the species also demonstrated a significant difference in two morphological variables, body size (SVL, Kruskal-Wallis: $\chi^2_1 = 8.85$, $P = 0.005$) and extent of black patterning on the dorsum (% black, Kruskal-Wallis: $\chi^2_1 = 24.69$, $P < 0.001$), but not in the other two morphological traits (z-yellow and z-black). Comparing the mean phenotypic variables among the ESUs in the separate species showed that ESUs in *A. varius* (ESUs 3-5, Fig. 2.1) differ in all four morphological variables (SVL, %

black, z-yellow, z-black, Kruskal-Wallis: $\chi^2_2 \geq 14.27$, $P \leq 0.001$) whereas the ESUs in *A. zeteki* (ESUs 1-2, Fig. 2.1) differ in all morphological variables (SVL, % black, and z-yellow, Kruskal-Wallis: $\chi^2_1 \geq 5.24$, $P \leq 0.022$) except black coloration (z-black, Kruskal-Wallis: $\chi^2_2 = 0.612$, $P = 0.434$).

Discriminant functions analyses (DFAs) showed that the morphological variables (SVL, z-yellow and z-black color values, and % black patterning, or % black) were moderately successful in discriminating among the five proposed ESUs with 77.5% of frogs being classed correctly (Table 2.5a). The majority of the mis-classifications (33/42) involved frogs assigned to an ESU in the opposite species. The first canonical discriminant function (CDF), which explains 61.8% of the variance, is strongly influenced by the extent of black patterning on the dorsum (% black) indicating the importance of this variable in discriminating among ESUs. Body size (SVL) was most strongly correlated with the second CDF (explaining 28% of the variance) and the color values (z-yellow and z-black) were most strongly correlated with the third and fourth CDFs (together explaining 10.2% of the variance). The DFA based on the same morphological variables also successfully discriminated among the two species - 77.1% of frogs were classed correctly as either *A. zeteki* or *A. varius* (Table 2.5b). For this model, only one CDF was extracted, which explains 100% of the variance and is most strongly influenced by % black. Separate DFAs of the two species were each very successful at classifying individuals to their respective ESUs; 91.6% of frogs were classed correctly to ESUs within *A. varius* (Table 2.5c), and 91.3% of frogs were assigned correctly to ESUs in *A. zeteki* (Table 2.5d).

Correlations among ecological, morphological, and genetic variation.

Pairwise comparisons across all populations showed that the magnitude of divergence in genetic, environmental and morphological variables differed (Table 2.6a). Only two of the morphological variables, SVL and % black, were correlated with environmental variables (Table 2.6a). The correlations between SVL and extent of black patterning with bioclim 1 and 2, respectively, and not genetic divergence, suggests that ecogenetic rather than phylogenetic factors account for these phenotypic population differences. Following these results, linear regressions revealed significant negative relationship between SVL and mean annual temperature ($r^2 = 0.282$, $P < 0.001$) and between % black and mean annual precipitation ($r^2 = 0.104$, $P < 0.001$).

The same relationship between SVL and mean annual temperature was also apparent in pairwise comparisons among populations of *A. varius* and *A. zeteki* considered separately, although the association was not as strong in *A. zeteki* (Table 2.6b and c). No significant correlations were found between genetic, morphological, or environmental variation among *A. zeteki* populations, which may reflect the smaller number of populations sampled (five versus 10 populations in *A. zeteki* and *A. varius*, respectively). Linear regressions of SVL and mean annual temperature remained significant in each species (*A. varius*: $r^2 = 0.250$, $P < 0.001$; *A. zeteki*: $r^2 = 0.267$, $P < 0.001$); the regression of % black and mean annual precipitation was also significant in each species (*A. varius*: $r^2 = 0.397$, $P < 0.001$; *A. zeteki*: $r^2 = 0.150$, $P < 0.001$).

Discussion

The analyses confirm that there are not only significant genetic differences among the ESUs (Tables 2.2 and 2.3), but also significant divergence in morphological variables among the ESUs sufficient for discriminating ESU membership of individual frogs (Table 2.5). These conclusions were robust when each species was analyzed separately, with the exception of *A. zeteki* which lacked significant genetic differentiation among ESUs. This lack of genetic structure most likely reflects the large amount of variation within population A, which contains highly divergent haplotypes characteristic of both species (Figure 2.2). Despite the taxonomic uncertainty surrounding these endangered species (which was the rationale for conducting the analyses across the five ESUs together, as well as within each species separately), the species themselves are also clearly differentiated based on genetic (Fig. 2.2 and Table 2.3) and morphological data (Table 2.5). The results clearly support the taxonomic status of *A. varius* and *A. zeteki* and the proposed ESUs exhibit both morphological and genetic differences (as detected from significant differences in frequency of haplotypes; reciprocal monophyly was not observed, but it would also not be expected for recently diverged ESUs and species, as discussed below). However, a general lack of correspondence in the magnitude of divergence among variables suggests that multiple processes are contributing to the observed differentiation. Possible explanations for the patterns of correlated change (and lack thereof) among genetic, phenotypic, and environmental variables, and potential implications for the conservation of the endangered Panamanian golden frogs are discussed below.

Patterns of differentiation and implications for the maintenance of variation in ESUs

Analyses of covariation among the genetic and phenotypic data revealed some complicated patterns across ESUs. The large amount of phenotypic differentiation observed between populations, ESUs, and species was apparent to varying degrees in the mtDNA phylogeny, the pairwise population F_{ST}-values, and AMOVAs. This genetic structure, given the fine geographic scale of this study, suggests the frogs have remained fairly isolated. For example, among six populations of *A. varius* separated by less than 10 km (populations F-J, Fig. 2.1), there are 30 haplotypes represented, 17 of which are exclusive to a single population (Table 2.2). Moreover, significant isolation-by-distance is also apparent across populations, again suggesting the importance of limited gene flow in maintaining variation in the frogs. Nevertheless, when the effect of geographic distance was controlled for (through the use of partial-correlation analyses), the ESUs remained genetically distinguishable (i.e., individuals from the same ESU were genetically more similar compared to individuals from different ESUs). This was true irrespective of whether all five ESUs were considered together or analyzed separately in the two species ($P < 0.05$, except for *A. zeteki* ESUs where fewer populations were compared and the relationship between ESU co-membership and genetic similarity was non-significant, $P = 0.099$). This again would support their long-term isolation, although it suggests that factors other than limited gene flow owing to the geographic distribution of populations may have contributed to the observed differentiation. The concordant boundaries of differentiation between the genetic and phenotypic data among ESUs and between species highlight the fact that patterns of differentiation across multiple characters have been strongly influenced by a common history. Yet the absence of a

strong correspondence between the degree of divergence in genetic and morphological variables (Table 2.6) suggests factors other than geographic constraints on gene flow contribute to the maintenance of variation in ESUs (see below).

Processes underlying divergence as revealed by consideration of multiple characters

In addition to avoiding the potential pitfalls involved with diagnosing conservation units based on neutral genetic data alone (Paetkau 1999; Gompert et al. 2006; Lorenzen et al. 2006), incorporating data from phenotypic traits is also integral if such units are to capture variation relevant to a species ability to contend with various threats through adaptive change. Two implicit assumptions when ESUs are defined from patterns of phenotypic variation are that the variation (1) is largely heritable (as opposed to environmental) and (2) is adaptive (rather than a consequence of drift or selection on a genetically correlated trait). Estimates of trait heritability require measurements of individuals with a known pedigree in a setting, such as a laboratory or common garden, where environmental factors can be controlled. Explicit tests that individuals with the putatively adaptive trait exhibit greater survival or reproduction, compared with those lacking the trait, are then necessary to provide direct evidence for the adaptive significance of the character. Such explicit tests are often unrealistic for ESU studies as time, resources, and access to animals are often limited. It is therefore no surprise that studies may rely on the untested assumption that phenotypic traits are both heritable and adaptive (e.g., Turgeon & Bernatchez 2003).

Intraspecific phenotypic variation falls into two distinct classes: ecogenetic and phylogenetic (Rosso et al. 2004; Thorpe et al. 1991). Ecogenetic variation can be generated by selection in response to current or recent ecological factors, as well as by adaptive phenotypic plasticity or selection acting on a genetically correlated trait. In contrast, phylogenetic variation is generated by dispersal and vicariance events experienced by populations historically. However, the relative roles of ecogenetic and phylogenetic factors in generating variation among ESUs are not generally distinguished. Here I used an alternative to explicit tests of heritable adaptive variation that also potentially provides insight into whether such variation reflects ecogenetic and/or phylogenetic factors. Specifically, comparisons of character variation across multiple populations were used to determine whether there is an association between traits and putative selective factors, thereby indicating the potential relevance of the characters to adaptive divergence among ESUs. The significant associations detected between body size and color patterning and environmental factors (Table 2.6) suggest that ESUs in the Panamanian golden frogs capture potentially adaptive variation (see details below). It is noteworthy that other anuran species also exhibit divergence in body size that covaries with ecological factors, such as temperature, elevation, and precipitation (Berven 1982; Camp & Marshall 2000; Nevo 1971, 1973). However, the general concordance of the ESU-boundaries with both patterns of genetic and phenotypic differentiation (Tables 2.3, 2.4, and 2.5), even if the magnitude of divergence in the multiple variables are not necessarily strongly correlated (Table 2.6), demonstrates that historical factors are also important in explaining golden frog variation.

Temperature and body size. In amphibians, body size (SVL) variation often reflects ecological differences among populations (reviewed in Ashton 2002). For golden frogs, body size and temperature are negatively correlated, meaning that smaller bodied frogs are found at higher temperatures. This negative correlation has been found in a number of amphibian species and is considered to be a special case of Bergman's rule, as reviewed by Ashton (2002). While the proximate mechanism (i.e. selection or plasticity) behind this correlation has yet to be studied for golden frogs, other studies showing similar correlations between amphibian adult body size and temperature have attributed them to phenotypic plasticity (Camp & Marshall 2000), selection (Nevo 1971, 1973), or both (Berven 1982) to avoid desiccation. Adult body size in amphibians depends on a number of factors which are likely subject to different levels of selection and degrees of phenotypic plasticity, including time and size at metamorphosis, growth rate pre- and post-maturity, age at maturity, and longevity (Augert & Joly 1993; Friedl & Klump 1997; Hemelaar 1988; Moravec 1990; Rosso et al. 2004; Semlitsch et al. 1988; Smith 1987). Mark-recapture and skeletochronology studies are currently underway to determine whether or how these factors vary among the golden frog populations.

Coloration and genetic variation. Two color variables (z-yellow and z-black) do not show strong support for either ecogenetic or phylogenetic forces in shaping their variation. In contrast, covariation in the extent of black patterning on the dorsum (% black) with environmental differences but not with mtDNA sequence differences (Table 2.6) indicates that the character is affected by ecogenetic, rather than phylogenetic factors. This variable was correlated with precipitation-related climatic variables (see

bioclim 2, Table 2.1) and a post-hoc linear regression revealed that precipitation and % black are negatively correlated so that frogs have less black patterning in areas of high annual precipitation.

Black patterning on the dorsum is an adaptation or plastic response to prevent the harmful effects of UV-B radiation, which for many organisms include immunosuppression and carcinogenic and mutagenic effects (Setlow 1974; Black & Chan 1977; Jagger 1985). Melanin, the black pigment found in amphibian skin, has been demonstrated to reduce the effectiveness of UV-B penetration of amphibian eggs, larvae, and adults (reviewed in Licht & Grant 1997). The amount of UV-B that reaches the earth is affected by a number of factors, including ozone, sunspot activity, latitude and the angle of the sun, which are not likely to vary appreciably over the small geographic range of Panamanian golden frogs. Cloud cover, on the other hand, can vary over small geographic areas and dramatically affects the incidence of UV-B radiation (Bjorn 1989; Wellburn 1994). As areas of high mean annual precipitation undoubtedly experience more cloudy days than areas of low precipitation, the inverse relationship between black patterning and precipitation is consistent with the idea that dorsal black patterning is an adaptation for reducing the harmful effects of UV-B.

Support for the separate taxonomic status of A. varius *and* A. zeteki

All the genetic analyses – pairwise population F_{ST}-values, AMOVAs, and the high degree of exclusivity of haplotypes to populations – consistently indicate that there is significant structuring of variation across populations and ESUs. Partitioning populations and

individuals by species also identified significant genetic differences between *A. varius* and *A. zeteki*; both species and ESU explained a greater amount of genetic variation than did the effect of population within the ESUs (Table 2.3), with the exception of *A. zeteki*, where genetic structuring is strong among populations within an ESU (likely due to the presence of the divergent '*A. varius* haplotypes' in population A of *A. zeteki*, Table 2.3d, Fig. 2.2). In addition to significant genetic differentiation among species, discriminant functions analyses based on multiple phenotypic differences provided accurate discrimination of the species, as well as the ESUs within each taxon (Table 2.5). This discriminatory ability was higher when *A. varius* and *A. zeteki* are considered to be distinct species, as opposed to treating them as a single taxon.

