DISSERTATION

MECHANISMS OF POPULATION DIVERGENCE ALONG ELEVATIONAL GRADIENTS IN THE TROPICS

Submitted by

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ABSTRACT

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Elucidating the mechanisms that give rise to population divergence and eventually initiate speciation is a key step for understanding the evolution of biodiversity. Most theories of differentiation and speciation have traditionally focused on geographically isolated populations. However, there is growing evidence that speciation can occur due to divergent selection despite initially high gene flow. My doctoral dissertation project investigates the effects of environmental heterogeneity and geography in promoting phenotypic and genetic divergence along elevation gradients in natural populations of a poison frog, *Epipedobates anthonyi*, across the landscape with a focus on environmental variation along elevational gradients. I studied populations distributed along a broad elevational gradient (0–1800 m above sea level) on the western slope of the Andes of southern Ecuador. First, I examined the relative roles of geographic distance and environmental gradients on genetic and phenotypic divergence. I found that populations are phenotypically divergent in size, color, and male advertisement calls, but they exhibit low genetic divergence at neutral loci. There is substantial gene flow between populations throughout the lowlands, but populations at higher elevations are relatively isolated. This is mainly due to a mountain ridge acting as a physical and possibly environmental barrier between northern and southern populations. Within elevational gradients, geographic distance corrected for topography is the main factor explaining both genetic and phenotypic divergence. However, when controlling for the effect of topographic distance, environmental conditions,

such as temperature and precipitation between sites best explain observed patterns of genetic divergence, whereas environmental conditions at a given site best explains differences in phenotypic traits, presumably due to divergent selection pressures.

To study the effect of temperature variation along elevational transects on adaptive divergence, I measured thermal tolerance of tadpoles across elevation. I found that populations from higher elevation had higher cold tolerance, suggesting that changes in temperature along elevation may cause divergent selection in thermal tolerance. Additionally, tadpoles from all sites have the ability to shift their thermal tolerance in response to previous exposure to different temperatures.

Finally, to examine the degree of local adaptation to environmental conditions at high and low elevations, I conducted a reciprocal transplant experiment. I evaluated populations from high and low elevations from two elevational transects. Overall, I found that all populations have higher reproduction rates at low elevation. In fact, at high elevation, populations had very low reproductive rates or did not reproduce at all. However, variation in life-history traits differed between transects. Populations from one transect revealed a pattern that was consistent with the expectation under local adaptation, namely, low elevation frogs had higher reproduction than high elevation frogs at the low elevation site. In contrast, populations from the other transect had a pattern that would be expected under countergradient variation, namely higher elevation frogs had higher reproduction at the low elevation site. Intriguingly, low elevation frogs had overall higher reproduction rates than high elevation frogs, suggesting that frogs from low elevation have higher fecundity than their counterparts at high elevation.

Overall, the findings of my dissertation suggest that (i) phenotypic divergence occurs in the face of gene flow, (ii) environmental variation along elevation, particularly temperature, is a force that drives population divergence, and (iii) the influences of environmental conditions on populations are variable at the intraspecific level.

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1. INTRODUCTION

Elucidating the mechanisms by which populations diverge is essential for understanding the origins of biodiversity. Population divergence is determined by the interplay between the homogenizing effect of gene flow, and differentiating processes such as genetic drift and adaptive divergence in response to different environmental selection pressures (Mayr 1963; Lenormand 2002; Bolnick and Fitzpatrick 2007). Due to the homogenizing effect of gene flow, most theories of differentiation and speciation have focused on geographically isolated populations, in which drift and selection interact to cause evolutionary change (Mayr 1963; Slatkin 1987; Coyne and Orr 2004). However, mounting evidence suggests that speciation can occur despite initially high gene flow (Lenormand 2002; Rundle and Nosil 2005; Nosil 2008; Fitzpatrick et al. 2014; Seehaussen and Wagner 2014). Through this "divergence with gene flow" process, divergent natural selection can cause differentiation in ecologically important characters when populations use different habitats (Schluter 2001; Gravilets 2003; Bolnick and Fitzpatrick 2007). Despite the large body of literature devoted to understanding the interplay between gene flow and adaptive divergence in natural populations, more empirical work is needed to understand the relative importance of geographic barriers and ecological factors as drivers of population divergence (Sexton et al. 2014). Likewise, there are many gaps in our knowledge about the conditions under which divergence with gene flow occurs in nature and the mechanisms involved (Bolnick and Fitzpatrick 2007; Sexton et al. 2014; Langin et al. 2015). The factors promoting or preventing adaptive divergence remain poorly characterized in most natural populations, as well as how consistently different species respond to similar selective pressure (Hereford 2009; Keller et al. 2013).

Among the environmental factors that can exert divergent selection pressures on organisms, temperature is arguably one of the most important (Keller and Seehausen 2012). Effects of temperature on biological processes are pervasive as it can affect organisms from the molecular to the organismal level (Angilleta et al. 2002; Navas et al. 2007; Pörtner et al. 2006). Temperature can also affect organisms indirectly by influencing resource availability and biotic interactions, such as predation, competition, and parasite load (Keller and Seehausen 2012). Changes in habitat temperature can vary across wide temporal and spatial scales. For example, temperature can fluctuate temporally across hours in a day or across multiple years, as well as spatially across altitudinal or latitudinal gradients, which can affect organisms' responses to fluctuations in temperature (Pörtner et al. 2006). Studies have shown that temperature can cause local adaptation, but those studies are still scarce and many gradients are relatively overlooked, such as elevational gradients (Hoffmann et al. 2002; Kavanagh et al. 2010; Keller and Seehausen 2012). Species inhabiting heterogeneous habitats, such as elevational gradients, are subject to spatially varying selective pressures. Therefore, elevational gradients are considered a particularly suitable system to study the interactions between environmental variation and population divergence over short geographical scales (Miaud and Merilå 2001; Keller et al. 2013).

Tropical mountains

Tropical mountains are hotspots of species richness, diversity, and endemism, and are among the most vulnerable ecosystems on Earth (Myers et al. 2000; Tewksbury et al. 2008). The Andes mountain range of South America provides a rich natural laboratory to answer questions about how geographic barriers and environmental factors promote population divergence and

speciation. Most explanations for speciation in the Andes invoke vicariance and environmental gradients as the main modes of differentiation (Moritz et al. 2000; Patton and Smith 1992; Graham et al. 2004; Guarnizo et al. 2009). Previous work has shown that many sister taxa within the Neotropics have parapatric or allopatric distributions, suggesting that geographic isolation should represent the null diversification hypothesis for vertebrates in the Neotropics (Brumfield and Edwards 2007; Patton and Smith 1992; Roy et al. 1997). Contrary to this hypothesis of speciation is the gradient model, which poses that strong divergent selection along ecological gradients causes divergence, and is followed by assortative mating that eventually reduces gene flow, leading to speciation. The frequent location of hybrid zones in ecotones and phenotypic differentiation between populations in adjacent habitats provides evidence for this model (Moritz et al. 2000). Indeed, some evidence shows that ecological gradients may influence differentiation in Andean anurans and insects (Lynch and Duellman 1997; Graham et al. 2004; Willmott et al. 2001).

Speciation along elevational gradients is thought to play a large role in the tropics, due to the greater seasonal uniformity in tropical mountains compared to those in temperate zones (Janzen 1967; Ghalambor et al. 2006; Kozak and Wiens 2010). This thermal stability is predicted to lead to the evolution of narrow thermal tolerance in organisms inhabiting these areas. In this case, if these narrowly-adapted organisms were to disperse above or below their natural range, they would more likely encounter a climate to which they are not adapted. From this conceptual model, it is implied that reduced dispersal across tropical mountains should lead in turn to greater genetic divergence between populations, potentially resulting in greater species turnover along elevational gradients. If selective pressures across elevational gradient are strong enough, they

could promote adaptive divergence even in the presence of gene flow. Hence, if ecological differences are significant, adaptive population divergence will be observed along the elevational gradient, which might or might not be correlated to levels of genetic differentiation in neutral molecular markers (Nosil 2008). Studies on the distribution of mountain species have revealed that, although some taxonomic groups (e.g. salamanders) exhibit niche conservatism in the temperate zone and niche divergence in the tropics, in many vertebrate taxa (e.g. birds and lizards, and when many groups are combined), tropical sister species have greater similarity in climatic distribution than temperate sister species (Kozak and Wiens 2007; Hua and Wiens 2009; Cadena et al. 2012). These contrasting results may imply that elevational zonation has different effects on organisms, depending on the taxa. While for some taxa, thermal niches are more conserved in the tropics, for other taxa, elevation zonation of climate in the tropics might be promoting local adaptation to thermal niches.

Study system

Amphibians are extremely diverse and vulnerable in tropical mountains, including the Andes (Stuart et al. 2004). Lynch and Duellman (1997) presented a speciation model for Andean anurans based on vicariance, suggesting that speciation is driven by a combination of isolation and divergent selection corresponding to differences in habitats occupied. Neotropical frogs are often sensitive to changes in temperature, precipitation, and the seasonality of both factors. Thus, those climatic conditions are likely important in limiting their distribution and, potentially, enhancing population divergence (Navas et al. 2008). High sensitivity to climate variables, relatively poor dispersal abilities (but see Funk et al. 2005), and high species richness and turnover in the Andean slopes make amphibians an excellent group to study the role of

environmental gradients along elevation in shaping gene flow, selection, and population divergence.

I studied the poison frog *Epipedobates anthonyi*. This frog species is abundant along the western slopes of the Andes in southern Ecuador and northern Peru and occurs from sea level to 1800 m (asl). Prior to my dissertation work, some observations of size, color, and acoustic variation had been described for a small number of populations and individuals (Santos et al. 2014). Variation in these traits is particularly interesting in the context of understanding how environmental variables promote population divergence and speciation, as color and male calls are important for behavioral and reproductive isolation (Summers et al. 1999; Ryan 2001; Funk et al. 2009; Twomey et al. 2014). Moreover, environmental variation along elevation and in average annual temperature might play an important role in differentiation in *E. anthonyi* (Graham et al. 2004). *Epipedobates anthonyi* has also been bred successfully in captivity (*pers. comm.*, Centro Jambatu for Research and Conservation of Amphibians). Together, these characteristics make this species an excellent study organism to develop a research program that combines analytical and experimental research approaches geared towards understanding the microevolutionary processes that underlie population divergence along elevational gradients.

Research overview

My dissertation work examines the mechanisms that underlie patterns of phenotypic and genetic divergence in natural populations of *Epipedobates anthonyi* across the landscape, with a focus on elevational gradients. To that end, I investigated the role of geographic features and environmental variation on putatively adaptive traits, and on neutral genetic divergence along

four elevational gradients. First, I investigated the levels and patterns of phenotypic and genetic divergence across the distributional range of the species in Ecuador, with a subset of populations corresponding to four elevational gradients. Then, I examined if the patterns of phenotypic and genetic divergence corresponded to patterns of isolation by distance, isolation by environmental resistance between populations, or isolation by environmental differences between populations (**Ch. 2**). Because temperature is the most obvious environmental variable causing divergent selection along elevational gradients, I examined the thermal limits of tadpoles across elevation in two elevational transects (**Ch. 3**). Finally, to investigate if populations were locally adapted to their local elevation, I conducted a reciprocal transplant experiment between populations from high and low elevation in which I monitored reproductive rates during seven months (**Ch. 4**).

Conclusions and significance

The main objective of my dissertation was to advance understanding of the mechanisms that drive population divergence, an initial step in the process of speciation, along environmental gradients. We took advantage of elevational gradients because they allow comparisons of populations exposed to different environments at a relatively small scale that could potentially be experiencing gene flow. Our results indicate that although geographic barriers and distance are primary actors in the differentiation process, at smaller scales, environmental variation along elevation also plays an important role. Remarkably, we found that the influence of environmental variation, mainly temperature and precipitation, on populations is two-fold: it reduces gene flow between populations, while exerting divergent selective pressures on phenotypic traits among sites.

Because temperature is the most obvious environmental variable along elevation gradients, we examined if variation in temperature across elevation causes divergent selection in the thermal limits of tadpoles. Overall, our findings suggest that temperature acts as a selective force along elevation and, contrary to the general expectations, revealed phenotypic plasticity in thermal limits in this tropical anuran. Finally, with the reciprocal transplant experiment, I found that environmental variation along elevation has an important effect on life history traits, with low elevation populations having higher reproductive rates than their high elevation counterparts.

Collectively, the results of my dissertation reveal striking patterns of phenotypic divergence in the face in gene flow, potentially determined by the concomitant effects of distance and environmental variation along elevational gradients. On top of that, they revealed that there is intraspecific variation in the effect of environmental conditions on populations' patterns of phenotypic divergence. Finally, I hope that the data presented here provide foundation for many subsequent studies aimed at understanding the mechanisms and implications of adaptive evolutionary divergence of natural populations, especially in vulnerable biodiversity hotspots.

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2. POPULATION DIVERGENCE WITH GENE FLOW IN THE ANDES: BARRIERS, DISTANCE, AND THE TWO-FOLD EFFECT OF ENVIRONMENTAL VARIATION[1]

Summary

Population divergence is shaped by the interplay between the homogenizing effects of gene flow and the differentiating processes of genetic drift and local adaptation to varying environmental selection pressures. We examined the mechanisms that underlie patterns of phenotypic and genetic divergence in populations of the poison frog, *Epipedobates anthonyi*, along four elevational gradients (0–1800 m above sea level) on the western slope of the Andes of southern Ecuador. We found that although populations are phenotypically divergent in size, color, and acoustic traits across the study area, genetic divergence is low at neutral microsatellite loci. There is substantial gene flow between populations through the lowlands, but populations at higher elevations are more isolated. This is mainly due to a cold, dry mountain ridge that prevents dispersal between adjacent drainages. Within elevational transects, topographic distance is the most important factor explaining patterns of genetic and phenotypic divergence. When controlling for topographic distance, we found that genetic divergence increases as a function of resistance to movement between populations mainly due to changes in temperature, whereas phenotypic divergence is mainly influenced by temperature differences among sites in the environment. These results indicate that although geographic barriers are important drivers of population divergence in this system, at a finer scale, environmental variation has a two-fold effect on populations divergence. On the one hand, environmental resistance in the intervening landscapes between sites reduces gene flow, while on the other hand, environmental differences among sites exert divergent selective pressures on phenotypic traits. This work shows that it is

[1] Co-authored by Daryl Trumbo and W. Chris Funk (Colorado State University)

important to study both genetic and phenotypic divergence to better understand the process of population divergence.