Nevertheless, *A. varius* and *A. zeteki* are not reciprocally monophyletic. The species boundaries correspond generally to two strongly supported clades. However, three populations within the Rio Cocle del Norte drainage contain a mix of haplotypes from both clades (populations A, G, and N). This pattern could have been generated by secondary contact with gene flow among formerly allopatric species, or alternatively, could reflect incomplete sorting of haplotypes among incipient taxa (Knowles 2004). Microsatellite markers are currently being developed to aid in distinguishing among these possibilities.

The lack of reciprocal monophyly in the mitochondrial gene tree itself is not necessarily inconsistent with the separate species status of *A. varius* and *A. zeteki*. Well-established population genetic theory raises significant concerns about the use of genetic thresholds

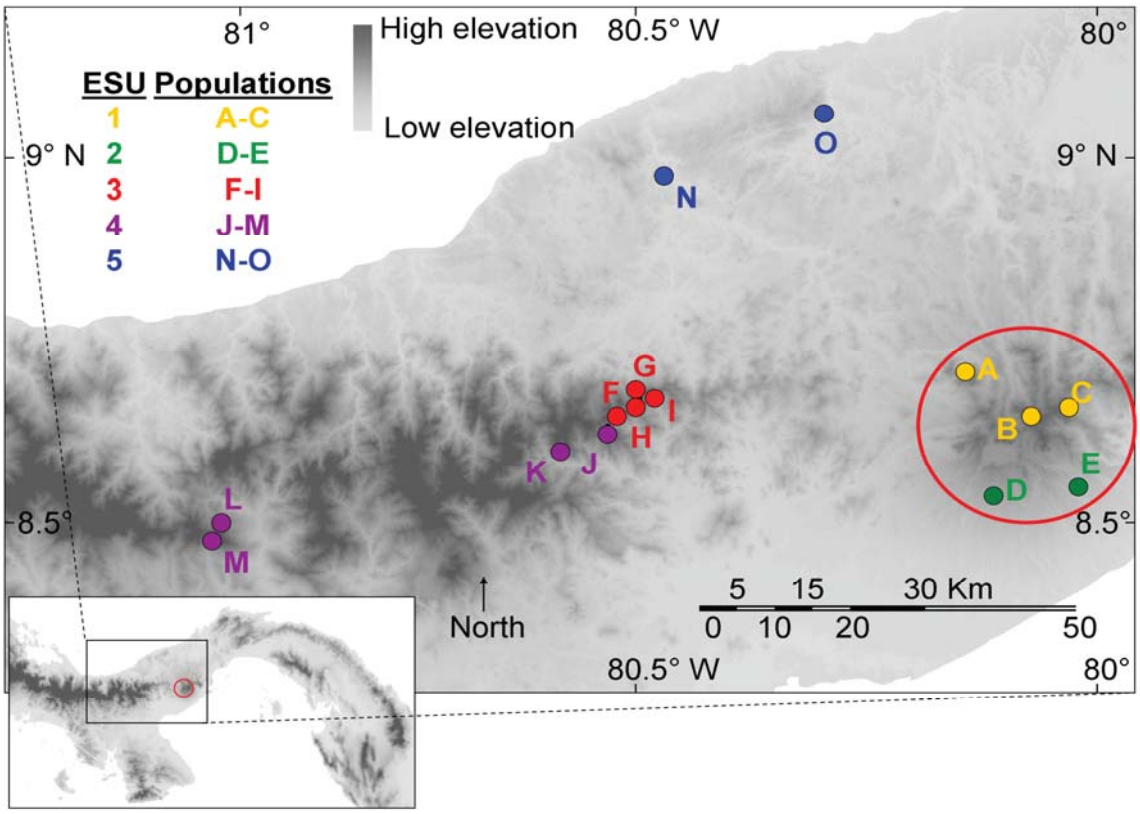
like reciprocal monophyly to delimit species (Takahata & Nei 1985; Hudson & Coyne 2002; Hudson & Turelli 2003; Moritz & Cicero 2004; Matz & Nielsen 2005; Knowles and Carstens 2007a, b). For example, expectations of reciprocal monophyly derived from population genetic theory (Hudson 1992; Wakeley 2006) indicate that a substantial amount of time is required after the initial divergence of species before there will be a high probability of observing reciprocal monophyly at a sample of multiple loci (Hudson & Coyne 2002). Recently derived species will tend to go undiscovered under a reciprocal monophyly criterion since species boundaries are not faithfully reflected in a gene tree until ancestral polymorphism has fully sorted (e.g., Hickerson et al. 2006; Maddison & Knowles 2006; Carstens & Knowles 2007).

Conclusions

This study demonstrates the utility of multiple character analyses, and more specifically tests for concordant variation among characters, in evaluating the support for proposed ESUs, especially in highly variable taxa such as Panamanian golden frogs. While many ESU studies include genetic data, fewer include ecological or morphological data. With the inclusion of such data, not only is it possible to evaluate whether ESUs are likely to preserve adaptive variation, but it also provides a framework to test for concordant variation among phenotypic and genetic variation that may give insights into the process(es) by which such variation has evolved. A general concordance between genetic and phenotypic differentiation among ESUs is evidence of the influence of historical factors on patterns of divergence, and supports the separate species status of *A. varius* and *A. zeteki*. The analyses also identified variation in body size and the extent of dorsal black

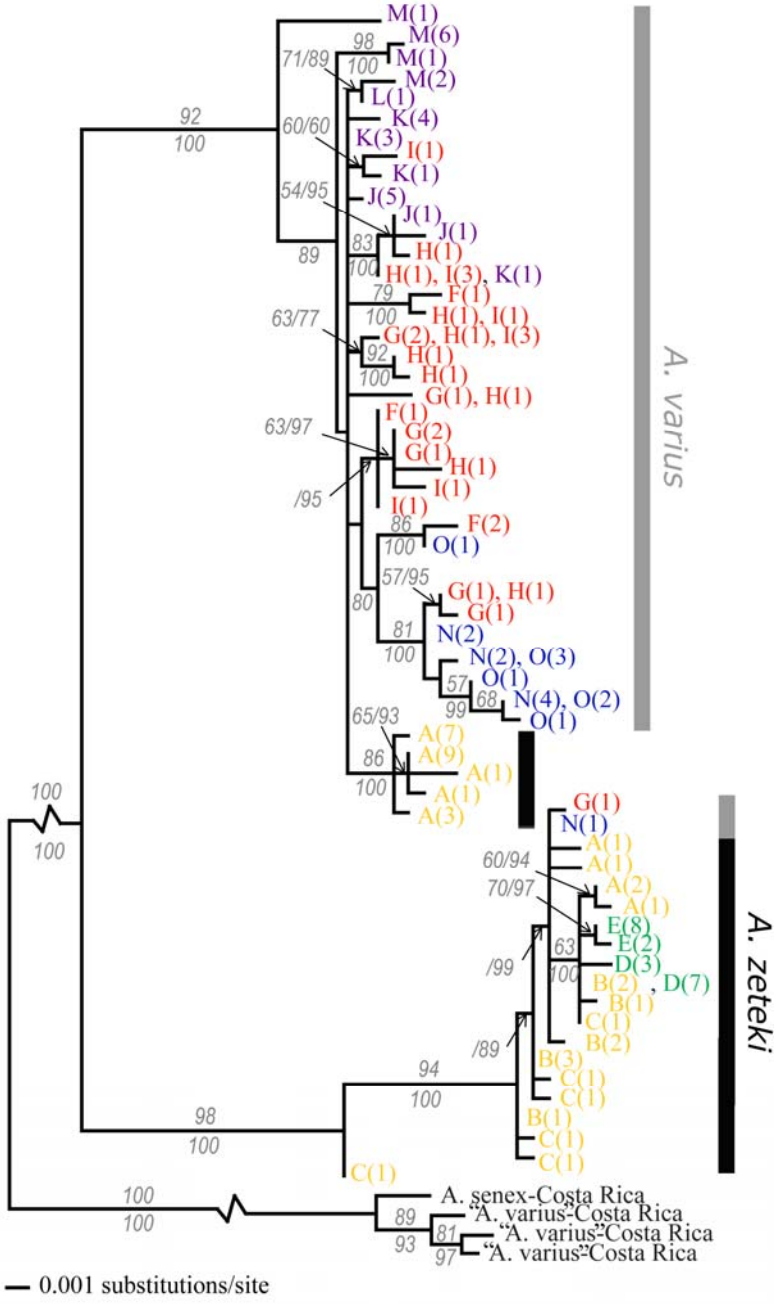
patterning as putative adaptations to climatic factors affecting the likelihood of desiccation and the deleterious effects of UV-B radiation, respectively. Overall, the analyses of multiple characters and tests of character concordance provide strong evidence in support of the conservation of the two critically endangered species, as well as their constituent ESUs.

FIGURE 2.1



Map of Panama (lower left) and enlargement (upper right) showing the approximate locations of populations sampled across the extant range of the golden frogs and proposed ESU membership (following Zippel et al. 2006). The red circle encloses the area of the El Valle crater, the putative range of *A. zeteki*. Populations outside of the circle are within the historical range of *A. varius* (Savage 1972).

FIGURE 2.2



ML tree showing the two clades recovered in all phylogenetic analyses. Haplotypes are colored by ESU and labeled by population in accordance with Fig. 2.1. The number of individuals with a particular haplotype is given in parentheses; colored bars differentiate *A. varius* and *A. zeteki* haplotypes. ML bootstraps are given first followed by Bayesian support values, or are shown above and below the branch, respectively (scores of 50 or less are not reported). Parsimony bootstrap scores (not shown) exceeded ML and Bayesian support values in every case. Haplotypes labeled as "*A. varius*" in the outgroup are from Monteverde, Costa Rica and are more similar to *A. senex* than any ingroup haplotype suggesting taxonomic confusion for these outgroup samples.

TABLE 2.1

Component matrix for climate PCA. Absolute values of component loadings indicate correlations between components (bioclim 1, 2 and 3) and the original variables. Bioclim 1 is strongly influenced by measures of temperature whereas Bioclim 2 and 3 are more strongly influenced by precipitation.

original variable	bioclim 1	bioclim 2	bioclim 3
mean annual temperature	0.935	0.224	-0.265
monthly temperature range	-0.892	0.072	-0.081
isothermality	-0.739	0.417	-0.153
temperature seasonality	0.873	0.285	0.258
maximum temperature of warmest month	0.881	0.320	-0.295
minimum temperature of coolest month	0.947	0.242	-0.206
annual temperature range	-0.841	0.012	-0.063
average temperature of wettest quarter	0.915	0.252	-0.300
average temperature of driest quarter	0.919	0.230	-0.312
average temperature of warmest quarter	0.940	0.240	-0.232
average temperature of coolest quarter	0.922	0.245	-0.285
mean annual precipitation	0.824	-0.264	0.487
precipitation of wettest month	0.220	0.832	0.483
precipitation of driest month	0.595	-0.792	0.096
precipitation seasonality	-0.604	0.783	-0.114
precipitation of wettest quarter	0.442	0.548	0.685
precipitation of driest quarter	0.667	-0.718	0.164
precipitation of warmest quarter	0.851	-0.485	0.159
precipitation of coolest quarter	0.751	0.332	0.466
elevation	-0.913	0.112	0.272

TABLE 2.2

Distribution of mtDNA haplotypes and genetic structure among populations (letters correspond to localities on Fig. 2.1). Number of individuals = N, number of haplotypes = h, number of haplotypes exclusive to that population = (excl.), and pairwise F_{ST}-values are given to the right of the dashed line. Boxes enclose within-ESU pairwise F_{ST}-values; significant values are marked with an asterisk (*), however, only values shown in bold are significant after sequential Bonferroni correction for multiple comparisons. Population L (N = 1, with one exclusive haplotype) was omitted from the matrix due to insufficient sample size; this population had recently declined due to disease and only one frog could be found.

Pop.	N	h (excl.)	A	B	C	D	E	F	G	H	I	J	K	M	N
A	26	9 (9)													
B	9	5 (5)	**0.689***												
C	9	6 (5)	**0.687***	0.063											
D	10	2 (1)	**0.704***	**0.421***	**0.642***										
E	10	2 (2)	**0.711***	**0.613***	**0.763***	**0.643***									
F	6	5 (3)	0.206*	**0.924***	**0.927***	**0.944***	**0.951***								
G	9	7 (4)	0.118*	**0.796***	**0.793***	**0.821***	**0.829***	0.041							
H	9	9 (4)	0.207*	**0.906***	**0.907***	**0.923***	**0.929***	0.071	0.009						
I	10	6 (3)	0.218*	**0.929***	**0.932***	**0.945***	**0.951***	0.132*	0.043	-0.039					
J	7	3 (3)	0.215	**0.953***	**0.958***	**0.970***	**0.977***	**0.269***	0.122*	0.082	0.131*				
K	9	4 (1)	0.219*	**0.957***	**0.961***	**0.972***	**0.978***	0.223*	**0.127***	0.094*	0.130*	0.275*			
M	10	4 (4)	**0.297***	**0.925***	**0.927***	**0.940***	**0.946***	**0.367***	0.246*	**0.299***	**0.356***	**0.411***	0.047		
N	8	4 (2)	0.271*	**0.831***	**0.829***	**0.852***	**0.861***	0.251*	0.091	**0.251***	**0.337***	**0.380***	**0.403***	**0.438***	
O	8	5 (3)	0.360*	**0.936***	**0.939***	**0.952***	**0.959***	0.424*	**0.218***	**0.383***	**0.510***	**0.619***	**0.637***	**0.600***	-0.042
Total	141	60 (49)													

TABLE 2.3

Analyses of molecular variance (AMOVAs). The four AMOVAs are based on different partitioning of variance across the five proposed ESUs (to accommodate taxonomic uncertainty; see methods for details): (a) partitions the variance across the five proposed ESUs and populations, treating all individuals as if sampled from a single species, (b) considers the contribution of putative species designations, *A. varius* and *A. zeteki*, on patterns of variation. In (c) and (d), the AMOVAs consider the two species separately: three *A. varius* ESUs (c) and two *A. zeteki* ESUs (d). Abbreviations are as follows: CT, variance among groups of populations, SC, variance among populations within groups, ST, variance among the individuals within a population.