Introduction

Population divergence can be an initial step in the process of speciation (Mayr 1963; Coyne and Orr 2004). Therefore, studying the drives and mechanisms by which population divergence is fundamental to advance our understanding how diversity arises. Numerous studies have focused on the patterns of genetic divergence among spatially separated populations, and how they can give us insights into the processes that drive population divergence. For instance, in the absence of selection, populations can be differentiated due to geographical barriers, or become differentiated with increasing distance, the latter pattern known as isolation-by-distance (IBD; Wright 1943; Sexton et al. 2014). Landscapes through which organisms disperse vary in their environmental suitability and resistance to movement; therefore, landscape resistance may predict the amount of gene flow between populations better than IBD (isolation-by-resistance, IBR; Cushman et al. 2006; McRae and Beier 2007). Aside from geographic distance and the landscape between populations, environmental conditions at which organisms are exposed where they occur can also play a crucial role in increasing genetic differentiation through selection, a pattern known as isolation-by-environment (IBE; Wang and Summers 2010; Bradburd et al. 2013; Sexton et al. 2014).

Patterns of phenotypic divergence among populations have received relatively less attention, and simultaneous examination of the role of distance, resistance to movement, and environmental selection on patterns of phenotypic divergence remain relatively scarce, yet important for more

deeply understanding the processes causing population divergence and speciation (but see Lowe et al. 2012; Richter-Boix et al. 2013). When phenotypic divergence is mainly determined by drift and gene flow, we would expect a correlation between neutral genetic divergence and phenotypic divergence, and that the landscape features explaining both are similar (Merila and Crnokrak 2001; Lowe et al. 2012; Sexton et al. 2014; but see McKay and Latta 2002). For instance, geographic barriers or the landscape through which organisms disperse, but not environmental differences among sites, would be explaining both measures of divergence. Conversely, if populations are exposed to different environmental conditions, which cause phenotypic divergence, a correlation between neutral genetic divergence, and phenotypic divergence is not necessarily expected (Storz 2002; Leinonen et al. 2006). Although a correlation could occur if adaptive divergence is strong enough to subsequently reduce gene flow, as in ecological speciation (Rundle and Nosil 2005; Schluter 2009). If different environmental conditions among sites cause phenotypic divergence, but not genetic divergence, that would indicate divergent selection on only phenotypic traits without impeding gene flow (Nosil 2008; Pinho and Hey 2010). Therefore, simultaneously testing hypotheses on the effect of landscape attributes behind both neutral genetic and phenotypic divergence can elucidate the interactions between gene flow and divergent selection across the landscape.

Tropical mountains are hotspots of species richness, diversity, and endemism (Myers et al. 2000), and are among the most vulnerable ecosystems on Earth (Tewksbury et al. 2008). Nonetheless, the evolutionary mechanisms underlying observed patterns of species richness in these regions remain poorly understood (Cadena et al. 2012). Recent work demonstrates that, in addition to geographic isolation, ecological differences along tropical elevational gradients can

drive diversification in montane groups, leading to local adaptation and, ultimately, promote speciation (Bridle et al. 2009; Guarnizo et al. 2009; Cadena et al. 2012). Tropical montane amphibians are exceptionally diverse, endemic, endangered, and generally thought to have poor dispersal abilities. Hence, their dispersal rates and patterns may be particularly sensitive to landscape features and environmental gradients. However, a few studies have shown amphibian dispersal may not always be so limited (Funk et al. 2005). High sensitivity to climate variables, relatively poor dispersal abilities, and high species richness and turnover make tropical amphibians an excellent group in which to study the role of environmental gradients in gene flow and selection.

We studied the role of landscape features on phenotypic and genetic divergence in populations of *Epipedobates anthonyi*. This is an abundant poison frog species distributed along the western slopes of the Andes in southern Ecuador and northern Peru that occurs from sea level to 1800 m (asl). Some observation of morphological and acoustic variation had previously been described in this species (Santos et al. 2014). However, a detailed characterization of these traits and genetic diversity among populations throughout most of the distributional range and testing specific hypothesis about their microevolutionary processes was lacking. Variation in these traits is particularly interesting in the context of the study of environmental variables promoting population divergence and speciation, as color and male calls are important for behavioral and reproductive isolation (Coyne and Orr 2004; Summers et al. 1999; Ryan 2001; Funk et al. 2009; Twomey et al. 2014). Moreover, environmental variation with elevation and average annual temperature might play an important role in differentiation in *E. anthonyi* (Graham et al. 2004). Together, these characteristics make this species an excellent study organism to develop a

research program geared towards understanding the microevolutionary processes that underlie observed population divergence along environmental gradients, namely elevation.

Here, we assessed (1) the degree of phenotypic divergence across the landscape in ecologically relevant traits: size, color, and male advertisement calls, and (2) genetic divergence across the landscape, using neutral microsatellite markers to examine to what extent gene flow and selection contribute to phenotypic and genetic divergence. We then investigated the relative roles of resistance vs. selection (IBR vs. IBE) in the observed patterns of population divergence (genetic and phenotypic). We tested two main hypotheses: (1) phenotypic divergence among populations occurs mainly due to differential environmental selection (IBE). Under this hypothesis, we predicted that different environmental conditions at site would best explain observed patterns of phenotypic divergence between populations. (ii) Population genetic divergence is primarily caused by resistance to movement among populations due to topography or barriers (IBR). Under this hypothesis, we predict that geographic distance and other barriers (e.g. mountain ridges) would best explain the observed patterns of genetic divergence between populations.

Methods

Data Collection

Field Sampling

We sampled a total 35 populations of *Epipedobates anthonyi* from 2012 to 2014 encompassing the species range in Ecuador (535 individuals; Table 2.1; Fig. 2.1). To focus on elevational gradients, a subset of these localities (n = 18) were located along four transects that spanned a

1500 m elevational gradient. On each transect, we sampled five localities at 400 m intervals from ~200 m to 1800 m (200, 500, 900, 1300, 1700 m asl, approximately), except the southernmost transect which only included three sites (~ 500, 900, 1300 m asl). The remaining localities were mainly in the lowlands, with a few mid-elevation populations scattered throughout the species' range. At each locality, we collected ~20 tissue samples and photographs, and recorded 1–5 male advertisement calls. Additionally, body size and environmental temperature were recorded. Tissue samples were stored in 95% ethanol. Every collected specimen was fixed and preserved following standard museum collection protocols and deposited in the scientific collection of Centro Jambatu for Research and Conservation of Amphibians (CJ, Quito-Ecuador). All procedures were approved by the Colorado State University Institutional Animal Care and Use Committee.

Phenotypic data

To assess phenotypic divergence, we focused on size, color, and two acoustic traits from male advertisement calls (a spectral parameter [maximum frequency] and a temporal parameter [note repetition rate]). For size, we measured snout-vent length (SVL) of 454 preserved specimens with an electronic Digital Caliper (0–150 mm). Sex was determined by dissection and the presence of vocal slits in adult males. Sexual maturity of females was determined by the presence of eggs or convoluted oviducts. In order to quantify individual color variation, photographs of 251 adults from 19 populations were taken with a black-white-gray standard QPcard 101 (standard color reference card) using an Olympus E-PL1 camera (Table S1). Ambient light correction was implemented using Adobe Photoshop CS version 8 (following Stevens et al. 2007). Corrected photographs were analyzed using ImageJ. To score color, we

used the RGB Measure plug-in to obtain the average red (R), green (G), and blue (B) scores for a standard area of the background dorsal skin (Dugas et al. 2015). Briefly, we obtained the scores of six 20 x 20 pixels, two at each side of the head dorsum, two at each side at mid-dorsum, and two at the posterior region of dorsum. We were careful not to include the light-colored dorsal stripes. We also scored a standard area of the background QPcard for standardization across photographs (Dugas et al. 2015). Given that photographs were taken in the field without standard lighting conditions, we calculated the residuals of a regression between the frog's dorsal skin scores and the QPcard scores, and used those residuals for subsequent analyses (Dugas et al. 2015).

Call recordings were made with a Fostex FR-2LE Field Memory digital recorder and a Sennheiser K6–ME 67 unidirectional microphone, with a sampling rate of 44 kHz. We recorded calls from the field at < 3 m from the calling male (n = 74). We documented environmental temperature and relative humidity and snout-vent length (SVL) of the recorded male. Recordings were processed in the Sound Analysis Software RAVEN 1.4 (Charif et al. 2004) on a MacOSX. Spectral parameters were obtained using *warbleR* package in R (R Development CoreTeam 2015, Araya-Salas and Smith-Vidaurre, 2016). We focused our analyses on one temporal parameter and one spectral parameter: note repetition rate (= number of notes/call length) and note dominant frequency, respectively.

Genetic data

In order to characterize patterns of genetic divergence, we developed 12 microsatellite loci for *Epipedobates anthonyi* at the EG Cornell Facility. Genomic DNA was extracted from tissue

using a Qiagen DNA Blood and Tissue Kit and eluted with 100 microliters AE buffer and quantified with a Qubit 2.0 Fluorometer. Genomic DNA was then digested with the restriction enzymes *AluI, RsaI,* and *Hpy166II*, in three separate reactions. After end polishing and adenylation, Y-adaptors were ligated with a T4 DNA ligase to the fragmented DNA. Digested and ligated fragments were then enriched for microsatellites by hybridization to biotinylated oligonucleotide repeat probes. Enriched genomic fragments were amplified and barcoded by PCR. Libraries with unique barcodes were pooled together and sequenced at the Sequencing and Genotyping Facility at the Cornell Life Sciences Core Laboratory Center (CLC) for 2 x 250 paired end sequencing on an Illumina MiSeq.

Microsatellites were scanned and primers designed using the program msatcommander 1.0.3 for Mac OSX. We prioritized tetramers, long repeats and annealing temperatures that would be amenable to multiplexing (van Asch et al. 2010). Loci that complied with these characteristics were compared against each other using Geneious version 8.0 (http://www.geneious.com; Kearse et al. 2012) to avoid selecting the same loci twice. We chose the ones that showed polymorphisms and amplified in a handful of individuals from a subset of the seven populations distributed throughout the study region. Loci that amplified were then scanned using the M13 protocol (Schuelke 2000), and tested in 20 individuals from two populations to check for null alleles, Hardy-Weinberg equilibrium, and linkage disequilibrium using GENEPOP (Raymond and Rousset 1995). Loci that passed this round of tests were chosen for genotyping all populations and individuals using 5' fluorescently labeled primers. Multiplex PCR was optimized using Multiplex Manager 1.0 (Holleley and Geerts 2009). Ten microsatellites were amplified in 10 μL multiplex reactions with four loci per reaction (Langin et al. 2015), using

Qiagen's Type-it Microsatellite PCR kit. PCR products were run on an Applied Biosystems 3730xl DNA Analyzer, and results were scored automatically and checked manually using the program Geneious version 8.0 (http://www.geneious.com; Kearse et al. 2012).

Environmental and geographic data

We collected environmental data from online GIS databases for 25 continuous variables hypothesized to affect *E. anthonyi* movement, gene flow, and phenotypes across the study area. These environmental variables included 19 temperature and precipitation variables at 1 km resolution, vegetation density (enhanced vegetation index), and percent tree cover at 250 m resolution, and elevation at 30 m resolution (Huete et al. 1994, 2002; Farr and Kobrick 2000; Rabus et al. 2003; Hansen et al. 2003; 2005; Hijmans et al. 2005; worldclim.org, modis.gsfc.nasa.gov, jpl.nasa.gov/srtm). Further, we calculated heat load index, topographic roughness, and compound topographic index of wetness from the digital elevation model at 30 m resolution using the Geomorphometric and Gradient Metric Toolbox v. 2.0 in ArcGIS v.10.3.1 (Moore et al. 1993; Gessler et al. 1995; Blaszczynski 1997; McCune and Keon 2002; ESRI 2015; arcgis.com).

Since many of these environmental variables were likely to show collinearity across the study area, we calculated Pearson's correlation coefficients among all variables using ENMTools v. 1.3 (Warren et al. 2010) and removed variables that were strongly correlated with more than one variable ($r > 0.7$). This resulted in a dataset of 12 uncorrelated environmental variables: annual mean temperature, annual precipitation, compound topographic index of wetness, enhanced vegetation index, heat load index, precipitation of coldest quarter, precipitation seasonality,

precipitation of the warmest quarter, temperature annual range, percent tree cover, topographic roughness, and isothermality (mean diurnal range/temperature annual range; Table 2.2).

To calculate environmental dissimilarity between pairs of sites, we used ArcGIS to extract environmental values at each study site and calculated an environmental dissimilarity matrix. These environmental dissimilarities represented the hypothesis of isolation due to environmental conditions at which populations are exposed to (IBE). To assess the intervening environmental conditions between pairs of populations, resistance surfaces were created from these environmental variables using the Raster Calculator in ArcGIS. Higher resistance to movement was assigned to habitats that were colder, drier, more seasonal, sparsely vegetated, steeply sloped, and with high heat load indices (i.e. solar radiation). We used Circuitscape v. 4.0 (McRae 2006; McRae et al. 2008) to calculate an environmental resistance matrix between all sites for each environmental variable. Circuitscape resistances can often be correlated due to the effects of shared geographic distances between sites (McRae 2006, McRae et al. 2008). Therefore, we removed resistance matrices with high Pearson correlation coefficients ($r > 0.7$). This resulted in a final resistance matrix dataset of five uncorrelated variables: annual mean temperature, annual precipitation, enhanced vegetation index, precipitation seasonality, and topographic roughness. These environmental resistances represented hypothesis of isolation by resistance from one population to another (IBR). For our isolation by distance (IBD) null model, we also calculated the topographically-corrected distance between all sites using the 3D Analyst toolbox in ArcGIS.

Analyses

Phenotypic analyses

We used linear models to test whether there were differences among populations in size, color, note dominant frequency, and note repetition rate. Model predictors were 'transect' and 'elevation' for all the models. For the models of size and color, we also included sex as a predictor, to control for known size differences between males and females, and to assess if the patterns of phenotypic variation across populations were similar in both sexes. For the two call traits, we included body size and the recording temperature as predictors. Body size and temperature are known to affect spectral and temporal parameters, respectively (Ryan 2001). Additionally, we ran a principal component analyses (PCA) on size and color scores (red, blue, and green scores) to compare overall patterns of phenotypic variation with patterns of genetic variation. We did not include call data in this analysis because sample size would have been too small, as calls were obtained from a subset of males. For subsequent analyses in which we wanted to explore the environmental and geographic differences that explained phenotypic differences between populations, we obtained Euclidean distances between pairs of populations for the four studied traits separately.

Genetic analyses

Standard populations genetic analyses were carried out to estimate variance within and among populations. Genotypes were checked for null alleles and scoring errors using MICROCHECKER 2.2.3 (van Oosterhout et al. 2004). Allele and genotype frequencies, exact probabilities for Hardy-Weinberg proportions, and exact probabilities for linkage disequilibrium were calculated with GENEPOP (Raymond and Rousset 1995) and *adegenet* package in R

(Jombart 2008). Bonferroni corrections were applied to determine the significance of Hardy-Weinberg and linkage results (Rice 1989). Expected heterozygosities, number of alleles per locus, allelic richness, and metrics of genetic differentiation among populations (F_{ST} and D_{PS}) were measured with *adegenet* and *PopGenReport* packages in R (Jombart 2008; Adamack and Gruber 2014). Effective population sizes (N_e) were calculated using OneSAMP and NeEstimator 2.01 using the molecular co-ancestry method (Tallmon et al. 2008; Do et al. 2014). We estimated if there was an isolation-by-distance pattern using IBD Web Service (IBDWS v. 3.23, Jensen et al. 2005).

To estimate the number of genetic demes (K) in a sample and to assign individuals to one or more of these demes based on multilocus genotypes, we used the program STRUCTURE 2.3.4 (Pritchard et al. 2000). STRUCTURE uses a Bayesian statistical approach to compute the likelihood $L(K)$ for deme assignment. This approach uses Hardy-Weinberg proportions and linkage equilibrium between loci within populations and then assigns individuals to one or more of these populations. We used the admixture model that assumes gene flow among populations and correlated allele frequencies. We used an initial burn-in of 100,000, followed by 3,000,000 repetitions. To infer the number of clusters we used the ΔK method using STRUCTURE HARVERSTER (Evanno et al. 2005; Earl and vonHoldt 2012). We performed 10 runs for each K, and calculated the mean ln $P(D)$ across runs for each K. Subsequent runs were executed for detecting the presence of sub-structure within each cluster when $K = 1$ was identified for a given deme.

Landscape effects on phenotypic and genetic divergence

We studied the relationship between environmental resistance between sites and environmental differences among sites (i.e. explanatory variables) with genetic and phenotypic distances among sites (response variables). We also included topographically-corrected distance between sites as an explanatory variable for genetic and phenotypic distance models. We used two methods: multiple linear regressions on distance matrices (MRDM) and linear mixed models (LMM). The main difference between the MRMD and LMM is that, in LMM, we used a random effect that accounted for the dependency between pairwise topographic distances (Clarke et al. 2002; Van Strien et al. 2012). For genetic distance, we used pairwise F_{ST} (genetic distance), and for phenotypic distance, we used four variables: size (snout-vent length), color (redness), note repetition rate (notes/second), and note dominant frequency (Hz).

For the two methods used (MRDM and LMM), we ran three sets of models for each response variable: (1) environmental differences as explanatory variables; (2) resistance-based distances as explanatory variables; and (3) the significant explanatory variables identified from the models selected in runs 1 and 2 together. For phenotypic data, we included genetic distance (F_{ST}) as an explanatory variable in runs 1 and 2 (and 3 if selected in any) to test whether phenotypic distance is mainly explained by neutral genetic distance, which would indicate phenotypic divergence caused by isolation. For call data, we included genetic distance, snout-vent length (of the calling male), and temperature (when recording the call) as explanatory variables for runs 1 and 2.

The first method employed, MRDM, is a nonparametric method designed for distance matrix analysis, which uses random permutations of the rows and columns to calculate regression

coefficients and significance values for the effects of multiple explanatory variables on a response variable (Legendre et al. 1994). This technique was shown by Balkenhol et al. (2009) to provide a good balance between type-1 error and power in a landscape genetics context. We conducted MRDM using PERMUTE 3.4 alpha 9 using a forward selection followed by a backward elimination procedure (Legendre et al. 1994; Balkenhol et al. 2009; Trumbo et al. 2013). Significant explanatory variables were retained in models using Bonferroni-corrected *P*-to-enter and *P*-to-remove values of 0.05, with 1000 random permutations of the dependent matrix per step.

For the second method, the linear mixed effects models (LMM), has been recently been used convincingly for landscape genetics analyses (van Strien et al. 2012; Dudaniec et al. 2013; Phillipsen et al. 2015; Row et al. 2015). We used the explanatory variables (environmental resistances and dissimilarities) as fixed effects, and the response variable was either the genetic or phenotypic distances. As we mentioned above, this method allowed us to include a random effect that accounted for the dependency between pairwise topographic distances (Clarke et al. 2002; Van Strien et al. 2012). The models were estimated using the 'lmer' package in R (Bates et al. 2014; R Core Team 2016). We used the ML estimation for model selection using Akaike's Informative Criterion (AIC), but the REML estimation to obtained unbiased estimates of the variance (Zuur et al. 2009). All explanatory variables were standardized and centered around their mean prior to the analyses for the REML estimates to be the same as in a linear regression (Clarke et al. 2002).

Results

Phenotypic divergence

Populations from *Epipedobates anthonyi* show phenotypic divergence in color, size, dominant frequency, and note repetition rate (Figs. 2.2 and 2.3). For color, populations had one of two major color morphs: red or brown. Red morphs were found along the two northern elevational transects (Transects 1 and 2), and brown morphs were found in the western lowland sites closer to the coast and southern two transects (Transects 3 and 4; Figs.2. 2 and 2.3). On average, frogs from northern transects 1 and 2 (red morphs, mean SVL in adults = 20.49 mm) were larger than frogs from the southern transects 3 and 4 (brown morphs; mean SVL in adults = 18.96 mm; $p < 0.001$, Table 2.3). Among red morphs, populations at higher elevations were larger, and exhibit brighter shades of red ($p < 0.001$; Fig. 2.3). Although females were considerably larger than males (mean SVL in adult females: = 20.71mm, mean SVL in adult males = 18.82), patterns of size variation across transects and populations did not differ between them (Figs. 2.4 and 2.5). As size is typically negatively related to dominant frequency, and note repetition rate is typically positively related to recording temperature, we included size and recording temperature as predictor variables when analyzing call data. Dominant frequency decreased with elevation only in red morphs, but it can be due to changes in size and recording temperature (Table 2.3). Note repetition rate, on the other hand, was only explained by recording temperature (Table 2.3; Fig. 2.3).

Genetic divergence

One of the ten original loci was removed due to missing values in a subset of populations (Table 2.4). Therefore, results presented here are based on nine microsatellite loci of 535 individuals.

All loci were independent and there was no evidence of departure from Hardy-Weinberg equilibrium (Table 2.4). Overall, we found low levels of genetic differentiation between populations (F_{ST} mean = 0.04, range = 0.01–0.12).

For the STRUCTURE analyses, the optimal number of clusters based on the ΔK method in STRUCTURE HARVERSTER was $K = 3$ (Evanno et al. 2005; Earl and vonHoldt 2012). However, high admixture occurred between all the clusters with generally decreasing admixture at higher elevations. The three major population groups corresponded to (i) the northernmost populations (blue in Transects 1 and 2); (ii) the higher populations of Transect 3 (yellow); and (iii) lowland western and southernmost populations (green lowlands and Transect 4; Fig. 2.1). Genetic diversity decreased at higher elevations ($p < 0.001$, adj. $r^2 = 0.46$ for allelic richness; $p < 0.001$, adj. $r^2 = 0.44$ for heterozygosity, Fig. 2.6, Table 2.4). There was also a weak positive correlation between geographic and genetic distance as measured using F_{ST} ($r = 0.22$, $p = 0.008$; Fig. 2.7).

Landscape effects on phenotypic and genetic divergence

We first run global MRDM and LMM analyses with all the studied populations. These results showed that both resistance variables and environmental differences had a positive relation with genetic and phenotypic distance, i.e. at greater environmental resistance or dissimilarity, greater genetic/phenotypic distance (Table 2.5). Environmental resistance due to annual mean temperature explained most of the genetic and color distances, likely due to the coldest environment at the mountain ridge between the northern (transects 1 and 2, red morphs) and the southern populations (3 and 4, brown morphs, Fig. 2.8). Environmental resistance due to annual

precipitation and annual mean temperature differences at site explained most of size variation. Annual mean temperature at site, along with differences in frog size, also explained most of the variation in dominant frequency, whereas recording temperature was the only predictor for note repetition rate variation. Topographic distance, included as a predictor variable in the MRDM, was not selected for any model (Fig. 2.9). Genetic distance (F_{ST}) only explained 0.01 % of dominant frequency, but was not included in any other well-supported models for phenotypic divergence (Table 2.5, Fig. 2.9).

Because we wanted to know the effect of environmental variation along elevation gradients, we then analyzed transects on each side of the mountain ridge separately. All effects are positive, unless otherwise noted. In the northern transects 1 and 2, our MRDM results showed topographic distance was the most important variable for explaining genetic and phenotypic distances, with the exception of dominant frequency and note repetition rate which were only affected by genetic distance and size, and by recording temperature, respectively (Table 2.5 and Fig. 2.9). When controlling for topographic distance with the LMM, genetic distance was explained by resistance related to annual mean temperature and enhance vegetation index, as well as environmental differences in heat load index. For phenotypic distances, only environmental differences at site explained variation. Differences in annual mean temperature consistently affected phenotypic variation (i.e. size, color, dominant frequency). Variables related to precipitation, heat load index, and vegetation also explained color variation. Dominant frequency was also explained by environmental differences in precipitation seasonality and recording temperature. Note repetition rate was only explained by differences in tree coverage. For the southern transects 3 and 4, genetic distance was explained by resistance due to annual mean temperature, annual

precipitation, precipitation seasonality, and precipitation of the warmest quarter. Size variation was explained by environmental differences in compound topographic index, temperature annual range, and tree coverage. No variables were selected for color and dominant frequency, and only recording temperature explained note repetition rate (Table 2.5 and Fig. 2.9).

Discussion

The goal of this study was to investigate the drivers of population divergence along multiple elevational gradients. We hypothesized that patterns of genetic differentiation would be explained mainly by resistance to movement between populations, whereas patterns of phenotypic variation would be mainly explained by environmental differences among sites. However, genetic divergence could also be explained by environmental differences among sites if phenotypic divergences has caused restricted gene flow (i.e. early stages of ecological speciation; Schluter 2001; 2009; Rundle and Nosil 2005). In order to test these hypotheses, we first characterized genetic and phenotypic variation across populations. Our results show remarkable divergence in phenotypes, particularly in color, size, and dominant frequency despite low genetic divergence, suggesting divergence in the face of gene flow (Nosil 2008; Pinho and Hey 2010). Overall, we found that a mountain ridge and topographic distance explained most of the observed phenotypic and genetic divergence across the study area. However, at a smaller scale within elevational gradients, we found that landscape resistance (mainly due to temperature), along with solar radiation (heat load index) predicted genetic divergence, whereas environmental differences among sites predicted phenotypic divergence. These findings suggest

that at smaller scales, environmental variation has a two-fold effect on promoting population divergence with elevation. The intervening environmental conditions between sites are likely reducing gene flow, while the environmental conditions within sites are exerting selective pressures on the phenotypes.

Phenotypic and genetic divergence

We found that populations along the two northern transects differed in coloration and were larger than populations along the southern transect and lowlands. Size of the calling male explained most of the variation in note dominant frequency, and note repetition rate variation depended solely on recording temperature. Interestingly, in the two northern transects, red coloration and size also increased with elevation, a pattern that was not observed in the southern transects. This finding reflects that even at the intraspecific level, different outcomes (i.e. patterns of genetic and phenotypic divergence) can occur across elevation. In terms of differences between sexes, we found that although females are considerable larger than males, the patterns of phenotypic variation across transects and elevation are remarkably similar among males and females, suggesting that mechanisms affecting phenotypic traits are the same in both sexes.