(a)

Source of Variation	df	Φ-statistic	% of total variance	P-value
Among 5 ESUs: (A-C)(D-E)(F-I)(J-M)(N-O)	4	$\Phi_{CT} = 0.465$	46.52	0.006
Among populations within ESUs	10	$\Phi_{SC} = 0.498$	26.54	<0.001
Among individuals within a population	126	$\Phi_{ST} = 0.732$	26.85	<0.001

(b)

Source of Variation	df	Φ-statistic	% of total variance	P-value
Among species: (A-E)(F-O)	1	$\Phi_{CT} = 0.511$	51.05	<0.001
Among populations within species	13	$\Phi_{SC} = 0.566$	27.69	<0.001
Among individuals within a population	126	$\Phi_{ST} = 0.787$	21.25	<0.001

(c)

Source of Variation	df	Φ-statistic	% of total variance	P-value
Among *A. varius* ESUs: (F-I)(J-M)(N-O)	2	$\Phi_{CT} = 0.241$	24.06	<0.001
Among populations within ESUs	7	$\Phi_{SC} = 0.096$	7.27	<0.001
Among individuals within a population	68	$\Phi_{ST} = 0.313$	68.67	<0.001

(d)

Source of Variation	df	Φ-statistic	% of total variance	P-value
Among *A. zeteki* ESUs: (A-C)(D-E)	1	$\Phi_{CT} = 0.110$	11.00	0.502
Among populations within ESUs	3	$\Phi_{SC} = 0.696$	61.92	<0.001
Among individuals within a population	58	$\Phi_{ST} = 0.729$	27.08	<0.001

TABLE 2.4

Identification of ESUs that differ significantly from each other in mean phenotypic variables, based on nonparametric multiple comparisons (Dunnett's C test). Listed variables were significant at the $\alpha = 0.05$ level, where 1 = SVL, 2 = % black, 3= z-yellow, and 4 = z-black. Boxes enclose *A. zeteki* (ESUs 1-2) and *A. varius* (ESUs 3-5) to demonstrate that SVL and % black (variables 1 and 2) vary among ESUs within each species.

ESU	2	3	4	5
1	1-3	2	2,3,4	1,2
2		1,3	1-4	1,2
3			1-4	1,2
4				1-3

TABLE 2.5

Results of discriminant function analyses (DFAs) for models containing all four morphological variables (SVL, % black, z-yellow and z-black). Correctly classified individuals are shown in dark gray along the diagonal, and N = number of individuals being classified for each category. The four tables represent analyses based on different partitionings (to accommodate taxonomic uncertainty, see methods for details): (a) the five proposed ESUs, considering all golden frogs to be one taxon, (b) *A. varius* versus *A. zeteki*, (c) the three proposed *A. varius* ESUs (*A. zeteki* individuals excluded), and (d) the two proposed *A. zeteki* ESUs (*A. varius* individuals excluded). In (b), light gray shading indicates incorrect ESU classifications within species.

(a) All five ESUs

		Classified as						
		1	**2**	**3**	**4**	**5**	**N**	**% correct**
True ESU	**1**	14	2	12	0	1	29	48.3
	2	2	47	2	0	0	51	92.1
	3	11	3	47	0	0	61	77.0
	4	0	0	2	24	2	28	85.7
	5	0	4	0	1	13	18	72.2
					Total		187	77.5

(b) *Atelopus varius* versus *A. zeteki*

		Classified as			
		A. varius	*A. zeteki*	**N**	**% correct**
Putative species	*A. varius*	66	15	81	81.5
	A. zeteki	24	82	106	77.4
			Total	187	79.1

(c) *Atelopus varius* ESUs

		Classified as				
		3	**4**	**5**	**N**	**% correct**
True ESU	**3**	58	0	3	61	95.1
	4	2	24	2	28	85.7
	5	1	1	16	18	88.9
			Total		107	91.6

(d) *Atelopus zeteki* ESUs

		Classified as			
		1	**2**	**N**	**% correct**
True ESU	**1**	26	3	29	89.7
	2	4	47	51	92.6
		Total		80	91.3

TABLE 2.6

Correlations among genetic, morphological, and environmental variation showing the *P*-values from the pairwise Mantel tests. Significant correlations are shown in bold and the corresponding R-values are also indicated.

(a) all populations together

	SVL	z-yellow	z-black	% black	bioclim 1	bioclim 2	bioclim 3
F_{ST}	0.124	0.869	0.926	0.503	0.138	0.219	0.096
bioclim 1	**0.035***	0.427	0.464	0.051			
bioclim 2	0.060	0.225	0.846	**0.007****			
bioclim 3	0.226	0.051	0.976	0.957			

* R = 0.398, ** R = 0.579

(b) *Atelopus varius* populations

	SVL	z-yellow	z-black	% black	bioclim 1	bioclim 2	bioclim 3
F_{ST}	0.059	0.258	0.736	0.261	0.091	0.095	0.805
bioclim 1	**0.025***	0.510	0.713	0.708			
bioclim 2	0.149	0.492	0.481	0.218			
bioclim 3	0.948	0.377	0.339	0.459			

* R = 0.724

(c) *Atelopus zeteki* populations

	SVL	z-yellow	z-black	% black	bioclim 1	bioclim 2	bioclim 3
F_{ST}	0.381	0.421	0.401	0.776	0.881	0.453	0.846
bioclim 1	0.072	0.310	0.921	0.820			
bioclim 2	0.457	0.334	0.450	0.167			
bioclim 3	0.170	0.062	0.673	0.314			

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CHAPTER III

QUANTIFYING THE CONTRIBUTIONS OF ALTERNATIVE LANDSCAPE FACTORS TO GENE FLOW IN PANAMANIAN GOLDEN FROGS

Abstract

Understanding how heterogeneous landscapes shape genetic structure not only sheds light on processes involved in population divergence and speciation, but can also guide management strategies to promote and maintain genetic connectivity of populations of endangered species. Several of the life history characteristics of amphibians, including their generally poor dispersal and use of both aquatic and terrestrial habitats during different life stages, suggest that landscapes are likely to have strong effects on dispersal and genetic structure. In this study, I examined the influence of landscape variables on gene flow among populations of one species of endangered Panamanian golden frog, *Atelopus varius*, by testing for correlations between alternative landscape-resistance scenarios and genetic distance. Alternative hypotheses about the influence of (1) riparian habitat corridors, (2) changes in elevation, and (3) climatic suitability on patterns of genetic structure were tested in a causal modeling framework, using Mantel and partial-Mantel tests, along with an analysis of molecular variation. These tests uniformly indicate that patterns of genetic variation among golden frog populations are affected by changes in elevation such that areas with steep slopes act as barriers to gene flow. In contrast,

areas of low slope, such as streams and mountain ridges, appear to be important corridors for gene flow, especially among high elevation populations.

Introduction

Understanding how landscape heterogeneity affects genetic structure can provide insight into important biological processes such as metapopulation dynamics, the formation of species distributions, population divergence, and speciation (Manel *et al.* 2003; Storfer *et al.* 2007). The ever-growing number of landscape genetic studies, which identify how landscape variables influence patterns of gene flow and genetic variation, have contributed to this understanding for a variety of taxa, including plants (e.g., Liepelt *et al.* 2002; Fievet *et al.* 2007), crustaceans (e.g., Michels *et al.* 2001), insects (e.g., Keyghobadi *et al.* 1999, 2005a, 2005b; Finn *et al.* 2006), mollusks (e.g., Pfenninger 2002; Arnaud 2003), birds (e.g., Piertney *et al.* 1998), mammals (e.g., Roach *et al.* 2001; Cegelski *et al.* 2003; Scribner *et al.* 2005; Vignieri 2005), fish (e.g., Poissant *et al.* 2001), and amphibians (e.g., Funk *et al.* 2005; Spear *et al.* 2005; Lowe *et al.* 2006; Giordano *et al.* 2007). Because landscape genetic analyses can identify specific barriers or corridors for gene flow and predict the effects of alternative management strategies on connectivity, they have an applied value as well (Storfer *et al.* 2007), especially for endangered species (e.g., Stevens *et al.* 2006; Wilmer & Wilcox 2007).

Several aspects of the life histories of amphibians suggest that landscapes are likely to have strong effects on dispersal and genetic structure (Funk *et al.* 2005; Spear *et al.*

2005). For example, their generally low vagility (Blaustein *et al.* 1994) and, in many cases, high rates of philopatry (Duellman & Trueb 1994) can lead to low levels of gene flow, even among geographically proximate populations (Garcia-Paris *et al.* 2000; Shaffer *et al.* 2000; Monsen & Blouin 2003; Spear *et al.* 2005). In addition, their biphasic life cycles, consisting in most cases of an aquatic larval phase and a terrestrial adult phase, suggest that both aquatic and terrestrial landscape features could play a role in the evolution of genetic structure (Spear *et al.* 2005). While these aspects of amphibian biology suggest a strong role for landscape features in structuring genetic diversity, the relative contributions of different types of landscape heterogeneity to patterns of population interconnectivity and isolation remains largely unclear. Because amphibians are often hard to detect outside of their aquatic breeding periods, terrestrial habitat use and movement patterns are difficult to measure directly and remain poorly understood for most species (MacKenzie *et al.* 2002; Semlitsch 2003). Landscape genetic analyses are therefore an important tool for understanding the relative influences of alternative landscape factors on patterns of amphibian divergence and gene flow (Spear *et al.* 2005). Additionally, because amphibian species are declining worldwide (Stuart *et al.* 2004) this information will be useful in designing effective conservation and management strategies.

The goals of this study are to (1) identify important barriers and corridors for gene flow among populations of *Atelopus varius*, and (2) measure the relative contributions of these landscape factors to patterns of genetic variation in a hypothesis testing framework. Understanding how landscape characteristics affect evolutionary processes is particularly

urgent for these frogs as *Atelopus* is among the most imperiled of all amphibian genera - thus far, 62 of 77 described species have been classified by the IUCN as extinct or critically endangered (La Marca *et al.* 2005, Pounds *et al.* 2006; Lötters 2007). Declines and extinctions in this group have been attributed to the frogs' extreme sensitivity to environmental perturbations (Lötters 1996), an emerging fungal disease called chytridiomycosis, and global warming (Pounds *et al.* 2006). In Panama, two sister species collectively called Panamanian golden frogs, are in critical danger of extinction due to these same threats (Zippel 2002; IUCN 2004). While *A. varius* was historically found throughout much of montane Costa Rica and western Panama (Savage 1972), it has disappeared from most of its range (Lips 1998; Pounds and Crump 1994; Zippel *et al.* 2006). Its sister species, *Atelopus zeteki* has also gone missing from parts of its range, which included the area in and around an extinct volcanic crater at El Valle de Anton, Panama (Dunn 1933; Zippel *et al.* 2006).

Panamanian golden frogs exhibit extreme morphological variation, even among geographically proximate populations (Richards & Knowles 2007). However, in contrast to their morphological diversity, the life histories of golden frogs vary little. Both species breed in and live around swiftly flowing streams in lowland rainforest and humid montane forest habitat (Savage 1972; Lötters 1996). As adults, both sexes have well-defined home ranges along these streams and show site fidelity, indicating that the dispersal of this life stage is probably low (Crump 1986; Lötters 1996). While, little is known about the movement of other life stages, the frogs' strong association with riparian habitat indicates that streams are likely important corridors for dispersal. Although their

exact climatic tolerances are unknown, several authors noted that *Atelopus* seem particularly sensitive to climatic perturbations, and declines and extinctions in this group have been linked to global warming (Rivero 1963; Pounds *et al.* 2006). Given this, it is possible that regional climatic differences influence golden frog distributions and movement patterns as well. Finally, the abrupt changes in elevation that characterize golden frog habitats may also affect dispersal and gene flow. The potential for limited gene flow among golden frog populations, even over short distances, is indicated by the fact that mitochondrial DNA variation shows a strong pattern of isolation by distance (Richards & Knowles 2007).