Despite the observed phenotypic differences, we found very little genetic divergence in neutral loci (F_{ST} and D_{PS}). In general, we did not identify discrete genetic clusters, and although there were high levels of admixture at low elevations, high elevation populations were more isolated. Others have found similar patterns of adaptive divergence with little or no genetic divergence (e.g. McKay et al. 2001; McCormack and Smith 2008; Mila et al. 2009; Richter-Boix et al. 2013; Muir et al. 2014; Fitzpatrick et al. 2014; Langin et al. 2015). Yet most studies of adaptively

divergent amphibian populations have found higher levels of genetic divergence, although most of these studies were carried out in temperate zones (Funk et al. 2005; Zancolli et al. 2014; Funk et al. 2016). Consistent phenotypic divergence in the face of gene flow suggests strong natural selection imposed by environmental differences (Kawecki and Ebert 2004).). This observed phenotypic divergence with gene flow could be caused by divergent natural selection driving differentiation in loci affecting these traits (Schluter 2001; Gravilets 2003; Bolnick and Fitzpatrick 2007). Alternatively, it could be caused through phenotypic plasticity (Ghalambor et al., 2007; Pfennig et al. 2010; Fitzpatrick, 2012). These divergent phenotypes, like size, color and male calls are known to be related to reproductive isolation in diurnal frogs (Ryan 2001; Summers et al. 1999), suggesting they could act as 'magic traits', traits under natural selection and directly related to mate choice that facilitate ecological speciation (Gravilets 2004; Servedio 2011). Nonetheless, low genetic divergence between populations contradicts this view, as populations seem to maintain high levels of gene flow (Servedio et al. 2011). Alternatively, we might be just catching very early stages of ecological speciation, in which case we would not necessarily expect high genetic divergence. We know that reproduction can occur between populations from high and low elevations at least in transect 1 (M. Páez-Vacas, unpubl. data). However, we do not know yet if there is preferential mating between frogs from the same populations, or variation in offspring viability and/or fertility. The differences in patterns of phenotypic variation, and the somewhat similar pattern of genetic variation among elevational transects provide further evidence that phenotype and genetic diverge can potentially be driven by different processes (Merila and Crnokrak 2001).

Populations at higher elevations had less genetic diversity in both allelic richness and

heterozygosity, which can be explained by their greater isolation and fewer surrounding populations that can act as sources of immigrants, as well as smaller N_e. The highest population of transect 3 (Paccha, 1712 m asl) was much less diverse than any other population, and also showed the highest F_{ST} values, which might be the due to recurrent landslides in that area, that were witnessed by one author (MPV) during the four years of the study, causing reduction in N_e and perhaps isolation.

Landscape effects on phenotypic and genetic divergence

Our results support the hypothesis that barriers to dispersal are important drivers of diversification in tropical mountains (Patton and Smith 1992; Lynch et al. 1997; Brumfield and Edwards 2007; Guarnizo et al. 2009). Most of the phenotypic and genetic divergence observed in this species is due to isolation between populations on either side of a large mountain ridge in the middle of the study area. The colder and relatively drier habitat in this mountain ridge, along with a higher topographic roughness just above the areas where the species occurs, act as a barrier to gene flow. We also found that in addition to the main role of the mountain ridge on patterns of phenotypic and genetic divergence, size and dominant frequency were also affected by environmental differences in annual mean temperature among sites, suggesting divergent selection acting on these traits.

Because one of our main goals was to investigate the mechanisms of divergence along elevational gradients, and most of the variation was explained by one particular geographic barrier (i.e. the mountain ridge), we decided to repeat the analyses on subsets of populations on either side of the mountain ridge. Our first finding was that the environmental variables that

explained observed genetic and phenotypic divergence were not the same in the northern transects vs. the southern ones. This was consistent with our previous finding that patterns of phenotypic variation differed between these two regions. Topographic distance was consistently an important variable for genetic divergence, size and color divergence in the northern transects (1 and 2; red morphs), but not for call traits. Call variables were affected by topographic distance and by size of the male and by recording temperature. Genetic distance in transects 3 and 4 (brown morphs) was explained mainly by precipitation seasonality and topographic roughness between sites. As expected, we did not find variables that explained phenotypic patterns of variation in transects 3 and 4, as those populations showed very little variation. As observed with the northern transects, only temperature affected note repetition rate, a result that is also consistent with our previous phenotypic analyses.

When controlling for topographic distance, environmental variables related to temperature and precipitation in the intervening area between sites reduced gene flow, whereas, environmental differences within sites in temperature and precipitation act as divergent selective pressures on phenotypes along elevation. In other words, environmental variation, mainly in temperature and precipitation reduces connectivity between populations and exerts selective pressures on phenotypic traits. However, although environmental variation reduces connectivity between populations, it does not strongly impede it, evidenced from low levels of genetic divergence. These results are consistent with our original hypotheses, that: (1) phenotypic divergence occurs mainly due to differential environmental selection (IBE); and (2) genetic divergence occurs mainly by resistance to movement across populations (IBR). Others studies on neotropical frogs on elevation gradients have found similar patterns of vicariance at a broader scale and selection

along gradients at a smaller scale, although only on genetic divergence (Guarnizo et al. 2009). Although the most well understood cause of ecologically-based divergent selection involves environmental differences (e.g. climate, habitat structure; Rundle and Nosil 2005; Keller and Seehausen 2012), other studies in poison frogs have shown that biotic factors such as ecological interactions with conspecifics (Wang and Summers 2011), predators (Willink et al. 2014), mimetic species (Twomey et al. 2015; 2016) and sexual selection might also be acting as forces of selection and drivers of divergence (Galeano and Harms 2016). Therefore, further exploration of biotic interactions in this species could also reveal their relative importance on processes of population divergence.

Correlative studies between genetic and phenotypic divergence hinders the drawing of causal inferences on the mechanisms responsible for the observed patterns of divergence. However, landscape genetics studies can be designed specifically to test these relationships and their directionality, as genetic divergence cannot cause environmental differences (Räsänen and Hendry 2008). We showed that including simultaneous examinations of the role of distance, resistance to movement, and environmental selection on patterns of phenotypic divergence, we obtain a more complete picture of the factors promoting population divergence and the underlying mechanisms.

Conservation implications

Effective conservation and management requires knowledge of dispersal and gene flow among populations, as well as knowledge of the factors to which species are most sensitive (Crandall et al. 2000; Funk et al. 2012). Our results show that environmental variation has a role in processes

of phenotypic and genetic divergence. The fact that we found evidence of phenotypic divergence due to environmental selective pressures along elevation, adds to growing evidence that elevational gradients are indeed engines of biodiversity, and should receive high conservation priority (Keller et al. 2013; Funk et al. 2016). Relative isolation of high elevation populations can reduce gene flow and hence increase genetic divergence, as shown in this study. Consequently, high elevation populations might be more vulnerable to changing climate and habitat conditions (Crandall et al. 2000; Hoffman and Sgrò 2011). Even in cases when gene flow is known among populations, caution is recommended as adaptive divergence could be determined by loci under genetic differentiation that might not be reflect at neutral loci and inferred demographic history (Funk et al. 2012; Shafer et al. 2015; Benestan et al. 2016).

We also found that the effect of the environment can vary at the intraspecific level, which suggests caution when attempting to generalize conservation measures across species and populations (Funk et al. 2012). Although this is an abundant species of poison frog, it shows sensitivity to subtle environmental changes in temperature, precipitation, and vegetation. This raises the question of how vulnerable other rarer tropical montane frogs are to climate change and habitat transformation. It might be the case that rare species with highly restrictive dispersal and gene flow might be even more susceptible to environmental variation. Further studies across taxa are necessary to understand the impact of environmental variation on evolutionary trajectories of species and their subsistence.

Tables and Figures

Table 2.1. Localities studied, transect, elevation, sample sizes for genetic, size (SVL), color and calls. Hardy-Weinberg *p*-value, observed heterozygocity (H_o), expected heterozygocity (H_e), effective population size (N_e).

Population	Transect	Elevation (m asl)	Genetic *n*	SVL *n*	Color *n*	Calls *n*	HW *p*-value	H_o	H_e	N_e
EA1	-	902	7	3	-	-	0.005	0.762	0.884	Infinite
EA2	1	1546	20	12	12	5	0.110	0.839	0.872	337.1
EA3	1	1327	19	17	5	3	0.025	0.813	0.862	142.2
EA4	1	579	20	16	10	3	0.473	0.894	0.89	Infinite
EA5	2	1319	20	20	20	3	0.409	0.872	0.877	Infinite
EA6	2	979	20	18	12	4	0.040	0.85	0.892	296.3
EA7	2	565	15	12	9	4	0.163	0.863	0.89	51.5
EA8	1	776	20	18	17	5	0.128	0.85	0.88	193.7
EA9	3	540	24	20	17	5	0.001*	0.777	0.872	158.4
EA10	3	946	20	19	19	5	0.341	0.856	0.868	372
EA11	3	1712	18	13	12	5	0.032	0.796	0.738	14.8
EA12	-	515	6	6	-	-	0.002	0.815	0.937	Infinite
EA13	-	756	12	11	-	-	0.052	0.87	0.913	92.6
EA14	3	276	20	17	13	1	0.004	0.835	0.903	35
EA15	2	1666	23	24	17	4	0.004	0.816	0.881	215.7
EA16	2	229	20	17	16	3	0.198	0.917	0.9	110.5
EA17	3	1304	7	6	3	1	0.005	0.968	0.856	24.2
EA18	4	1250	20	22	22	4	0.410	0.911	0.906	760.8
EA19	4	470	8	2	2	3	0.025	0.94	0.861	11.9
EA20	4	894	20	24	22	4	0.122	0.9	0.921	262.6
EA21	-	72	6	6	5	-	0.006	0.852	0.954	Infinite
EA22	1	230	20	24	18	1	0.012	0.866	0.904	371.1
EA23	-	1570	4	-	-	-	0.169	0.778	0.852	Infinite
EA24	-	1386	10	-	-	-	0.430	0.889	0.899	1498.6
EA25	-	1550	10	5	-	3	0.034	0.767	0.838	Infinite
EA26	-	1130	6	-	-	3	0.012	0.722	0.857	69.1
EA27	-	962	5	3	-	-	0.299	0.8	0.761	Infinite
EA28	-	192	3	-	-	-	0.314	0.926	0.861	Infinite
EA29	-	591	20	-	-	-	0.593	0.903	0.903	207.6
EA30	-	110	20	20	-	-	0.001*	0.839	0.917	169.9

EA31	-	51	20	20	-	-	0.007	0.865	0.915	63.2
EA32	-	13	20	20	-	-	0.249	0.894	0.91	61
EA33	-	494	20	20	-	-	0.251	0.891	0.897	120.9
EA34	-	1023	18	18	-	-	0.002	0.839	0.911	Infinite
EA35	-	65	19	18	-	-	0.014	0.869	0.922	1265.2
TOTAL			**540**	**454**	**251**	**69**				

Table 2.2. Environmental variables used, description and source.

Process	Category	Variable	Code	Description	Source	Calculation	Ecological Justification
IBD	Distance	Topographic distance	td	Distance between sites corrected for topography	Jpl.nasa.gov/srtm	ArcGIS, 3D Analyst	Null model of isolation by distance
IBR	Temperature	Annual mean temperature	amt	Monthly mean temperature per year (°C)	Worldclim.org	CircuitScape	Lower mean temperatures, predominately found at higher elevations, restricts breeding and dispersal.
	Precipitation	Annual precipitation	ap	Total precipitation accumulation per year (mm)	Worldclim.org	CircuitScape	Dry habitats with low precipitation accumulation limit breeding site availability and restricts dispersal.
		Precipitation seasonality	ps	Coefficient of variation of monthly precipitation	Worldclim.org	CircuitScape	Habitats with highly seasonal precipitation limit breeding site availability and dispersal during the dry season.
		Precipitation of the warmest quarter	pwq	Total precipitation accumulated during warmest 3 months (mm)	Worldclim.org	CircuitScape	Dry habitats with low precipitation accumulation limit breeding site availability and restricts dispersal.
	Vegetation	Enhanced vegetation index	evi	Density of vegetation calculated from	Modis.gsfc.nasa.gov	CircuitScape	Low vegetation density limits leaf litter cover and soil

				chlorophyll reflectance in visual and near-infrared spectra			wetness, restricting dispersal.
	Topography	Topographic roughness	tr	Topographic complexity based on scaled variance in elevation within moving window	Jpl.nasa.gov/srtm	CircuitScape, ArcGIS, Geomorphometric and Gradient Metric Toolbox	Fine scale topographic complexity restricts dispersal due to energetic costs.
IBE	Temperature	Annual mean temperature	amt	Monthly mean temperature per year (°C)	Worldclim.org	ArcGIS, Spatial Analyst	Lower mean temperatures, predominately found at higher elevations, restricts breeding and dispersal.
		Isothermality	i	Mean diurnal temperature range divided by temperature annual range	Worldclim.org	ArcGIS, Spatial Analyst	Highly seasonal temperatures limit breeding and dispersal during the cold season.
		Temperature annual range	tar	Maximum temperature of the warmest month minus minimum temperature of the coldest month	Worldclim.org	ArcGIS, Spatial Analyst	Highly seasonal temperatures limit breeding and dispersal during the cold season.
	Precipitation	Annual precipitation	ap	Total precipitation accumulation per year (mm)	Worldclim.org	ArcGIS, Spatial Analyst	Dry habitats with low precipitation accumulation limit breeding site availability and restricts dispersal.
		Precipitation of the coldest	pcq	Total precipitation	Worldclim.org	ArcGIS, Spatial Analyst	Dry habitats with low precipitation

	quarter		accumulated during coldest 3 months (mm)			accumulation limit breeding site availability and restricts dispersal.
	Precipitation seasonality	ps	Coefficient of variation of monthly precipitation	Worldclim.org	ArcGIS, Spatial Analyst	Habitats with highly seasonal precipitation limit breeding site availability and dispersal during the dry season.
	Precipitation of the warmest quarter	pwq	Total precipitation accumulated during warmest 3 months (mm)	Worldclim.org	ArcGIS, Spatial Analyst	Dry habitats with low precipitation accumulation limit breeding site availability and restricts dispersal.
Solar radiation	Heat load Index	hli	Total incident solar radiation as a function of slope, aspect, and latitude	Jpl.nasa.gov/srtm	ArcGIS, Spatial Analyst, Geomorphometric and Gradient Metric Toolbox	Solar radiation is associated with warmer, drier intervening habitat that restricts dispersal.
Vegetation	Enhanced vegetation index	evi	Density of vegetation calculated from chlorophyll reflectance in visual and near-infrared spectra	Modis.gsfc.nasa.gov	ArcGIS, Spatial Analyst	Low vegetation density limits leaf litter cover and soil wetness, restricting dispersal.
	Tree coverage	tc	Percent tree cover (%)	Modis.gsfc.nasa.gov	ArcGIS, Spatial Analyst	Low tree cover limits leaf litter cover and soil wetness, restricting dispersal.

	Compound topographic index	cti	Steady state soil wetness index as a function of slope and upstream catchment area	Jpl.nasa.gov/srtm	ArcGIS, Spatial Analyst, Geomorphometric and Gradient Metric Toolbox	Amphibians rely on moisture gradients for dispersal; so wetter intervening habitat increase dispersal and potentially breeding site availability.
Topography	Topographic roughness	tr	Topographic complexity based on variance in elevation within moving window	Jpl.nasa.gov/srtm	ArcGIS, Spatial Analyst, Geomorphometric and Gradient Metric Toolbox	Fine scale topographic complexity restricts dispersal due to energetic costs.

Table 2.3. Summary of the best linear models that explain phenotypic variation across transects and elevation. SVL = snout-vent length (mm); REP = note repetition rate; DOM = note dominant frequency (Hz). Temperature refers to recording temperature. SVL as a predictor of dominant frequency corresponds to the size of the calling male.

Trait	Model	R^2
Size	SVL ~ Elevation * Transect + Sex	0.735
Color	RED ~ Elevation * Transect + Sex	0.620
	GREEN ~ Elevation * Transect	0.156
	BLUE ~ Transect	0.216
Calls	REP ~ Temperature	0.233
	DOM ~ Elevation * Transect + Temperature + SVL	0.718

Table 2.4. Microsatellites information. T_a = temperature of annealing, H_e = expected heterozygocity, H_o = observed heterozygocity.

Locus	Primer Sequence (5'– 3')	GeneBank accession number	Repeat motif	Size range	Multiplex group	Number of alleles	T_a (°C)	H_e	H_o
EAN002	AAAGGAAGCAGCAAGTTAGACAG	-	AGAT	135 – 387	1	41	63.2	0.934	0.840
EAN006	AGGTGAATGCTGGGTAATATCAC	-	AGAT	140 – 268	2	32	63.0	0.920	0.884
EAN008	AGGGTCCTTACTCCAAAGACTTG	-	AGAT	148 – 228	1	21	63.3	0.906	0.868
EAN009	ATTGCACAGACGGAGAATGAATC	-	AGAT	143 – 263	2	21	62.8	0.904	0.857
EAN010	CCAACTGCTCTGTTCTACTGTAC	-	AGAT	142 – 246	1	24	63.6	0.917	0.863
EAN016	GGGAAGAGTGAGAGGGATTTCC	-	AGAT	143 – 255	3	29	64.0	0.929	0.840
EAN028	CATCCAATGTCTGATGTGCAATG	-	AGAT	176 – 296	1	29	63.4	0.911	0.880
EAN030	AAGTCAGCTGAAAGAACTAAGGC	-	AGAT	203 – 391	3	40	63.7	0.932	0.848
EAN036	ACTGTCAATCATACCTGTTGCTG	-	AGAT	180 – 356	2	23	63.0	0.912	0.709
EAN037	GTGTGAAATCATGTCCAATTCGC	-	AGAT	243 – 359	3	26	63.9	0.900	0.743

Table 2.5. Variables selected as predictors of variation in genetic and phenotypic distances, obtained from multiple regressions on distance matrices (MRDM) and linear mixed models (LMM). Predictors are resistances layers or environmental differences. Amt = annual mean temperature, tar = temperature annual range, iso = isothermality, ap = annual precipitation, ps = precipitation seasonality, pwq = precipitation of warmer quarter, evi = enhanced vegetation index, cti = compound topographic index of wetness, hli = heat load index, tc = percentage of tree coverage, tr = topographic roughness, SVL = snout-vent length, TempCall = environmental temperature at moment of calling. SVL as a predictor of dominant frequency corresponds to the size of the calling male.

n	PREDICTOR	F_{ST}		SVL		Color		Dominant Frequency		Note repetition rate	
ALL POPULATIONS		MRDM	LMM	MRDM	LMM	MRDM	LMM	MRDM	LMM	MRDM	LMM
	F_{ST}								0.01		
RESISTANCES	amt	0.41	0.11		0.03	0.55	0.27				
	ap		0.09	0.35		0.48	0.18				
	ps		0.01			0.23	0.12				
	pwq		0.10		0.06						
	evi				0.04						
	tr							0.25	0.05		
ENVIRONMENTAL DIFFERENCES	amt		0.04	0.32	0.09			0.47	0.18		
	tar		0.03		0.007						
	ps		0.03				0.02				
	pwq		0.01								
	hli				0.04						
	cti				0.03						
	evi		0.01		0.02						
CALL PREDICTORS	SVL							0.47	0.33		
	TempCall								0.02	0.25	0.05
Model R^2		0.16		0.19		0.57		0.54		0.06	
Northern (Transects 1 and 2)		MRDM	LMM	MRDM	LMM	MRDM	LMM	MRDM	LMM	MRDM	LMM
	Topographic distance	0.76		0.61		0.76					
	F_{ST}							0.22			

RESISTANCES	amt		0.28								
	ap										
	ps										
	pwq										
	evi		0.16								
	tr					-0.20					
ENVIRONMENTAL DIFFERENCES	amt				0.22		0.26		0.45		
	iso	0.14			0.48						
	hli		0.23				0.32				
	evi						0.07				
	ps						0.14		0.17		
	tc										
CALL PREDICTORS	SVL							0.58			
	TempCall								0.18	0.30	0.13
Model R^2		0.66		0.37		0.71		0.37		0.09	
Southern (Transects 3 and 4)		MRDM	LMM	MRDM	LMM	MRDM	LMM	MRDM	LMM	MRDM	LMM
	Topographic distance	0.07									
	F_{ST}										
RESISTANCES	amt		0.16								
	ap		0.08								
	ps	0.03	0.28								
	pwq										
	tr	0.67	0.45								
ENVIRONMENTAL DIFFERENCES	tar				0.16						
	cti				0.04						
	tc				0.50						
CALL PREDICTORS	SVL										
	TempCall									0.52	0.24
Model R^2		0.85								0.27	

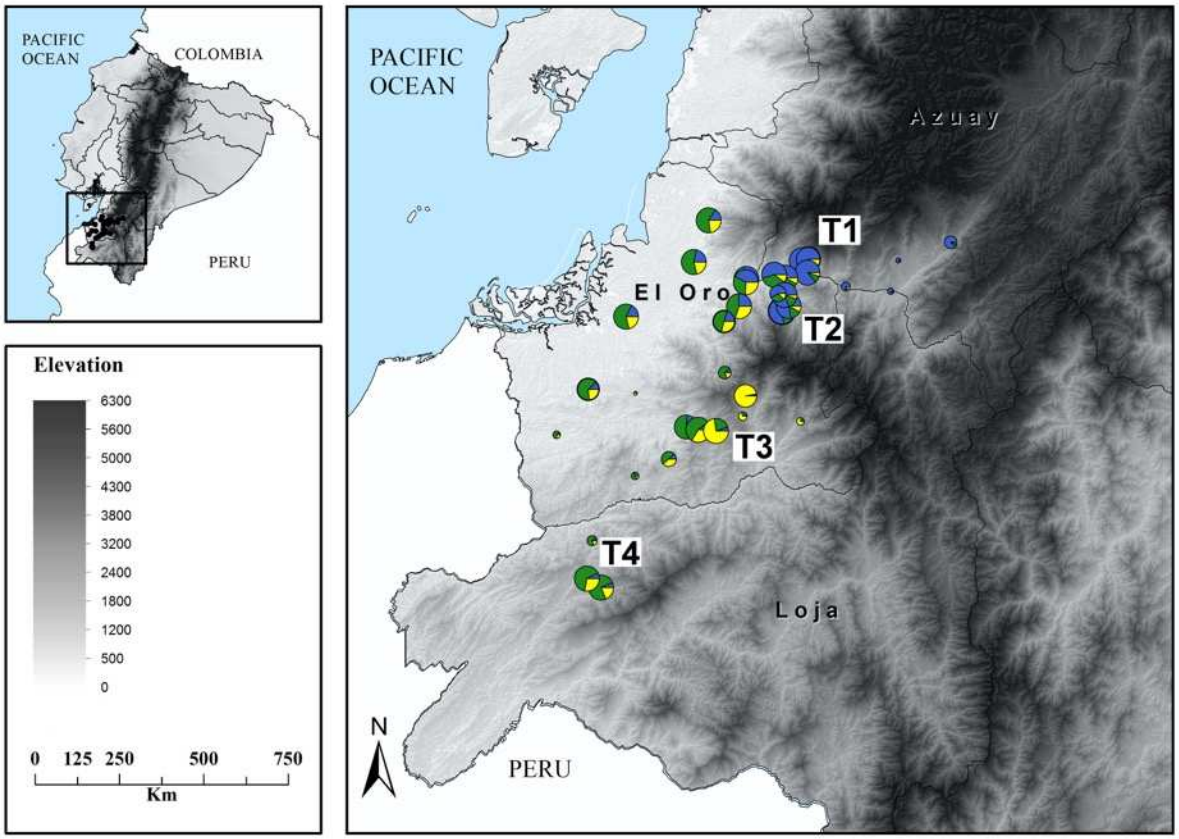

Figure 2.1. Localities of *Epipedobates anthonyi* showing patterns of genetic admixture with neutral microsatellite loci. Each color represents the percentage of genotypes assigned to a given genetic cluster. Elevational transects are shown (T1-T4). Size of pie charts corresponds to sample size. Note the mountain ridge between transects 2 and 3.

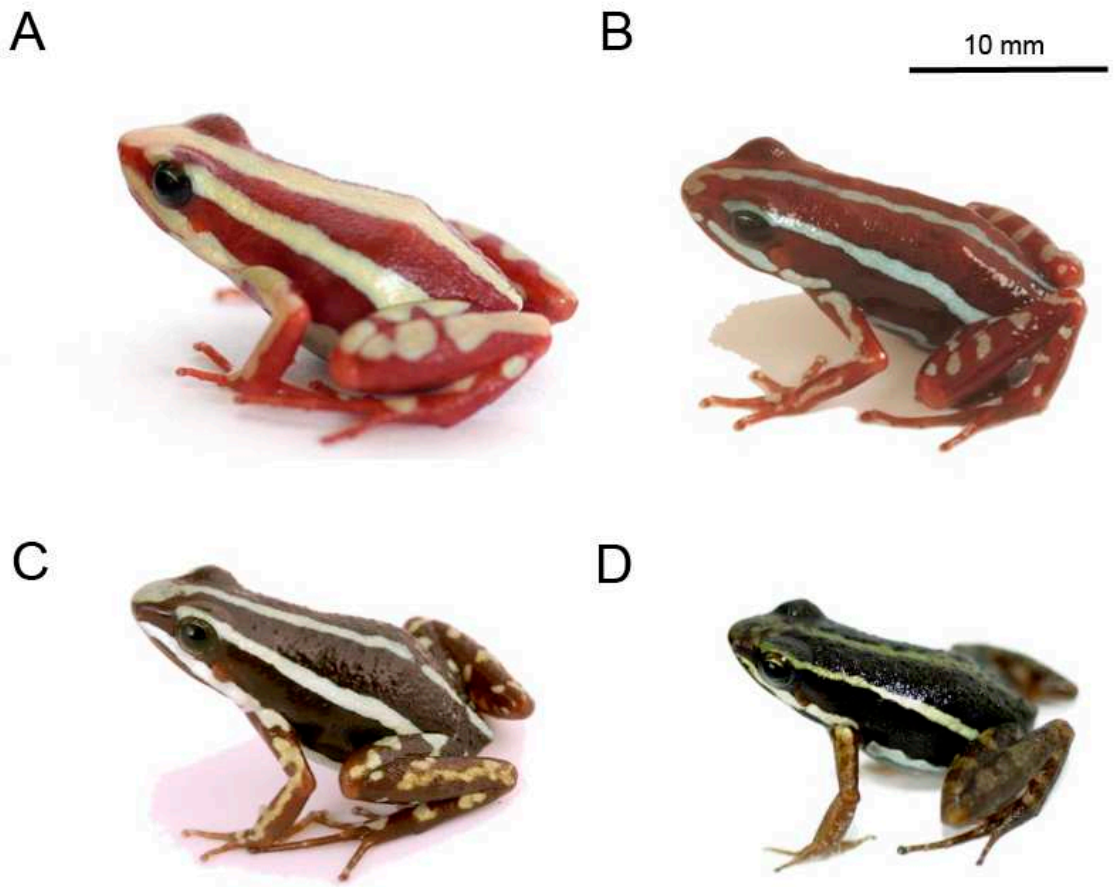

Figure 2.2. Phenotypic variation in size and color in populations of *Epipedobates anthonyi*. (A) high elevation red morph, (B) low elevation red morph, (C) high elevation brown morph, (D) low elevation brown morph.