In this study, I quantify the effect of landscape heterogeneity on gene flow and genetic differentiation among endangered *A. varius* populations by comparing the strengths of correlations between alternative landscape-resistance scenarios and genetic distance among nine populations from western Panama (Fig. 3.1). To test the hypothesis that golden frog gene flow has been facilitated by riparian habitat corridors but impeded by changes in elevation and areas of less-suitable climate, correlations between landscape distances, Euclidian (straight-line) distance, and the genetic distance among mitochondrial DNA haplotypes were compared using Mantel tests and an analysis of molecular variation (AMOVA). Partial-Mantel tests and a causal modeling framework were used to evaluate 15 alternative hypotheses about the relationship between landscape factors and gene flow. Given that the number of healthy golden frog populations has continued to dwindle since the initiation of this study (Richards, unpublished), the decision may someday be made to reintroduce individuals from captively-managed

populations to Panama (Zippel 2002). As such, this study not only contributes to our understanding of how landscape heterogeneity influences the formation of genetic structure, but has potential applications for the future management of these highly endangered frogs as well.

Materials and Methods

Sample collection and sequencing

Toe-clip tissue samples were collected from *A. varius* populations throughout their extant range in Panama (Fig. 3.1). These frogs were historically found throughout much of montane Costa Rica and western Panama, but by the time of collection (February to August, 2004) their extant range had been reduced to the area of western Panama sampled here, which includes parts of the Veraguas, Cocle, and Colon provinces. All animals were captured by hand and released at the point of capture within five minutes of data collection. The latitude and longitude for each population were recorded in the field using a hand-held GPS unit. The sampled populations range in elevation from 92 to 1124m and span four drainage basins as well as the continental divide. Tissues were preserved in a salt-saturated DMSO and EDTA solution in the field and stored at room temperature until the time of DNA extraction.

A 755 base pair fragment of CytB and 630 base pairs of COI were sequenced from the mitochondrial genome for each of the 76 individuals in this study. Sample sizes ranged from six to ten individuals per population with a median sample size of eight individuals

per population. Extraction of genomic DNA, primers, polymerase chain reaction (PCR) conditions, and sequencing were as described in Richards and Knowles (2007).

Genetic analyses

The sequence data from the two mitochondrial genes were concatenated into a single haplotype for each individual (GenBank Accession numbers for CytB: EF494922 - EF494948 and COI: EF494967 - EF494995). Population pairwise F_{ST} values were estimated in ARLEQUIN version 2.000 (Schneider *et al.* 2000). An analysis of molecular variance (AMOVA) with individuals nested by (a) population and (b) drainage basin was performed in ARLEQUIN 2.0 (Excoffier *et al.* 1992; Schneider *et al.* 2000) using 1000 permutations, and a Kimura 2-Parameter distance matrix with $\Gamma = 0.2226$.

Landscape friction gradients

Three landscape factors - climatic suitability, changes in slope, and the distribution of riparian habitat - were identified a priori as potentially influencing golden frog population structure. Based on these predictions, a series of landscape friction surfaces were generated to account for the difficulty golden frogs would potentially face in dispersing through (1) changes in slope, (2) sub-optimal climates, and (3) non-riparian habitat. For each factor, alternative friction surfaces representing different levels of resistance to dispersal were investigated.

Slope and riparian habitat. Friction maps for slope and riparian habitat were built from a 90m resolution digital elevation map (DEM) produced by the CGIAR Consortium for

Spatial Information (CGIAR-CSI: http://csi.cgiar.org). The slope at each cell within the study area was calculated using the Spatial Analyst extension of ArcGIS Desktop 9.2. To convert the slope map into a series of friction gradients, slope values were standardized so that areas with zero slope had a friction value of one and friction increased linearly with slope. Slope gradients were developed with maximum friction values ranging from 5 to 100. A stream map of the study area was generated from the DEM using the Arc Hydro 1.1 extension of ArcGIS Desktop 9.2. Stream friction gradients were developed from this map by assigning stream cells a friction value of one and non-stream cells a larger friction value, which varied from 5 to 100.

Climate. Climate suitability gradients were developed using the output of a species distribution model generated in Maxent version 3.0.4-beta (Phillips *et al.* 2006). The species distribution model was developed using a series of 19 bioclimatic layers at a spatial resolution of 30 arc-seconds (Hijmans *et al.* 2005; see also http://www.worldclim.org/) and 113 localities where *A. varius* is known to have occurred. These localities were compiled from species records at the American Museum of Natural History, California Academy of Sciences, Field Museum, Florida Museum of Natural History, Kansas University Museum of Natural History, Natural History Museum of Los Angeles County, Museum of Comparative Zoology, Museum of Vertebrate Zoology, University of Michigan Museum of Zoology, National Museum of Natural History, and the nine populations sampled for this study. Performance of the species distribution model was evaluated using receiver operating characteristic (ROC) analysis. For this analysis, 25% of species occurrence records were randomly selected as test data

and 10,000 randomly selected pixels from the study area were used as background points. The area under the ROC curve (AUC) for the test data was 0.874, indicating good discrimination between golden frog presence and absence (Phillips *et al.* 2006). The species distribution modeling algorithm in Maxent uses the set of climate layers and species occurrences to predict the climatic suitability of each cell of the study area. These suitabilities are reported in the form of a GIS layer with values ranging from zero to one, one being most and zero being least suitable. To convert the Maxent output layer to a series of climate suitability gradients, these output values were algebraically transformed so that the most suitable climate areas had a friction value of one and friction increased linearly with decreasing climatic suitability. A series of climate suitability gradients was developed with maximum friction values ranging from 5 to 100.

Isolation by distance

For each landscape friction gradient, the least-cost distances between populations were calculated using Pathmatrix (Ray 2005) in ArcView 3.3. The least-cost distance is calculated as the sum of the friction values for each cell along the least-cost path between populations. Pathmatrix was also used to compute the straight-line, or Euclidian distances between all populations. The strength of the association between the log of each landscape factor's resulting least-cost path distance and the log of genetic distance (measured by F_{ST}) was compared across multiple friction levels using Mantel tests (Mantel 1967) in IBD version 1.5 (Bohonak 2002) with 10^4 randomizations. The strength of the association between Euclidian (straight-line) and genetic distances was assessed in the same way. Since Euclidian distance does not take into account landscape

heterogeneity, the strength of the pattern of isolation by Euclidian distance served as a null model against which the performance of alternative landscape gradients was compared – only landscape factors that showed tighter correlations with genetic distance than did Euclidian distance were considered as potential influences on genetic structure. For each landscape factor (slope, climate, and streams), the friction level that resulted in the largest Mantel correlation coefficient (r) was used for further analysis and hypothesis testing as it best explains the pattern of among population genetic structure.

Landscape factors and hypothesis testing

To assess the relative support for each of the three landscape factors as drivers of genetic structure, the strength of the association between genetic distance and Euclidian distance was compared to those of genetic distance and the alternative landscape distances. Because each landscape factor could affect gene flow independently, in concert with others, or not at all, 15 alternative patterns of causality were possible. Following Cushman *et al.* (2006), each of these were tested as separate hypotheses (Table 3.1) and causal modeling (Legendre & Troussellier 1988; Legendre 1993) was used to identify the landscape hypothesis with the strongest support. Each hypothesis has a corresponding set of diagnostic, statistical predictions (Table 3.1) regarding the relationship between genetic distance and alternative landscape distances. Under this framework, only the hypothesis with the strongest support will have all its predictions upheld. To test each prediction, the strength of the association between two distance matrices (e.g., log of genetic and log of Euclidian distances) after removing the effect of a third (e.g., the log of the least-cost distance for the slope gradient, or "slope distance") was measured using a

partial Mantel test. These were again carried out in IBD version 1.5 with 10^4 randomizations.

Results

Performance of alternative friction gradients

The strength of the pattern of isolation by distance was significant across 21 of the 22 alternative landscape gradients after Bonferroni correction ($P < 0.0023$; Table 3.2). The only landscape gradient that had a non-significant correlation with genetic distance was the stream-distance gradient with a maximum friction value of 100 (St_{100}; $P = 0.0082$). When the 22 distance matrices were ranked by Mantel correlation coefficient (*r*), Euclidian distance ranked 12th and only stream and slope distance matrices ranked higher (Table 3.2, Fig. 3.2). The strongest correlation between genetic distance and stream distance was found when the friction value for non-stream habitats was set to 10 (St_{10}: Fig. 3.3). However, several of the slope-distance matrices had stronger correlations with genetic distance than did St_{10}. The strongest correlation between any distance matrix and genetic distance came when a slope gradient was used and the maximum friction value was set to 100 (Sl_{100}; Fig. 3.2). The distance matrices for climate gradients were never more strongly correlated with genetic distance than was Euclidian distance (Fig. 3.2).

Support for alternative landscape hypotheses

For only one of the 15 alternative hypotheses – genetic isolation by slope distance - were all statistical predictions upheld (Table 3.3). This indicates that gene flow among golden

frog populations is influenced by changes in elevation with no significant, independent relationships with Euclidian distance, riparian habitat, or climate. The routes between populations that minimize changes in slope (i.e., the least-cost paths) tend to follow streams and rivers, as well as mountain ridges, suggesting that these landscape features are important corridors for gene flow. The AMOVA with individuals nested by (a) population and (b) drainage basin further supports the role of streams as important corridors for gene flow as a significant proportion of golden frog genetic variation was found among drainage basins (Table 3.4).

Discussion

Of the 22 alternative landscape resistances investigated, half resulted in a pattern of isolation by landscape distance stronger than the null model of isolation by Euclidian, or straight-line distance (Table 3.2). Only landscape gradients attributing resistance to (a) dispersal across non-riparian habitats (stream distances) or (b) dispersal across changes in elevation (slope distances) explained more of the genetic variation than did Euclidian distance. For elevation, this result was not dependent upon the range of friction values used (i.e., the rate at which resistance to movement increased with slope). However, for streams, only the landscape gradients attributing low friction values (5 to 20) to travel through non-riparian habitat resulted in a stronger correlation with genetic distance than did Euclidian distance. These results highlight the importance of considering a range of relative friction values when calculating landscape distances using least-cost path analyses. This technique is especially useful when little information about the ecology

and movement behavior or the study organism is available from which to empirically derive or estimate these relative friction values (Ray *et al.* 2002; Ray 2005), as is the case with golden frogs.

The strongest correlations with genetic distance were found when the resistance to dispersal across changes in elevation was high (Sl_{100}; Fig. 3.2). For these slope gradients, least-cost paths between populations followed streambeds and rivers almost exclusively, moving over land only for short distances, most often along mountain ridges (Fig. 3.3). This was especially true at high elevations, where the majority of *A. varius* populations are found. Paths among low elevation populations, where changes in slope are not as dramatic, tended not to follow streams and ridges as closely.

Causal modeling also supported the hypothesis that gene flow among golden frog populations is influenced predominately by changes in elevation. No significant, independent relationships with stream distance, climate distance, or Euclidian distance were found. However, the fact that the least-slope paths among populations tend to follow streams and rivers, suggests that these features, in addition to mountain ridges, represent important corridors for gene flow among populations. The AMOVA results further highlight the importance of contiguous riparian corridors by demonstrating that a significant proportion of golden frog genetic variation is found among drainage basins, which are by definition not connected by riparian habitat (Table 3.4). Conversely, only a small fraction of the total genetic variation is found among populations within the same drainage basin. Taken together, these results are consistent with the idea that patterns of

genetic variation among golden frog populations are affected by changes in elevation such that areas with steep slopes act as barriers to gene flow. In contrast, areas of low slope, such as streams and mountain ridges, appear to be important corridors for gene flow, especially among high elevation populations.

Changes in elevation have been found to isolate populations of temperate amphibian species as well (Funk et al. 2005; Spear et al. 2005; Lowe et al. 2006; Giordano et al. 2007). However, because most of these studies have focused on pond-breeding amphibians, the role of riparian habitat corridors as facilitators of gene flow among amphibian populations has not often been explored. A notable exception to this was the study of Columbia spotted frogs (*Rana luteiventris*) by Funk et al. (2005), which found the pattern of isolation by river distance to be stronger than that of Euclidian distance over some parts of the species' range. The distribution of riparian habitat has been linked to patterns of gene flow among other, stream-associated taxa, such as the Pacific jumping mouse (*Zapus trinotatus*) (Vignieri 2005). However, this is not always the case (e.g., an alpine stream fly: Finn et al. 2006).