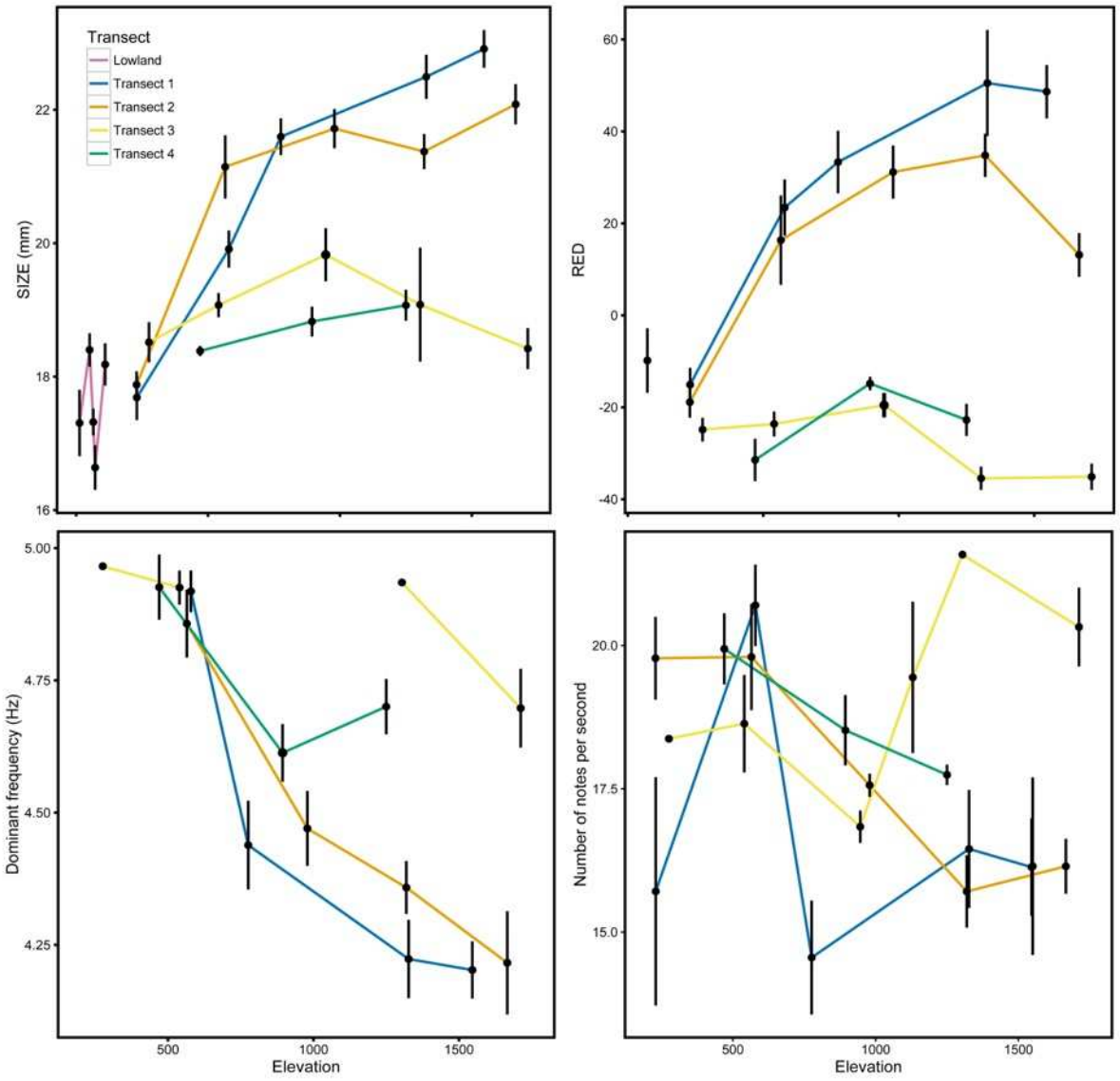

Figure 2.3. Phenotypic variation across transects and elevation. Size (snout-vent length in mm), redness, note dominant frequency (Hz), and note repetition rate (numbers of notes per second).

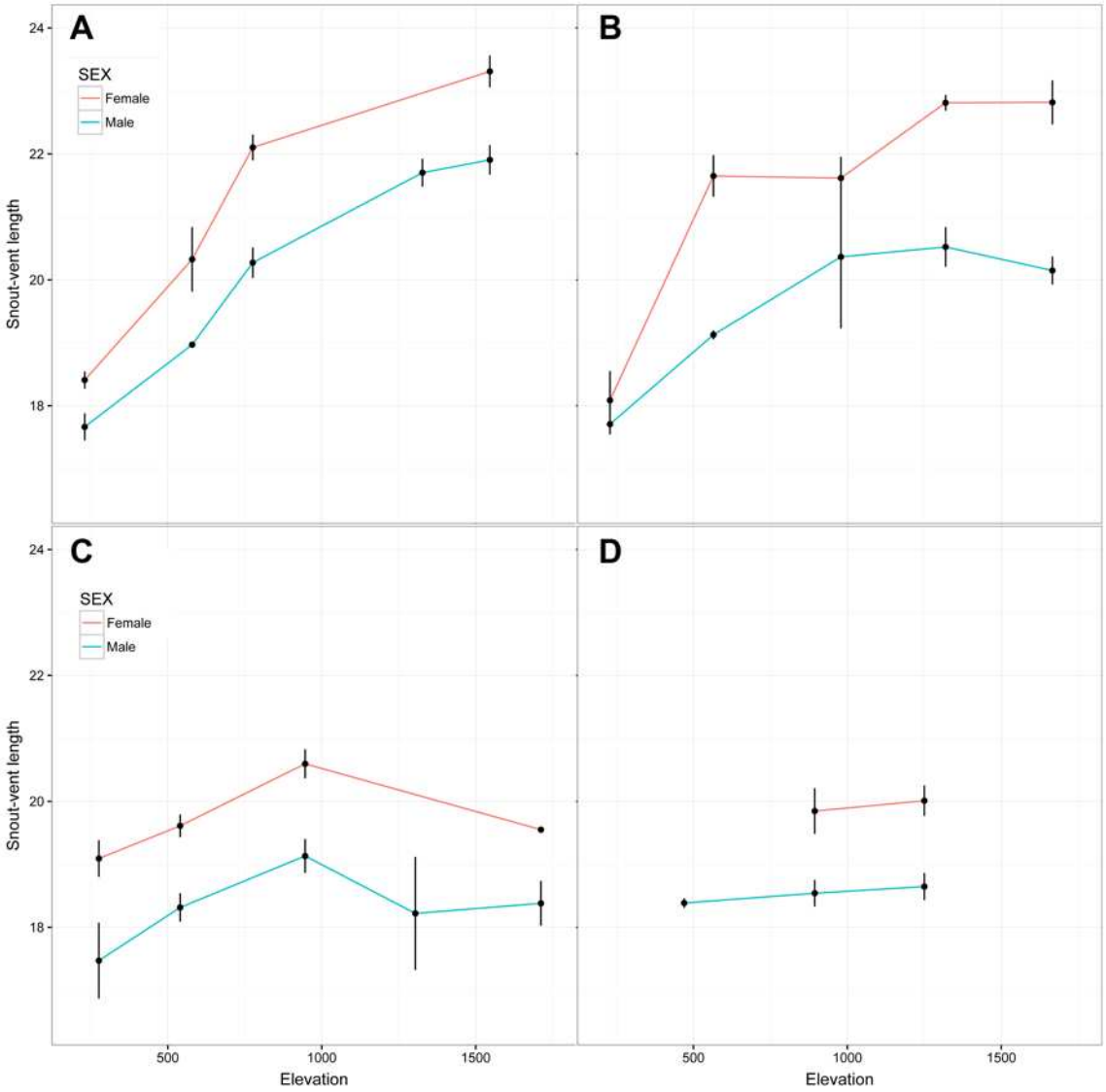

Figure 2.4. Size variation (mm) across elevation and sex. (A) Transect 1, (B) Transect 2, (C) Transect 3, and (D) Transect 4.

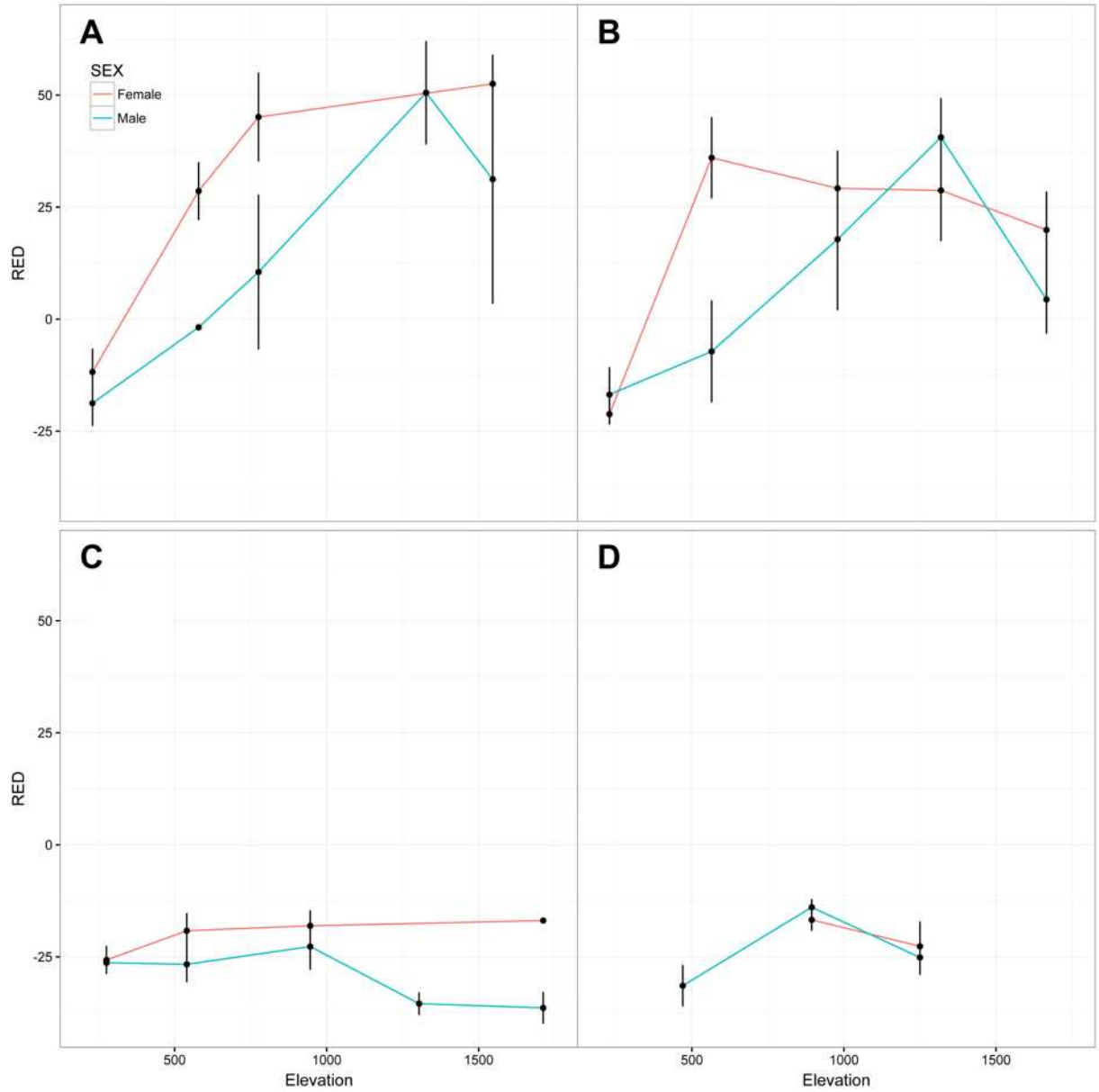

Figure 2.5. Red color variation across elevation and sex. (A) Transect 1, (B) Transect 2, (C) Transect 3, and (D) Transect 4.

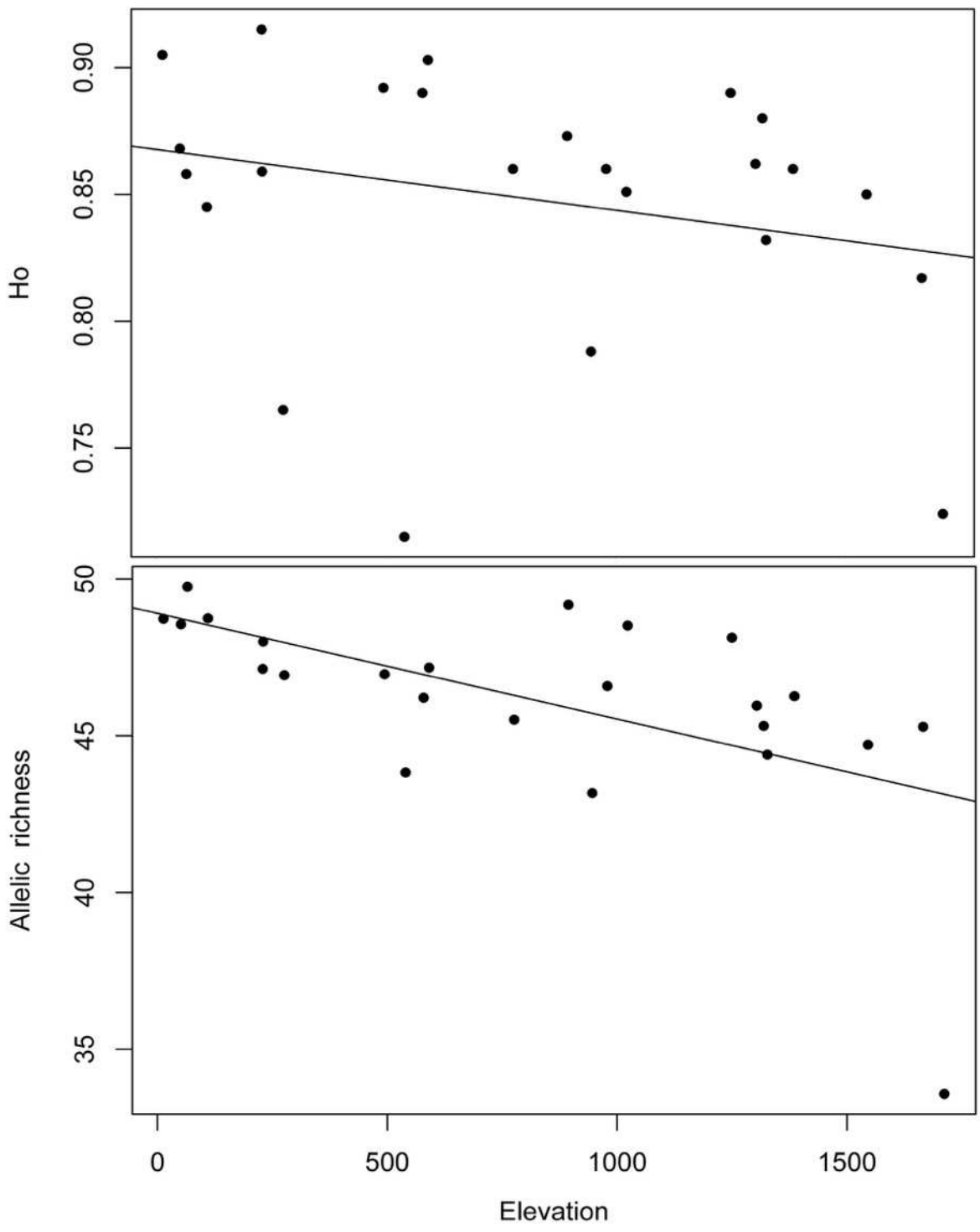

Figure 2.6. Genetic diversity along elevation in (A) observed heterozygocity (H_o), and (B) allelic richness.