For golden frogs, landscape distances attributing resistance to movement through sub-optimal climates (climate distances) were less strongly correlated with genetic distance than was Euclidian distance (Table 3.2, Fig. 3.2). This result is consistent with the idea that regional climatic variation has little or no effect on gene flow among golden frog populations. However, it is also possible that climatic heterogeneity does influence gene flow but the species distribution model used to quantify relative climatic suitability failed

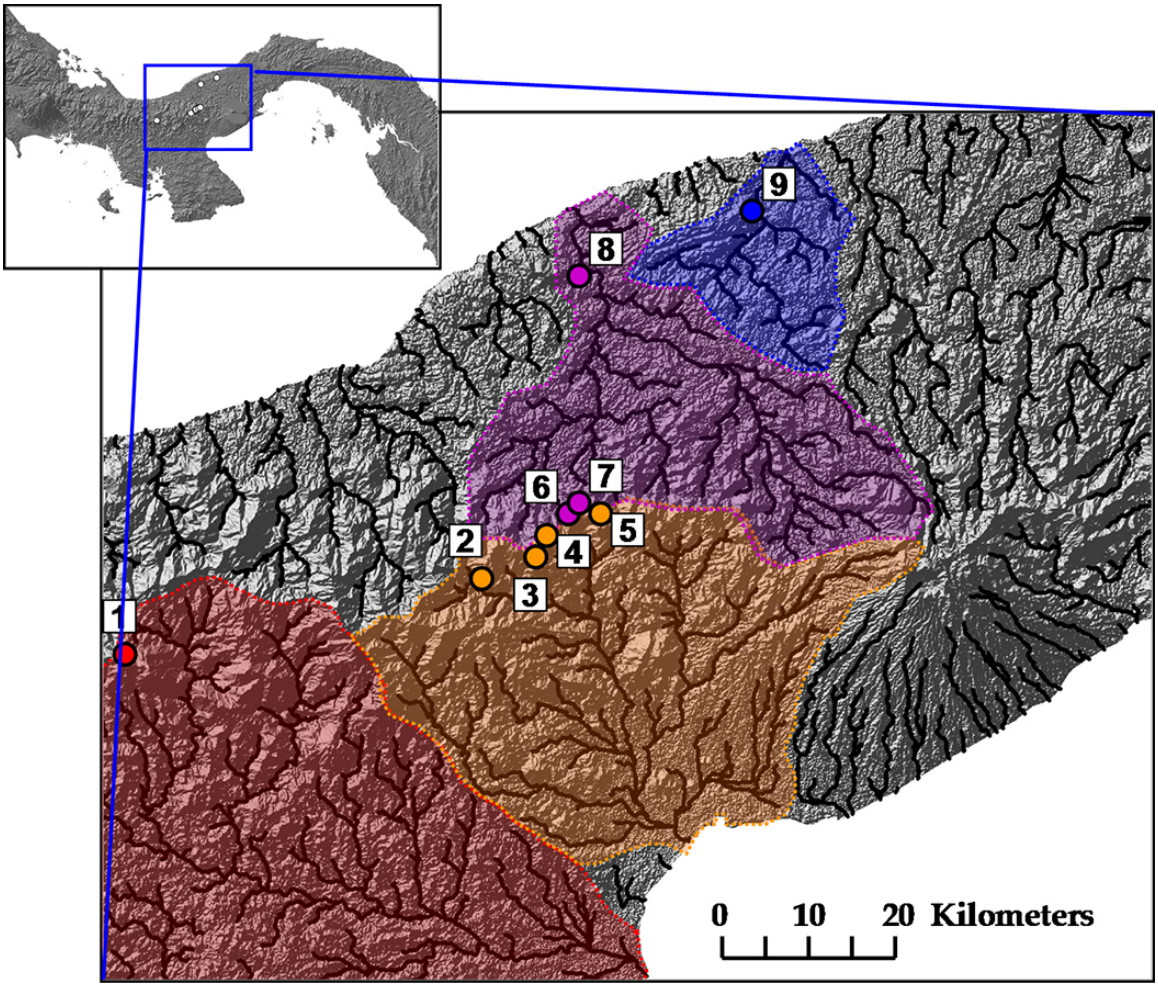
to capture its effect. Unlike changes in elevation and the locations of riparian corridors, which have remained relatively stable in the region for thousands of years, the climate of western Panama may not have been consistent enough for a measurable correlation with the mitochondrial genetic distances among populations to have developed. The WorldClim bioclimatic variables used to build the climatic suitability gradients were derived from temperature and precipitation measurements averaged over the time period from 1950 – 2000. It is possible that patterns of gene flow are more strongly correlated with the climate landscape of earlier time periods (e.g., during the Pleistocene) than with present climate heterogeneity. Similar influences of past climatic landscapes on patterns of genetic connectivity have been found in other Neotropical riparian species (e.g., white piranha: Hubert *et al.* 2007) as well as a host of temperate taxa (e.g., frogs: Green *et al.* 1996, birds: Avise & Walker 1998; spiders: Ayoub & Riechert 2004, grasshoppers: Knowles & Richards 2005, and fish: Stepien *et al.* 2007).

Conclusion

The comprehensive landscape genetic approach of this study not only identified landscape factors affecting gene flow among golden frog populations, but also permitted the relative contributions and interactions of these factors to be evaluated in a hypothesis testing framework. Mantel tests, causal modeling, and AMOVA uniformly support the strong role of areas of low slope, such as streams and mountain ridges as conduits for gene flow among populations.

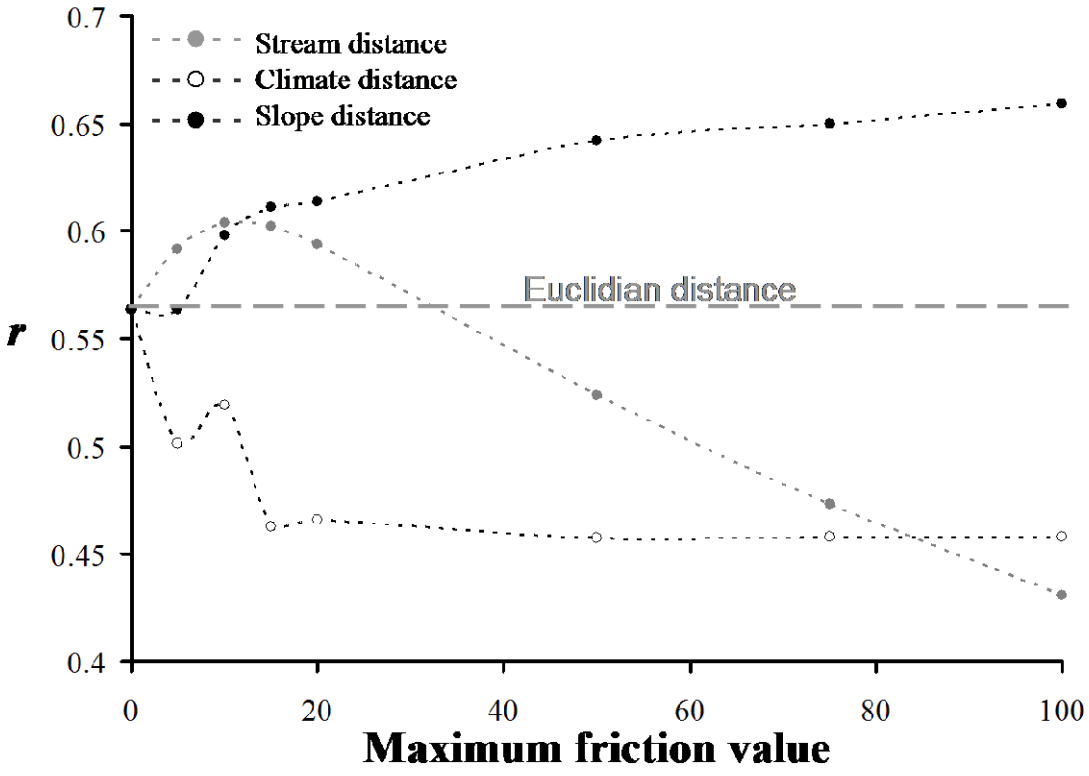
These findings not only contribute to our understanding of the ongoing evolutionary processes shaping golden frog variation, but also engender important considerations for the management and conservation of these critically endangered species. For example, because gene flow is dependent upon riparian connectivity, the construction of dams, introduction of potentially predatory fish, contamination of streams by agricultural runoff, and other anthropogenic changes to streams and rivers are likely to affect the future evolution and ecology of these organisms. Likewise, the fragmentation of habitat along mountain ridges is likely to adversely affect the connectivity of golden frog populations. Understanding how the elevation profile and affects dispersal and gene flow will be critical if, down the line, the decision is made to reintroduce captively raised golden frogs to Panama. Not only can this information help guide the selection of suitable locations for reintroductions, given the captively-bred frogs' populations of origin, but it will allow for informed predictions of the pattern of recolonization the frogs are likely to exhibit as well.

FIGURE 3.1



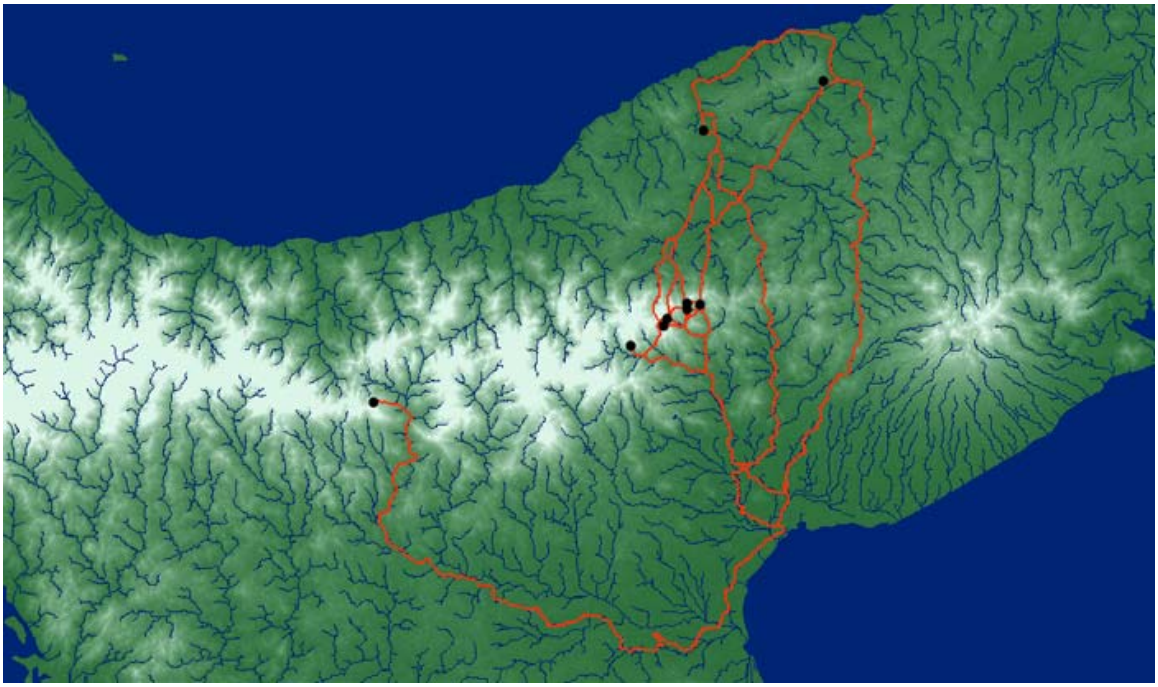
Study area and sampled populations. The four sampled drainage basins are indicated by differently colored areas on the shaded relief map (refer to inset for the position of the sampling area within Panama).

FIGURE 3.2



Mantel correlation coefficients (*r*) across landscape gradients and friction ranges. The x-axis indicates the maximum friction value for the variable.

FIGURE 3.3



Map of the least-cost paths between populations (denoted by red lines) for the landscape friction gradient most strongly correlated with genetic distance ($slope_{100}$). The background is an elevation map (light colors indicate high elevation).

TABLE 3.1

Description of the 15 alternative landscape genetic hypotheses tested. The statistical predictions are a list of the partial Mantel tests used to evaluate each hypothesis and the expected pattern of significance if the model is correct. Abbreviations are as follows: G = genetic distance; D = log of Euclidian distance; Sl = "slope distance", the log of the least-cost distance with changes in slope as the source of friction; C = "climate distance", the log of the least-cost distance with changes in climate as the source of friction; St = "stream distance", the log of the least-cost distance with movement outside of streams as the source of friction. A period (.) separates the main distance matrices (on the left) from the covariate matrix (on the right), whose effect is removed in the partial Mantel tests (e.g, DG.Sl is the partial Mantel test between log Euclidian and genetic distance after removing the effect of "slope distance").

Hypothesis	**Statistical predictions**					
(Genetic isolation by...)	**positive correlation (r > 0)**			**no correlation (r = NS)**		
Distance	DG.Sl	DG.C	DG.St	SlG.D	CG.D	StG.D
Slope	SlG.D	SlG.C	SlG.St	DG.Sl	StG.Sl	CG.Sl
Climate	CG.D	CG.Sl	CG.St	DG.C	SlG.C	StG.C
Stream	StG.D	StG.C	StG.Sl	DG.St	CG.St	SlG.St
Distance and slope	DG.Sl	DG.St	SlG.C	StG.D	StG.Sl	CG.D
	DG.C	SlG.D	SlG.St	CG.Sl		
Distance and climate	DG.Sl	DG.St	CG.Sl	SlG.D	SlG.C	StG.C
	DG.C	CG.D	CG.St	StG.D		
Distance and stream	DG.Sl	DG.St	StG.C	SlG.D	SlG.St	CG.St
	DG.C	StG.D	StG.Sl	CG.D		
Slope and climate	SlG.D	SlG.St	CG.Sl	DG.Sl	DG.C	StG.C
	SlG.C	CG.D	CG.St	StG.Sl		
Slope and stream	SlG.D	SlG.St	StG.C	DG.Sl	DG.St	CG.St
	SlG.C	StG.D	StG.Sl	CG.Sl		
Climate and stream	CG.D	CG.St	StG.C	DG.C	DG.St	SlG.C
	CG.Sl	StG.D	StG.Sl	SlG.St		
Distance, slope and climate	DG.Sl	DG.C	DG.St	StG.D	StG.C	StG.Sl
	SlG.D	SlG.C	SlG.St			
	CG.D	CG.Sl	CG.St			
Distance, slope and stream	DG.Sl	DG.C	DG.St	CG.D	CG.Sl	CG.St
	SlG.D	SlG.C	SlG.St			
	StG.D	StG.C	StG.Sl			
Distance, climate and stream	DG.Sl	DG.C	DG.St	SlG.D	SlG.C	SlG.St
	CG.D	CG.C	CG.St			
	StG.D	StG.C	StG.Sl			
Slope, climate, and stream	SlG.Sl	SlG.C	SlG.St	DG.Sl	DG.C	DG.St
	CG.D	CG.C	CG.St			
	StG.D	StG.C	StG.Sl			
Distance, slope, climate and stream	DG.Sl	DG.C	DG.St			
	SlG.Sl	SlG.C	SlG.St			
	CG.D	CG.C	CG.St			
	StG.D	StG.C	StG.Sl			

TABLE 3.2

Mantel test results for 22 alternative distance matrices, ranked by correlation coefficient (*r*). All but one (St_{100}) were significant after Bonferroni correction ($P < 0.0023$). Abbreviations follow Table 3.1. Subscripted numbers indicate the maximum friction value of the landscape gradient.

Rank	**Distance matrix**	**(*r*)**
1	Sl_{100}	0.659
2	Sl_{75}	0.650
3	Sl_{50}	0.642
4	Sl_{20}	0.614
5	Sl_{15}	0.611
6	St_{10}	0.604
7	St_{15}	0.602
8	Sl_{10}	0.598
9	St_{20}	0.594
10	St_{5}	0.592
11	Sl_{5}	0.564
12	***Euclidian***	***0.564***
13	St_{50}	0.524
14	C_{10}	0.519
15	C_{5}	0.501
16	St_{75}	0.473
17	C_{20}	0.466
18	C_{15}	0.463
19	C_{75}	0.458
20	C_{100}	0.458
21	C_{50}	0.457
22	St_{100}	0.431 (NS)

TABLE 3.3

Evaluation of alternative landscape hypotheses. Abbreviations and Partial Mantel test nomenclature follow Tables 3.1 and 3.2. Predictions upheld by the partial Mantel tests are in bold. Values in parentheses are partial Mantel correlation coefficients (r) for tests that were significant ($P < 0.05$) after Bonferroni correction for experiment-wise error rates. Non-significant tests are denoted by (NS).

Hypothesis	**Statistical predictions**					
(predictions upheld / total)	**- - - - - - - - - - - - positive correlation (r > 0) - - - - - - - - - - - -**			**- - - - - - - - - - - - - - no correlation (r = NS) - - - - - - - - - - - - - -**		
Distance (2/6)	DG.Sl_{100} (NS)	DG.C_{10} (NS)	DG.St_{10} (NS)	Sl_{100}G.D (0.527)	**C_{10}G.D (NS)**	**St_{10}G.D (NS)**
Slope (6/6)	**Sl_{100}G.D (0.527)**	**Sl_{100}lG.C_{10} (0.609)**	**Sl_{100}G.St_{10} (0.545)**	**DG. Sl_{100} (NS)**	**St_{10}G. Sl_{100} (NS)**	**C_{10}G.Sl_{100} (NS)**
Climate (2/6)	C_{10}G.D (NS)	C_{10}G.Sl_{100} (NS)	C_{10}G.St_{10} (NS)	**DG.C_{10} (NS)**	Sl_{100}G.C_{10} (0.609)	**St_{10}G.C_{10} (NS)**
Stream (2/6)	St_{10}G.D (NS)	St_{10}G.C_{10} (NS)	St_{10}G.Sl_{100} (NS)	**DG.St_{10} (NS)**	**C_{10}G.St_{10} (NS)**	Sl_{100}G.St_{10} (0.545)
Distance and slope (7/10)	DG.Sl_{100} (NS)	DG.St_{10} (NS)	**Sl_{100}lG.C_{10} (0.609)**	**St_{10}G.D (NS)**	**St_{10}G.Sl_{100} (NS)**	**C_{10}G.D (NS)**
	DG.C_{10} (NS)	**Sl_{100}G.D (0.527)**	**Sl_{100}G.St_{10} (0.545)**	**C_{10}G. Sl_{100} (NS)**		
Distance and climate (2/10)	DG.Sl_{100} (NS)	DG.St_{10} (NS)	C_{10}G. Sl_{100} (NS)	Sl_{100}G.D (0.527)	Sl_{100}lG.C_{10} (0.609)	**St_{10}G.C_{10} (NS)**
	DG.C_{10} (NS)	C_{10}G.D (NS)	C_{10}G.St_{10} (NS)	**St_{10}G.D (NS)**		
Distance and stream (2/10)	DG.Sl_{100} (NS)	DG.St_{10} (NS)	St_{10}G.C_{10} (NS)	Sl_{100}G.D (0.527)	Sl_{100}G.St_{10} (0.545)	**C_{10}G.St_{10} (NS)**
	DG.C_{10} (NS)	St_{10}G.D (NS)	St_{10}G.Sl_{100} (NS)	**C_{10}G.D (NS)**		
Slope and climate (7/10)	**Sl_{100}G.D (0.527)**	**Sl_{100}G.St_{10} (0.545)**	C_{10}G. Sl_{100} (NS)	**DG.Sl_{100} (NS)**	**DG.C_{10} (NS)**	**St_{10}G.C_{10} (NS)**
	Sl_{100}lG.C_{10} (0.609)	C_{10}G.D (NS)	C_{10}G.St_{10} (NS)	**St_{10}G.Sl_{100} (NS)**		
Slope and stream (7/10)	**Sl_{100}G.D (0.527)**	**Sl_{100}G.St_{10} (0.545)**	St_{10}G.C_{10} (NS)	**DG.Sl_{100} (NS)**	**DG.St_{10} (NS)**	**C_{10}G.St_{10} (NS)**
	Sl_{100}lG.C_{10} (0.609)	St_{10}G.D (NS)	St_{10}G.Sl_{100} (NS)	**C_{10}G.Sl_{100} (NS)**		
Climate and stream (2/10)	C_{10}G.D (NS)	C_{10}G.St_{10} (NS)	St_{10}G.C_{10} (NS)	**DG.C_{10} (NS)**	**DG.St_{10} (NS)**	Sl_{100}G.C_{10} (0.609)
	C_{10}G. Sl_{100} (NS)	St_{10}G.D (NS)	St_{10}G.Sl_{100} (NS)	Sl_{100}G.St_{10} (0.545)		
Distance, slope and climate (6/12)	DG.Sl_{100} (NS)	DG.C_{10} (NS)	DG.St_{10} (NS)	**St_{10}G.D (NS)**	**St_{10}G.C_{10} (NS)**	**St_{10}G.Sl_{100} (NS)**
	Sl_{100}G.D (0.527)	**Sl_{100}lG.C_{10} (0.609)**	**Sl_{100}G.St_{10} (0.545)**			
	C_{10}G.D (NS)	C_{10}G.Sl_{100} (NS)	C_{10}G.St_{10} (NS)			
Distance, slope and stream (6/12)	DG.Sl_{100} (NS)	DG.C_{10} (NS)	DG.St_{10} (NS)	**C_{10}G.D (NS)**	**C_{10}G.Sl_{100} (NS)**	**C_{10}G.St_{10} (NS)**
	Sl_{100}G.D (0.527)	**Sl_{100}lG.C_{10} (0.609)**	**Sl_{100}G.St_{10} (0.545)**			
	St_{10}G.D (NS)	St_{10}G.C_{10} (NS)	St_{10}G.Sl_{100} (NS)			
Distance, climate and stream (0/12)	DG.Sl_{100} (NS)	DG.C_{10} (NS)	DG.St_{10} (NS)	Sl_{100}G.D (0.527)	Sl_{100}G.C_{10} (0.609)	Sl_{100}G.St_{10} (0.545)
	C_{10}G.D (NS)	C_{10}G.Sl_{100} (NS)	C_{10}G.St_{10} (NS)			
	St_{10}G.D (NS)	St_{10}G.C_{10} (NS)	St_{10}G.Sl_{100} (NS)			
Slope, climate, and stream (6/12)	**Sl_{100}G.D (0.527)**	**Sl_{100}lG.C_{10} (0.609)**	**Sl_{100}G.St_{10} (0.545)**	**DG. Sl_{100} (NS)**	**DG.C_{10} (NS)**	**DG.St_{10} (NS)**
	C_{10}G.D (NS)	C_{10}G.Sl_{100} (NS)	C_{10}G.St_{10} (NS)			
	St_{10}G.D (NS)	St_{10}G.C_{10} (NS)	St_{10}G.Sl_{100} (NS)			
Distance, slope, climate and stream (/12)	DG.Sl_{100} (NS)	DG.C_{10} (NS)	DG.St_{10} (NS)			
	Sl_{100}G.D (0.527)	**Sl_{100}lG.C_{10} (0.609)**	**Sl_{100}G.St_{10} (0.545)**			
	C_{10}G.D (NS)	C_{10}G.Sl_{100} (NS)	C_{10}G.St_{10} (NS)			
	St_{10}G.D (NS)	St_{10}G.C_{10} (NS)	St_{10}G.Sl_{100} (NS)			

TABLE 3.4

Results of the AMOVA with individuals grouped by drainage basin and population.

Source of Variation	df	Φ-statistic*	% of variance	P-value
Among drainage basins	3	$\Phi_{CT} = 0.184$	18.44	0.012
Among populations within drainage basins	5	$\Phi_{SC} = 0.146$	11.91	<0.001
Among individuals within a population	68	$\Phi_{ST} = 0.303$	69.65	<0.001

* CT = variance among groups of populations, SC = variance among populations within groups, and ST = variance among the individuals within a population.

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CHAPTER IV

PANAMANIAN GOLDEN FROGS COMBAT AN EMERGING FUNGAL EPIDEMIC BY INDUCING A "BEHAVIORAL FEVER"

Abstract

Evidence is mounting that recent, global amphibian declines and extinctions have been caused by the emerging, pathogenic fungus *Batrachochytrium dendrobatidis*. In Central America, the appearance of *B. dendrobatidis* has been linked to mass mortality events and dramatic losses of amphibian biodiversity. Temperature appears to affect the distribution and severity of *B. dendrobatidis* infection, suggesting that amphibians may be able to defend themselves against infection by adjusting their thermoregulatory behavior. Using a combination of mark-recapture and diagnostic *B. dendrobatidis* assays, I demonstrate that Panamanian golden frogs (*Atelopus zeteki*) can induce a "behavioral fever" in response to a *B. dendrobatidis* epidemic, modifying their thermoregulation behavior to increase body temperatures above their normal set point. This behavioral response appears to be effective in combating the fungus as infection prevalence decreased with increasing body temperature. However, comparisons of the frogs' body conditions before and during the epidemic suggest that the benefit of maintaining a high body temperature in terms of pathogen avoidance might be tempered by the physiological cost of this behavior. These results represent the first evidence that ectotherms can mount

an effective, behavioral fever response to natural infections in the wild. Furthermore, the thermal dependency of the relationship between *B. dendrobatidis* and its amphibian hosts demonstrates how the progression of an epidemic can be influenced by complex interactions between the genotypes of both host and pathogen and the environment in which they are found.

Introduction

In recent decades, amphibian populations have undergone rapid, global declines and extinctions such that over 30% of all amphibian species are now considered threatened and as many as 122 species may have become extinct (Stuart *et al*. 2004). In many cases this can be attributed to threats amphibians share with other taxa, including land-use change, overexploitation and the introduction of exotic species. However, the declines and extinctions of as many as 200 frog species across the globe appear to have been caused by a newly-emerged, fungal pathogen called *Batrachochytrium dendrobatidis* (Skerratt *et al*. 2007). This fungus infects the keratinized layers of an amphibian's skin, causing a potentially fatal disease called chytridiomycosis (Longcore *et al*. 1999). Chytridiomycosis can be transmitted by direct contact with infected frogs or indirectly via contaminated substrates or water (Berger *et al*. 1998; Parris & Cornelius 2004; Rachowicz & Vredenburg 2004; Rowley *et al*. 2007). In Central America, a north-to-south epidemic wave of this disease has been linked to declines and mass die-offs, resulting in dramatic losses of amphibian biodiversity (Lips *et al*. 2006).