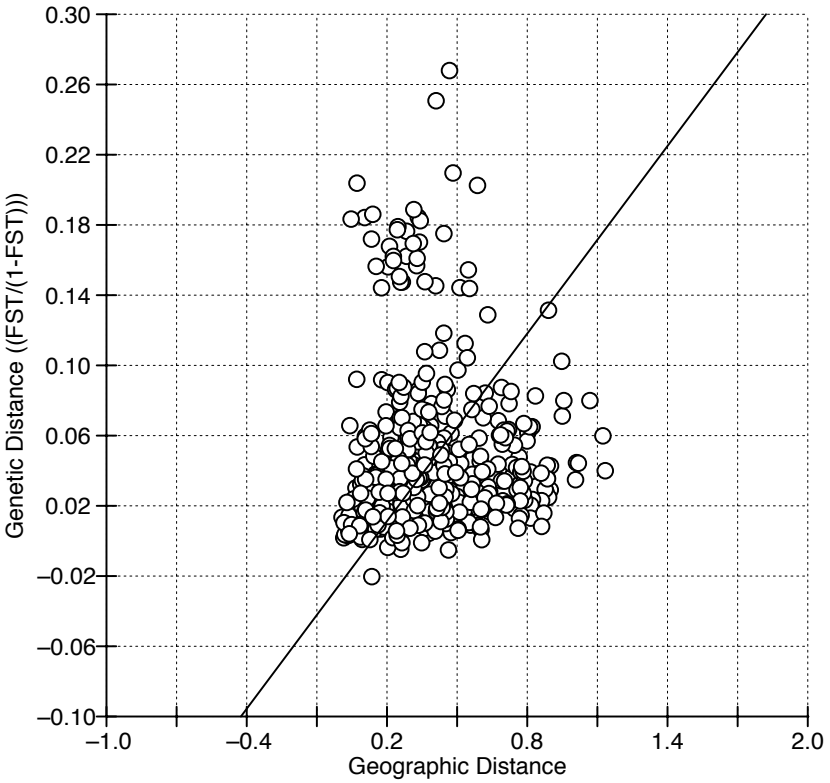

Figure 2.7. Isolation by distance.

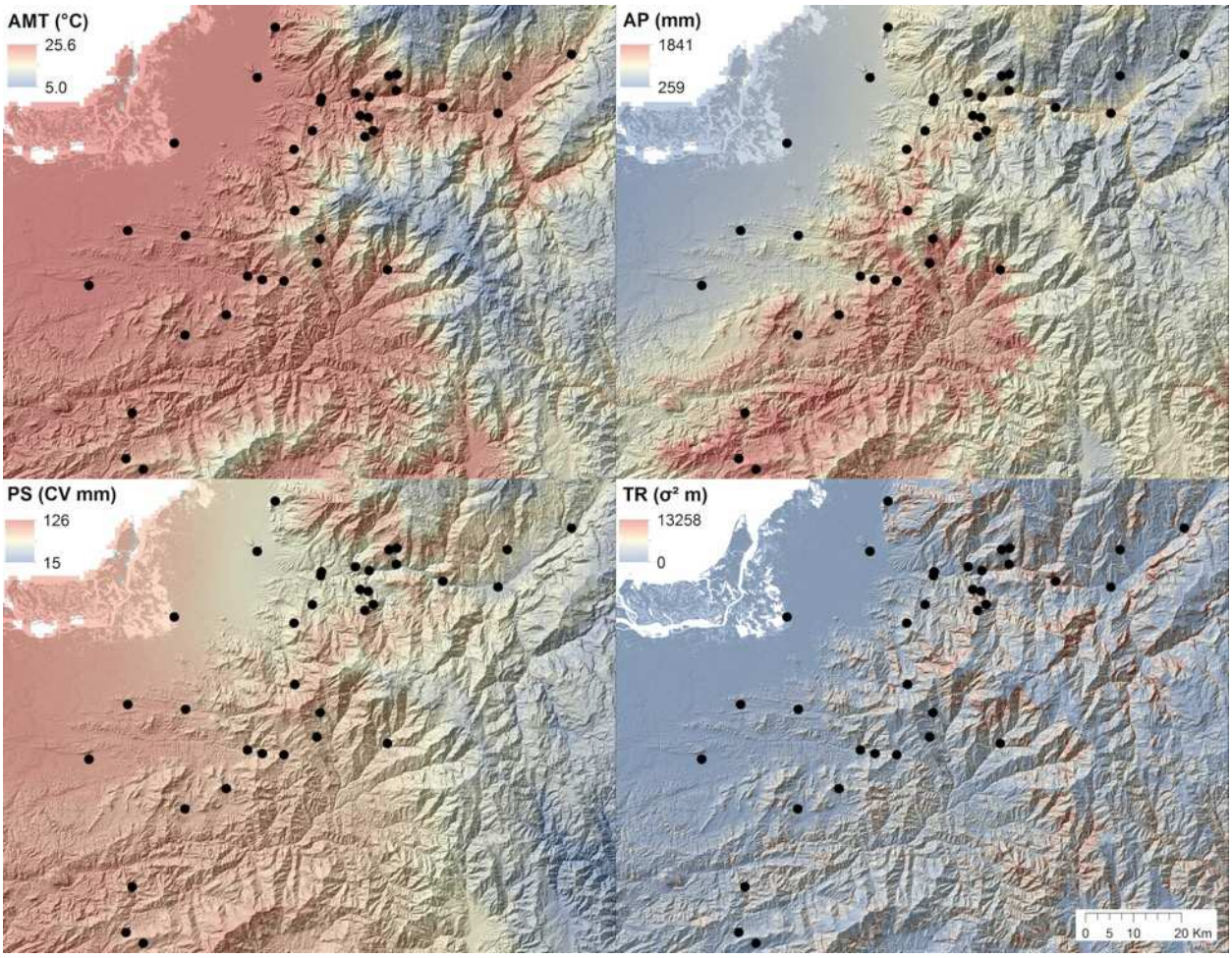

Figure 2.8. Variation in annual mean temperature (AMT), annual precipitation (AP), precipitation seasonality (PS), and topographic roughness (TR) across the study area. Note the cold and relatively dry east-west mountain ridge in the middle of the study area between transects 2 and 3, and how topographic roughness increases just above the edge of populations' distribution (black dots) on that ridge.

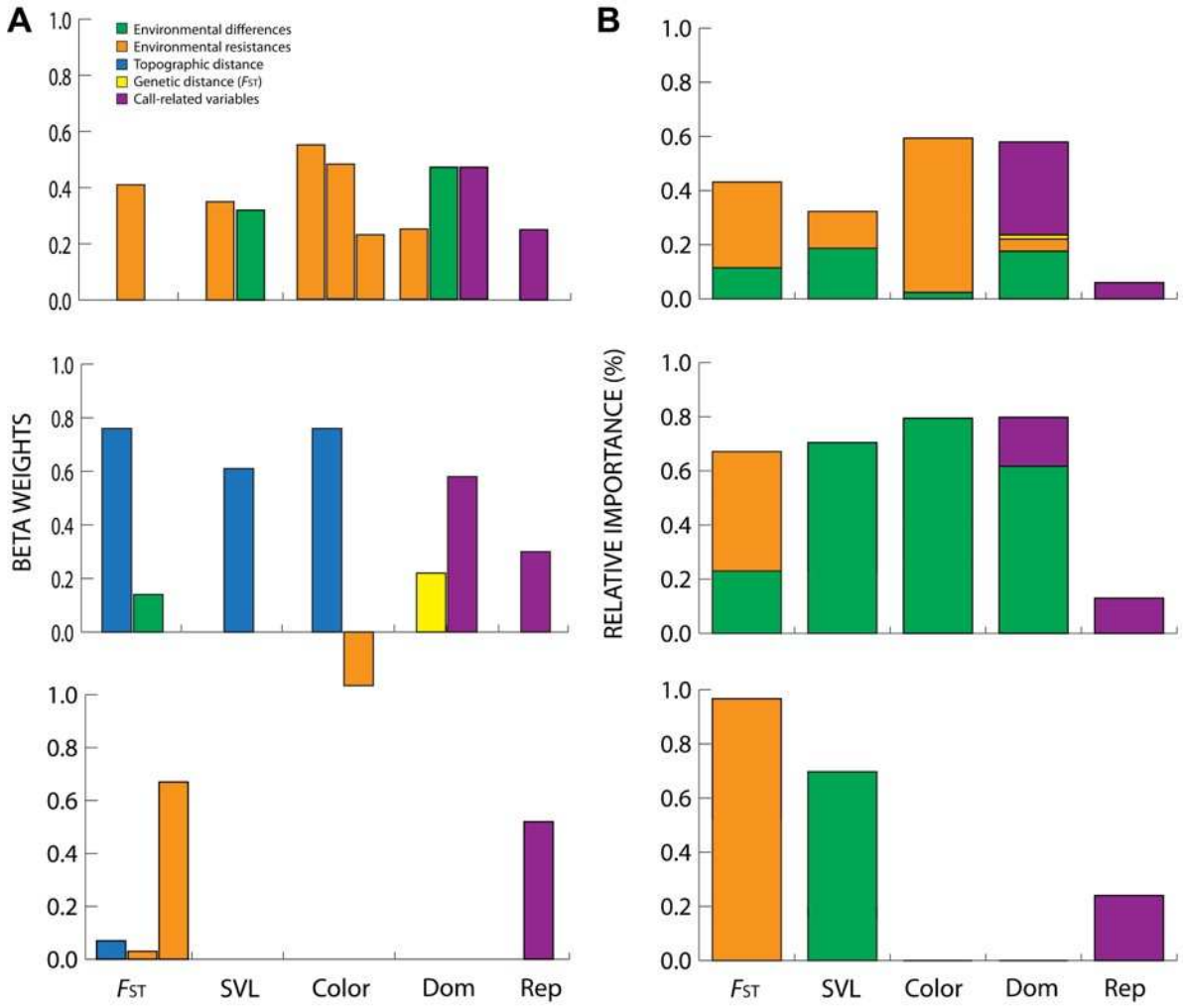

Figure 2.9. Predictors explaining divergence in genetic and phenotypic traits. All populations (top panels), northern transect 1 and 2 (middle panels), and southern transect 3 and 4 (bottom panels). (A) Results of multiple regressions on distance matrices (MRDM), shown as beta-weights of variables selected in the models. As these values are not additive, we showed each selected variable separately. (B)Results of linear mixed models (LMM) shown as the relative importance of categories of variables in the model. SVL = snout-vent length, Dom = dominant frequency, and Rep = Note repetition rate.

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3. CRITICAL THERMAL LIMITS ALONG ELEVATION AND ACCLIMATION ABILITIES

Summary

Variation in temperature across elevation may cause divergent selection in organisms' thermal tolerance. Generally, high elevation populations are predicted to tolerate lower temperatures than low elevation populations. Organisms can also respond to changes in temperature along elevation through phenotypic plasticity, although theory and empirical evidence suggests that tropical ectotherms have limited abilities to acclimate. We measured the thermal limits (CT_{MAX} and CT_{MIN}) of tadpoles of a poison frog species (*Epipedobates anthonyi*) along two elevational gradients (200 – 1700 m asl) in southeastern Ecuador to investigate their thermal tolerance across elevation. We also tested if tadpoles could shift their thermal limits in response to exposure to different temperatures (20 °C, 24 °C, and 28 °C). Overall, we found that CT_{MIN} was lower at higher elevations, suggesting that elevational variation in temperature causes divergent selection on this thermal trait across elevation. In contrast, CT_{MAX} did not change along elevation. All populations shifted their thermal tolerances for CT_{MAX} and CT_{MIN}, according to the treatment (i.e. higher tolerance to cold temperatures if maintained in colder temperatures), in response to treatment temperature, demonstrating phenotypic plasticity in thermal limits. Overall, differences in CT_{MIN} among high, mid, and low elevation populations were maintained despite plastic responses to treatment temperature. These results demonstrate that low temperature acts as a selective force along elevation, even when populations show acclimation abilities.

Introduction

Although geographic isolation is one important factor causing differentiation and speciation in several montane groups, recent studies have shown that divergent ecological selection can also drive diversification along elevation gradients (Cadena et al. 2012; Lavrenchenko 2011; Funk et al. 2016). The gradient model of speciation contends that strong divergent ecological selection causes adaptive divergence, assortative mating follows, and gene flow is eventually reduced, leading to speciation (Bridle et al. 2009; Wilson et al. 2012; Funk et al. 2016). The relative seasonal uniformity in temperature in tropical mountains has been hypothesized to lead to the evolution of narrow thermal tolerance in tropical montane organisms relative to their temperate zone counterparts (Janzen 1967; Ghalambor et al. 2006). Since tropical ectotherms are exposed to relatively stable and narrow thermal regimes in a given elevational zone, they are expected to have relatively narrow thermal tolerances (Janzen 1967; Pörtner et al. 2006; Sunday et al. 2010) and a limited ability to shift their response when exposed to different temperatures (i.e. insects, amphibians, and reptiles; Brattstrom 1968; Gunderson and Stillman 2015). If the selective pressures exerted by thermal regimes along elevational gradients are strong enough, they could promote adaptive divergence, even in the presence of gene flow (Smith et al. 1997; Orr and Smith 1998; Scheinder et al. 1999; Keller and Seehausen 2012). Consequently, if tropical organisms were to disperse above or below their natural elevational zone, they would be more likely to encounter temperatures to which they are not adapted. This conceptual model implies that reduced dispersal in tropical mountains should, in turn, lead to greater genetic divergence between populations, higher speciation rates, and greater species turnover along elevation gradients (Ghalambor et al. 2006).