The effect of temperature on the ability of *B. dendrobatidis* to grow and infect amphibians has been demonstrated in the laboratory, where the fungus grows best between 17 and 25°C and achieves peak growth and pathogenicity at 23°C (Johnson *et al*. 2003; Piotrowski *et al*. 2004). At 28°C the fungus stops growing and at 30°C it dies. Disease surveys of wild amphibians also suggest that the prevalence and severity of chytridiomycosis infections tend to decrease during warmer months (Berger *et al*. 2004; Woodhams & Alford 2004; Retallick *et al*. 2004; Kriger & Hero 2006, 2007a, 2007b). However, a direct link between amphibian body temperatures and *B. dendrobatidis* infection has yet to be established in the wild.

As ectotherms, the body temperatures of amphibians are constrained by the temperature of their surroundings. But by choosing particular microclimates within a spatially and temporally variable environment, ectotherms can, to some extent, regulate their body temperature behaviorally and buffer themselves from negative effects of temperature on physiological performance (Huey 1991). If body temperature affects an amphibian's susceptibility to *B. dendrobatidis*, it stands to reason that individuals may be able to avoid or reduce the severity of infections by behaviorally increasing their body temperatures (Woodhams *et al*. 2003). In laboratory studies, many ectotherms respond to injections of pathogens by inducing a "behavioral fever" (Sherman *et al*. 1998; Kluger 1991; Gardner & Thomas 2002). By altering thermoregulation behavior to sustain a body temperature above that usually preferred, these organisms are better able to fight infection. However, this behavior increases the metabolic rate, and hence the energetic requirements of amphibians (Sherman & Stephens 2006), and has been associated with fitness costs in

some taxa (Gardner & Thomas 2002; Elliot *et al*. 2005). Regardless, the occurrence and effectiveness of ectotherms inducing a behavioral fever in response to natural infections has yet to be documented in the wild.

Using a combination of mark-recapture and diagnostic *B. dendrobatidis* assays, I aimed to determine whether critically endangered Panamanian golden frogs (*Atelopus zeteki*) alter their thermoregulation behavior in response to an epidemic of *B. dendrobatidis* and if so, whether this change in behavior can reduce their chance of infection. The results of this three-year study show that not only did the body temperatures of the frogs increase during the epidemic, but that the odds of *B. dendrobatidis* infection decreased with increasing body temperature. However, changes in the frogs' body conditions during the epidemic suggest the existence of a fitness tradeoff between avoiding *B. dendrobatidis* infection and overall physiological performance.

Materials and Methods

Mark-recapture studies were conducted on three 200m transects within a 3km stretch of the Rio Mata Ahogado, in Panama Province, Panama (elevation 290m) where mean annual air temperature is 26.0°C with an annual range of 21.4 – 31.6°C (Hijmans *et al*. 2005). Each transect was surveyed between 1000 and 1800 hours on five days during five time periods: 20 January - 2 February 2004, 10-16 December 2004, 20-27 January 2005, 8-15 December 2005, and 22-26 January 2006. Body temperature, weight, and body size were recorded for each *A. zeteki* encountered. Body temperature was measured prior to

capture using a non-contact infrared thermometer (Rowley & Alford 2007). Weight was measured using a spring scale and body size was measured using dial calipers. Each frog was given an identifying toe-clip mark upon first capture and released at the point of capture.

During January 2005, December 2005, and January 2006, the dorsum, venter, and feet of each frog were swabbed with a sterile cotton swab. Swabs were stored in a salt-saturated DMSO solution at room temperature prior to extraction. The 482 *A. zeteki* swab samples and 100 negative controls were randomized and tested for *B. dendrobatidis* using Taqman diagnostic quantitative PCR (q-PCR) (Boyle *et al*. 2004). DNA was extracted from each sample and negative control following Hyatt *et al*. (2007) and q-PCR assays were performed in triplicate following Boyle *et al*. (2004). Samples containing PCR inhibitors were detected using VIC_{TM} Exogenous Internal Positive Controls (Applied Biosystems) and inhibition was overcome by dilution following Hyatt *et al*. (2007) Samples were scored as positive if all three replicates indicated the presence of *B. dendrobatidis*. Samples testing positive in one or two replicates were re-assayed once. If the second assay produced a negative or positive result in all three replicates the sample was scored as negative or positive, respectively. Samples testing positive in one or two replicates of the second assay were not included among the negative or positive samples. One of the 100 negative controls tested positive for *B. dendrobatidis*, indicating a false positive rate for DNA extraction and *B. dendrobatidis* assay of 1%.

Statistical analyses were performed in SPSS 11.0. Because body temperatures and body conditions were sometimes non-normally distributed (Shapiro-Wilk: $P < 0.05$), non-parametric tests were used to compare means across sampling periods and infection classes and body conditions were log transformed prior to linear regression.

Results

B. dendrobatidis was not detected in any of the 139 frogs tested in January 2005. However, in December 2005, 19 (13.5%) of 141 frogs were infected and by late January 2006, the infection prevalence had risen to 47.0% (94 of 200 frogs infected). No dead *A. zeteki* were found in December 2005 but eight were found dead along the stream during January 2006, all of which tested positive for *B. dendrobatidis*.

The frogs' body temperatures were higher during the epidemic than during three previous sampling periods (Kruskal-Wallis: $N = 1225$, $\chi^2_4 = 536.9$, $P < 0.001$; Dunnett's C: $P < 0.05$) despite the fact that the average air temperature did not differ among sampling periods (Kruskal-Wallis: $N = 32$, $\chi^2_4 = 9.46$, $P = 0.051$) (Fig. 4.1). Infected frogs had significantly lower body temperatures than uninfected frogs during both December 2005 (Mann-Whitney: $N = 119$, $U = 659.5$, $Z = 2.1$, $P = 0.036$) and January 2006 ($N = 128$, $U = 1383$, $Z = 2.42$, $P = 0.016$) (Fig. 4.2). Furthermore, the odds of a frog being infected decreased by 60.5% with each 1 °C increase in body temperature in December 2005 (logistic regression: $N = 119$, $\chi^2_1 = 4.10$, $P = 0.025$) and by 28.4% with each 1 °C

increase in body temperature in January 2006 (logistic regression: N = 128, $\chi^2_1 = 5.89$, P = 0.01).

The body condition of the frogs (measured as weight / snout-vent length) was lower during the epidemic than during the pre-epidemic sampling periods (Kruskal-Wallis: N = 867, $\chi^2_4 = 60.88$, $P < 0.001$; Dunnett's C: $P < 0.05$) and the log of body condition was inversely related to body temperature (linear regression: N = 931, $F_{1,929} = 70.25$, $P < 0.001$, $r^2 = 0.069$; Fig. 4.3). However, the body conditions of infected and uninfected frogs did not differ in December 2005 (Mann-Whitney: N =115, U = 813.5, Z = 0.154, $P_{1\text{-sided}} = 0.439$) or January 2006 (Mann-Whitney: N = 136, U = 2111, Z = 0.005 $P_{1\text{-sided}} = 0.498$). Body condition did not differ among sampling periods prior to December 2005 (Kruskal-Wallis: N = 867, $\chi^2_4 = 60.88$, $P < 0.001$; Dunnett's C: $P > 0.05$).

Discussion

The arrival and progression of *B. dendrobatidis* infections at the study site, which is located 60 km east of the latest documented decline, is consistent with the hypothesized north-to-south wave of epidemic infections in Central America since the late 1980s (Lips *et al*. 2006). In the population studied here, golden frogs only spend time along the river during their breeding season, which occurs from early December until late January each year. During the remainder of the year they inhabit dry, grassy uplands on either side of the river. As *B. dendrobatidis* was not detected in the population toward the end of the 2004-2005 breeding season, and was detected in only 13.5% of the frogs at the beginning

of the 2005-2006 breeding season, it is likely that the golden frogs encountered the pathogen for the first time as they arrived at the river to breed in December 2005. Just over one month later, the infection rate had risen to 47%, which is consistent with the rapid onset of infection observed during other *B. dendrobatidis* epidemics (Lips *et al*. 2006).

While air temperatures did not differ among sampling periods, the average body temperature of the golden frog population increased with infection prevalence; it was 1.05°C higher in December 2005 and 2.35°C higher in January 2006 than the average of the pre-infection sampling periods (Fig. 4.1). During the three pre-epidemic sampling periods, only 10% of frogs (85 of 811) had body temperatures outside of the range where *B. dendrobatidis* achieves peak growth and infectivity (17 - 25°C) and fewer than 1% of these (8 of 811) had body temperatures high enough to stop the fungus from growing (28°C). However, by January 2006, 77% of frogs (273 of 354) had body temperatures above 25°C and over 9% (33 of 354) had body temperatures above 28°C. The upward shift of the population's body temperature distribution during the epidemic suggests a population-wide "behavioral fever" response. This is consistent with the idea that the majority of frogs were exposed (and behaviorally responding) to the fungus and supports the hypothesis that stream habitats can become saturated with *B. dendrobatidis* zoospores during an epidemic (Lips *et al*. 2006).

While both uninfected and infected frogs had higher body temperatures during the epidemic than the pre-epidemic population averages, uninfected frogs had higher body

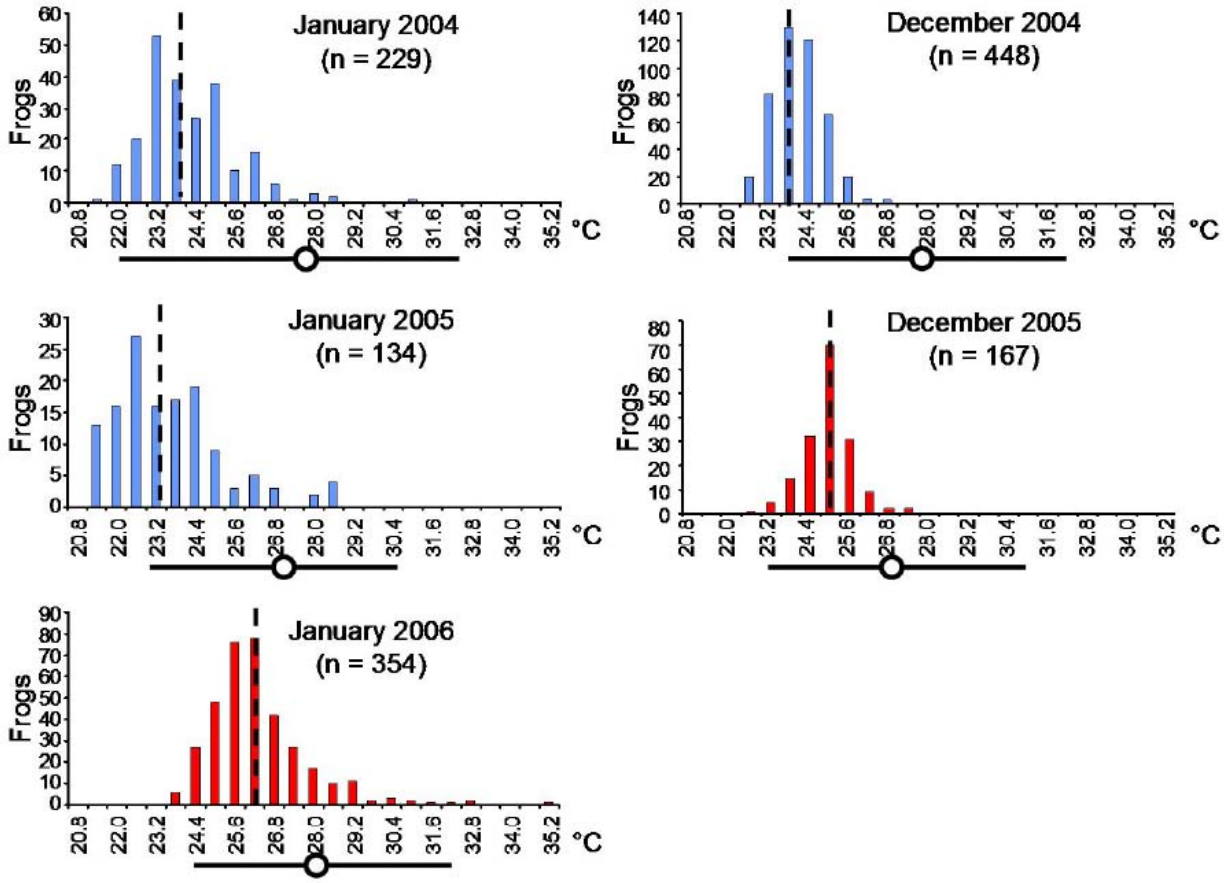
temperatures than infected frogs during both December 2005 and January 2006 (Fig. 4.2). This, coupled with the decrease in odds of infection with increasing body temperature, suggests a strong link between body temperature and susceptibility to *B. dendrobatidis* infection. While most, if not all frogs responded to the pathogen by inducing a behavioral fever, those with the highest body temperatures were more likely to be free of *B. dendrobatidis*. Taken together, these findings indicate that amphibians can mount an effective, behavioral fever response to *B. dendrobatidis* infection in the wild.

Given the relationship between temperature and the standard metabolic rate of amphibians (average $Q_{10} = 2.21$: White *et al*. 2006) the increase in body temperature observed during the *B. dendrobatidis* epidemic would have resulted in an 8.7% (December 2005) and a 20.5% (January 2006) increase in metabolic energy expenditure over pre-infection rates. An increase in energy expenditure could account for the lower body condition of frogs during the epidemic if they were not able to replace lost energy from available environmental resources (Wikelski & Cooke 2006). This would result in a fitness tradeoff between avoiding *B. dendrobatidis* infection and overall physiological performance. While it is also possible that *B. dendrobatidis* infection itself led to the decrease in body condition, a previous study failed to find a link between body condition and infection prevalence (Woodhams & Alford 2004) and no difference in body condition between infected and uninfected frogs was seen in this study. A third possibility is that the decrease in body condition preceded the epidemic and was related to other environmental stressors. This would be consistent with one study, which found an increase in fluctuating asymmetry (a developmental indicator of stress) two years prior

to *B. dendrobatidis*-related declines (Alford *et al*. 2007). However, if body condition did begin to decline prior to the epidemic described here, its onset must have come fewer than 11 months prior to the arrival of *B. dendrobatidis* as body condition did not differ among sampling periods prior to the end of January 2005.

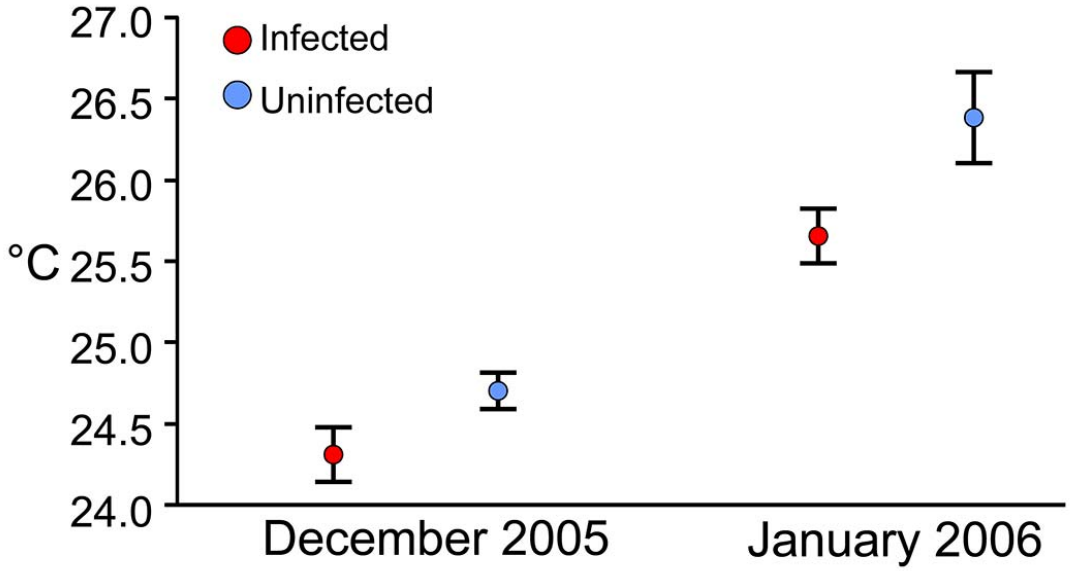
The results of this study provide the first evidence that even small changes in body temperature in the field can dramatically alter levels of susceptibility to *B. dendrobatidis* infection in wild populations. Furthermore, this work demonstrates that ectotherms are capable of mounting a population-level, behavioral response to an epizootic and that this response can be effective in reducing the odds of infection. However, this form of behavioral immunity may incur a fitness cost in terms of an increase in metabolic energy expenditure. The thermal dependency of the relationship between *B. dendrobatidis* and its amphibian hosts demonstrates how the progression of an epidemic can be influenced by complex interactions between the genotypes of both host and pathogen and the environment in which they are found.

FIGURE 4.1



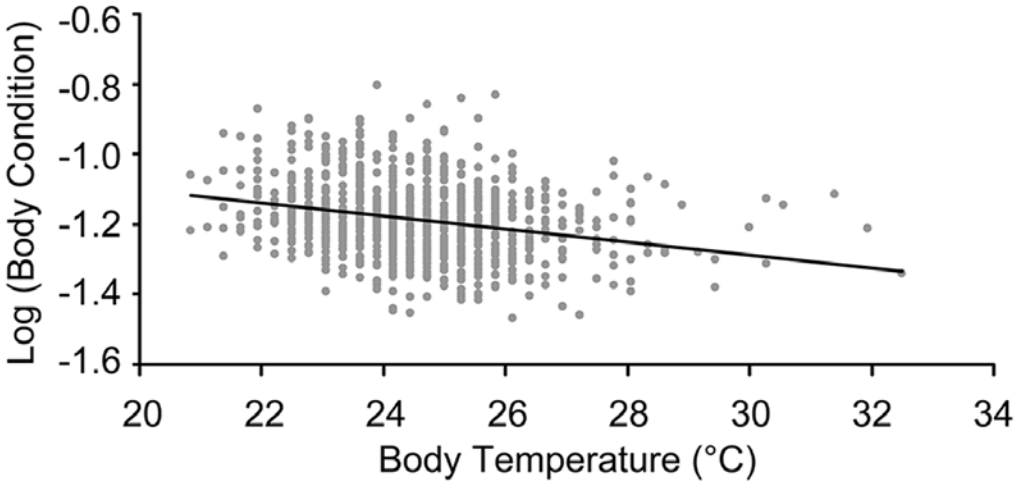
Distribution of *A. zeteki* body temperatures across sampling periods. Body temperatures are higher during the *B. dendrobatidis* epidemic (red bars) than before the epidemic (blue bars). Vertical dashed lines indicate population means. Solid horizontal lines indicate the range of air temperatures and white circles indicate the mean air temperature during each sampling period.

FIGURE 4.2



Average body temperatures of infected frogs are lower than those uninfected for each sampling period. Error bars indicate the standard error of the mean.

FIGURE 4.3



Inverse relationship between the log of body condition and body temperature. Body condition was measured as weight / snout-vent length.

TABLE 4.1

Sampling period	Sample size	Mean	Standard error
January 2004	229	23.92	0.094
December 2004	448	23.82	0.038
January 2005	134	23.36	0.142
December 2005	167	24.65	0.060
January 2006	354	26.04	0.081

Sample sizes, means, and standard errors for body temperature measurements (°C) taken from all frogs over the five sampling periods, regardless of infection status.

TABLE 4.2

Sample	Sample size	Mean	Standard error
Uninfected, December 2005	100	76.46	0.147
Infected, December 2005	19	75.74	0.263
Uninfected, January 2006	45	79.50	0.498
Infected, January 2006	83	78.20	0.274

Sample sizes, means, and standard errors for body temperature measurements (°C) from infected and uninfected frogs during the two epidemic sampling periods.

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CHAPTER V

CONCLUSION

Implications for the conservation of Panamanian golden frogs

Before this research began, it had already become abundantly clear that Panama's golden frogs and many of their Neotropical congeners were in serious trouble. The populations of *A. varius*, *A. chiriquiensis*, and *A. senex* in Costa Rica and far western Panama had already declined and for the most part, disappeared, and evidence implicating the amphibian chytrid fungus, *Batrachochytrium dendrobatidis*, in this south and eastward moving wave of disappearances was building. However, the link between this epizootic and the loss of lower Mesoamerican amphibian diversity was further fortified by the work of Karen Lips and collaborators in El Cope, Panama before and during that community's chytridiomycosis epidemic (Lips *et al*. 2006).

Realizing that the continued progress of this disease through Panama threatened Central America's remaining *Atelopus* species with extinction, members of the conservation group, Project Golden Frog (PGF) sprang into action, establishing a captive breeding program for golden frogs in U. S. zoos and dividing the remaining wild populations into five management units based on qualitative phenotypic differences. However, because no phylogeographic information was available for these amazingly diverse populations, PGF

members had no way of knowing the extent to which these units and captive populations captured the range of genetic diversity among golden frog populations, or even how many species they were managing.

My work (Chapter II) not only demonstrated the genetic and phenotypic distinctness of the five proposed ESUs by quantifying variation in multivariate phenotypes and mtDNA haplotypes among them, but also supported the current status of *A. varius* and *A. zeteki* as separate species. This study's results also suggest that two of the phenotypic characters (body size and extent of dorsal black patterning) that vary among these populations may be associated with adaptive divergence with respect to temperature and UV exposure. Should golden frogs go extinct in the wild, some comfort can be taken in the knowledge that the populations being managed in captivity represent three of the five ESUs (ESUs 1-3), both species, and a wide range of both of these potentially-adaptive phenotypes. If the decision is someday made to re-introduce golden frogs to the wild, this phenotypic and genetic diversity will likely be beneficial in terms of the frogs' continued viability and adaptability to changing environmental conditions.

The extent to which patterns of gene flow and genetic differentiation among golden populations are influenced by properties of the Panamanian landscape was the topic of my second study (Chapter III). The results of this work highlight the importance of riparian habitat corridors as conduits for gene flow among populations and, to a lesser extent, changes in slope along those streams as barriers. If the decision should be made to return captively-bred frogs to the wild, this information will be useful in selecting

suitable locations for reintroductions, and allow for informed predictions of the pattern of recolonization the frogs are likely to take. In addition, this work suggests that anthropogenic changes that alter the connectivity of riparian habitat are likely to affect the viability and evolutionary potential (Moritz 2002) of the remaining wild populations.

While my first two studies implicitly assume that genetic and phenotypic variability are beneficial in terms of golden frogs' surviving and adapting to environmental change, my third study (Chapter IV), in which I examine the relationship between individual behavior and susceptibility to chytridiomycosis infection, clearly demonstrates the effect of such variation on a population level. While most, if not all golden frogs responded to a *B. dendrobatidis* epidemic by inducing a behavioral fever, those that reached the highest body temperatures were more likely to be free of infection. Thus, it appears that the progression of a *B. dendrobatidis* epidemic is influenced not only by interactions among the phenotypes of both the amphibians and the fungus, but also by the environment in which they are found.

Applicability to the conservation of other *Atelopus* species

While members of the genus *Atelopus* exhibit a wide array of morphological diversity, their life histories are much less variable. Most species are diurnally active, locally abundant (at least historically) and found in close association with the streams in which they breed (Lötters 1996). While *Atelopus* occur across a wide range of elevations and latitudes in the Neotropics, most species are found in montane habitats with cool climates

and are thus likely to be susceptible to *B. dendrobatidis* epidemics (Lötters 1996; La Marca *et al.* 2005; Ron 2005). In fact, chytridiomycosis infection (La Marca *et al.* 2005) and an interaction between chytridiomycosis and climate change (Pounds et al. 2006) have been implicated in the declines and extinctions of many *Atelopus* species from undisturbed habitats.

To the extent that the similarities in life history traits among *Atelopus* species translate into similar effects of landscape and climate characteristics on gene flow and susceptibility to *B. dendrobatidis*, the results of this research will be useful in guiding the establishment of conservation priorities for other endangered *Atelopus*. For instance, the results of Chapter II suggest that in the absence of phylogeographic information, conservation efforts should focus on preserving a wide range of phenotypic diversity as phenotypes and genotypes tend to vary concordantly among populations. The results of Chapter III further indicate that conservation efforts should aim to conserve populations from many river drainages, as these are more likely to be genetically distinct from one another. Furthermore, this study highlights the importance of maintaining the integrity of riparian habitat corridors for maintaining patterns of gene flow among *Atelopus* populations. The results of Chapter IV demonstrate that in some cases, *Atelopus* may be able to combat *B. dendrobatidis* infection by behaviorally raising their body temperatures above the pathogen's range of optimal virulence. Maintaining within-population genetic variation, and thus, the opportunity for selection on thermoregulation behavior, may therefore be important in facilitating the frogs' own abilities to persist in the face of an epidemic.

Studies of the genetic, morphological, and ecological variation of other endangered *Atelopus* would undoubtedly improve the ability of conservation initiatives to preserve the functionality of the ecological and evolutionary processes generating these patterns. However, given the rapidity of recent declines and the fact that many species are now represented by a small number of individuals in one or a few populations (La Marca *et al.* 2005), this is not a realistic goal for most *Atelopus*. It is my hope that the type and detail of information amassed in this dissertation with respect to Panamanian golden frogs will be useful in guiding conservation efforts for other *Atelopus* as well.

Directions for future research

The idea that golden frogs can combat this potentially devastating fungal disease on their own represents an uncommon ray of hope for these charismatic frogs. However, the work described in Chapter IV took place in a population of *A. zeteki* with an exceptionally warm and seasonally-dry habitat that appears to be on the very edge of niches of both the frogs and the pathogen (Lötters 1996; Ron 2005). The behavioral fever response may be less effective in countering chytridiomycosis infection in cooler, wetter amphibian populations. In addition, even in populations where behavioral fevers are effective in reducing the odds of infection, there may be a fitness tradeoff due to the metabolic cost of this behavior. Future work clarifying the environmental conditions under which the behavioral fever response is effective in combating chytridiomycosis and the fitness costs associated with this behavior will be critical in generating accurate predictions of the

ultimate geographic extent and biodiversity implications of this emerging, global pathogen.

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