In addition to genetically-based adaptive responses to spatial variation in temperature, organisms can also respond by phenotypic plasticity, the ability of a genotype to produce different phenotypes in different environments. Phenotypic plasticity is ubiquitous in nature and its role in adaptive divergence is contentious (Pfenning et al. 2010). Traditionally, it has been considered irrelevant for evolution, since by definition, it does not cause changes in allele frequencies across generations. Another traditional view is that it shields genetic variation from selection (Pfenning et al. 2010). However, more recent perspectives argue that plasticity can contribute to evolution by allowing establishment and persistence in new environments, and by exposing cryptic genetic variation on which selection can act (Ghalambor et al. 2007; Pfennig et al. 2010; Fitzpatrick 2012). Therefore, evaluating the degree of plasticity in thermal physiology can elucidate its potential importance in evolutionary changes along elevation, as well as the degree to which organisms can cope with environmental change, and thus their resilience or vulnerability to climate change (Gunderson and Stillman 2015).

Amphibians are at the forefront of the current biodiversity crisis, particularly in tropical montane regions (Stuart et al. 2004; Wake and Vredenburg 2008). In tropical mountains, anurans are exceptionally diverse with most species occupying narrow elevational ranges (Duellman 1999; Amphibiaweb.org). Neotropical frogs tend to have poor dispersal abilities and are considered sensitive to environmental change (Navas et al. 2007). Combined, these characteristics might limit their distribution and promote population divergence, potentially increasing their vulnerability to landscape and climate change. Moreover, one of the projected effects for climate change for ectotherms is a shift to higher elevations (Tewksbury et al. 2008). Hence, understanding the extent of local adaptation to variation in temperature across elevation and the

ability to respond plastically to changes in temperature is important for predicting the response of amphibians to climate change.

The poison frog *Epipedobates anthonyi* is distributed along the western slopes of the Andes from 80 to 1769 m above sea level in southern Ecuador and northern Peru. This is an abundant species that has been successfully maintained and bred in captivity, characteristics that make it ideal for experimental approaches (L. Coloma, *pers.comm.* Centro Jambatu for Research and Conservation of Amphibians, Quito, Ecuador). *Epipedobates anthonyi* also exhibits population divergence in color, size, and male advertisement calls along replicated elevational transects (Chapter 2). By combining phylogenetics and niche modeling, Graham et al. (2004) found evidence that the elevational gradient of the Andes might have played a role in speciation within the genus *Epipedobates*. Their results also suggest that, in *E. anthonyi*, elevation and annual mean temperature are more correlated with genetic divergence than other climatic variables. However, a mechanistic approach that evaluates the degree of divergence in thermal biology is required to more thoroughly test the role of temperature in promoting population divergence.

Our goal here was to understand the selective pressures exerted by thermal gradients across elevation and the effect of those pressures on local adaptation in *E. anthonyi*. To that end, we first measured temperature of the stream microhabitats used by *E. anthonyi* tadpoles, and second, measured the critical thermal limits (CT_{MAX} and CT_{MIN}, for Critical thermal maximum and minimum temperatures, respectively) of tadpoles across multiple populations along two independent elevational gradients. Critical thermal limits are widely used in the study of thermal physiology and correspond to the temperatures at which motor function ceases (Coyles and

Boggert 1944; Brattstrom 1968; Lutterschmidt and Hutchison 1997a; Angilleta 2009). Thermal limits depend on complex interactions at the biochemical, cellular, and systematic levels (Angilleta 2009), such as oxygen limitation inducing mitochondrial anaerobic metabolism and loss of enzymatic functioning (Pörtner 2001; 2002; Verberk et al. 2011). On the one hand, cold temperatures can inactivate mitochondrial enzymes involved in adenosine triphosphate (ATP) supply, as well as slow circulation and ventilation systems causing a mismatch between oxygen demand and supply, leading to collapse of physiological function (Angilleta 2009). On the other hand, high temperatures can increase energy demand, and cause a breakdown in enzymatic function (Pörtner 2002; Angilleta 2009; Verberk et al. 2011). We used tadpoles rather than adults because: (i) upper thermal tolerance is not influenced by dehydration in tadpoles, whereas CTmax estimates are thought to be affected by dehydration in the terrestrial adult stage (Rezende et al. 2011); (ii) tadpoles have limited ability to use behavioral compensation (Bogert effect; Bogert 1949; Huey et al. 2003); and (iii) body temperature in tadpoles is considered the same as water temperature (Lutterschmidt and Hutchison 1997b).

We used CT_{MAX} and CT_{MIN} to test two hypotheses: (i) variation in temperature drives local adaptation in thermal tolerance across elevation (Fig. 3.1a); and (ii) organisms are able to shift their thermal tolerance depending on the environmental conditions they are exposed to (Fig. 3.1b). The first hypothesis predicts that CT_{MAX} and CT_{MIN} will differ between populations from different elevations, in accordance with the environmental temperatures populations experience. For example, populations from lower elevations should tolerate higher temperatures (higher CT_{MAX}), and populations from higher elevations should tolerate lower temperatures (lower CT_{MIN}), suggesting local adaptation due to divergent selection. The second hypothesis predicts

that individuals from the same population will exhibit higher CT_{MAX} and CT_{MIN} when acclimated to higher temperatures, whereas CT_{MAX} and CT_{MIN} will be lower when exposed to lower temperatures, suggesting physiological plasticity in thermal tolerance. Importantly, these two hypotheses are not mutually exclusive, as there may be both adaptation to the local temperature regime, as well as the ability to respond plastically to temperature.

Materials and methods

Sampling design

Six populations were used in this study, and were located at low, mid, and high elevations within each of two elevational transects (200–1700 m asl) in southwestern Ecuador (Fig. 3.2, Table 1). Sampling was conducted in April-May 2014 (Transect 1) and January-March 2015 (Transects 1 and 2; note that Transect 2 here is the Transect 3 of Chapter 2). To determine the thermal regime to which tadpoles are exposed in their natural habitat, we placed temperature data loggers (HOBO U22-001, Water Temperature) in the ponds, streams, or ditches where tadpoles were collected. Habitat temperature was recorded every ten minutes for approximately two months (sampling days ranged from 54–62 days) while the experiments were carried out in 2015. We do not have data for the low elevation (300 m) site at Transect 2, as the data logger disappeared. Tadpoles (total $n = 936$) from low, mid, and high elevation ($n = 297$, 306, and 331, respectively; Table 3.1) were collected and were taken immediately to mid elevation (~800 m asl) to a field station where the experiment was conducted.

Experimental procedures

Tadpoles from each population were randomly assigned to one of three different temperature treatments for 1-2 weeks before measuring their thermal limits. The three temperature treatments were 20 °C, 24 °C, and 28 °C. The lowest treatment temperature (20 °C) corresponded to stream temperature at high elevation sites at the moment of tadpole collection and was maintained using an IceProbe Thermoelectric Aquarium Chiller. Mid temperature (24 °C) corresponded to room temperature at the field station located at mid elevation where the experiments were conducted. High temperature (28 °C) was chosen to maintain the 4ºC difference between the two previous temperatures, and was maintained using an Inster Submergible Aquarium Heater. Tadpoles were starved for 1-2 days prior to the experiments to avoid expenditure of energy for digestion during thermal tolerance experiments. Only tadpoles in developmental Gosner stages 25–38 (Gosner, 1960) were used to avoid effects of metamorphosis on thermal tolerance.

To measure critical thermal limits, tadpoles were placed in a different tank with an individual mesh container in a water bath. Initial temperature was the same as the respective treatment temperature. After 1-minute baseline period, temperature was increased or decreased at a constant rate of 0.5 °C min^{-1}. Because of possible decay of physical condition while measuring critical thermal limits, we did not use a slower rate of temperature change, as it could affect estimates of thermal limits, although faster rates seem to show more variation among individuals (Chown et al. 2009; Rezende et al. 2010). Given the small size of these ectothermic animals, water temperature was measured as a proxy of tadpole body temperature. Temperature was recorded with a digital temperature logger (Omega, HH804) connected to an Ultra precise RTD sensor (Omega, P-M-A-1/8-12-O-G-3). It is generally recommended to consider the onset of

spasms as a response to reaching CT_{MAX} (Lutterschmidt and Hutchison 1997). However, given the erratic movements of tadpoles, we recorded the temperature at which there was absence of response to a mechanical stimulus, which consisted of squirting water at the tadpole with a turkey baster. For CT_{MIN}, we recorded the temperature when cessation of movement was observed (Lutterschmidt and Hutchinson 1997). After reaching CT_{MAX} or CT_{MIN}, tadpoles were immediately placed in another water bath maintained at room temperature to allow recovery. We recorded total length, head length, and Gosner stage for each tadpole. Only data from tadpoles that appeared to recover immediately and that did not show signs of damage after one hour of observation were included in analyses. Each individual was tested only once.

Data analysis

To evaluate if there were differences in thermal limits among populations from different elevations, and if populations had the ability to shift their thermal limits in response to treatment temperature, we used linear mixed models. Models included population of origin (*i.e.* elevation) and treatment as fixed effects. Size (head length, HL) and time in the treatment temperature (in days) were included as random effects. We chose to use only head length as a metric for size because it was highly correlated with total length and Gosner stage (Pearson's $r = 0.92$, $r = 0.85$, respectively). For Transect 1, we also included year (2014 or 2015), mean habitat temperature, and standard deviation of habitat temperature as random effects. Models were run using the *lmer* function in the lme4 package in R (R Development Core Team). Models were ranked using Akaike's Information Criterion (AIC; Akaike 1973). A model was considered to be the best model if its AIC score was a minimum of 4 less than the second best model, and was

significantly different to the second best in an ANOVA. If there were models with similar support, we chose the one with fewer parameters as the best model.

Results

Habitat temperature

As predicted, habitat temperature decreased with elevation ($p < 0.001$, Fig. 3.3). However, when mean and standard deviation of habitat temperature were included in the mixed models on CT_{MAX} or CT_{MIN} (only for Transect 1), this variable did not have a significant effect on the models, nor decrease the AIC of the models. Therefore, these variables were not included in subsequent analyses.

Critical maximum temperature (CT_{MAX})

The best models for both transects included population of origin (elevation) and treatment temperature as fixed effects, with time in treatment temperature (days) as a random effect. In general, treatment temperature had a consistent positive effect on $CT_{MAX,}$ whereas there were no consistent differences in CT_{MAX} related to population of origin (elevation; Fig. 3.3). However, because the transects differed in whether there was an interaction or additive effect between population and treatment, and for ease of interpretation, we present results for transects separately. For transect 1, the model with an interaction between population and treatment had the highest support, whereas, for transect 2, the additive model had the highest support (Fig. 3.4; Table 3.2). For transect 1, the mid elevation population differed from the other two populations in that it had the lowest CT_{MAX} values when exposed to the 24 °C treatment, and the highest CT_{MAX} values when exposed to 28 °C (Fig. 3.4). Controlling for number of days of temperature

treatment and year improved the models ($p < 0.001$ when comparing models with an ANOVA; Table 3.2). However, including head length and mean habitat temperature had little or no effect. For transect 2, treatment temperature had a larger effect on CT_{MAX} values than population (*i.e.* elevation). There was a slight difference in how the mid elevation population responded compared with the low and high elevation populations, although models with and without interaction had similar AIC values (Fig. 3.4). In this transect, controlling only for size (head length) improved the models.

Critical minimum temperature (CT_{MIN})

As for CT_{MAX}, the best CT_{MIN} models included population of origin (elevation) and treatment (temperature) as fixed effects, with only time in the treatment temperature (days) as a random effect. The best models also varied between transects ($p < 0.001$ when comparing models with an ANOVA; Table 3.2). For transect 1, populations showed differences in their thermal tolerances, and could shift their response to environmental cues, but all three populations responded in a similar way, so the effect was additive. The high elevation population could resist colder temperature (i.e. lower CT_{MIN}) as hypothesized, followed by the low elevation population; whereas the mid elevation population showed higher CT_{MIN} (*i.e.* less resistant to colder temperatures compared to the high and low elevation populations; Fig. 3.3 and 3.4). For transect 2, populations had different thermal tolerances with the high elevation showing the lower CT_{MIN}, followed by the mid and the low elevation populations, as hypothesized. The three were able to shift their response to the treatment in similar ways, although the low elevation population showed lower CT_{MIN} when exposed to colder temperatures (20 °C) compared to the mid elevation population (Fig. 3.4).

Discussion

Our overall results showed that cooler temperatures are likely acting as a driving force leading to local adaptation, primarily with regards to $CT_{MIN.}$ Although we found differences in thermal limits between the two transects, the general trends are similar: populations from colder environments tolerate colder temperature, suggesting local adaptation to environmental temperature along elevational gradients. Our findings are consistent with previous studies in which that CT_{MAX} does not vary along elevational gradients in tropical anurans (e.g. Howard et al. 1983; Christian et al. 1978; Heatwole et al. 1968). Therefore, narrow thermal tolerances observed in tropical amphibians (e.g. Snyder and Weathers 1975; this study), lizards (e.g. Van Berkum 1988), and insects (e.g. Addo-Bediako et al. 2000) relative to temperate taxa are generally driven by differences in CT_{MIN} (Sunday et al. 2010). Tropical amphibians at higher elevations not only have broader thermal tolerances than lowland populations because CT_{MIN} declines with increasing elevation, but also because performance improves over broader ranges of temperatures at higher elevations (Brattstrom 1968; Christian et al. 1988; Navas 1996; Bernal and Lynch, 2013). The observation that there is variation along elevation in CT_{MIN} in the direction expected suggests that frogs from different elevations might have lower fitness than local frogs if they were to move into the nonnative thermal niche, particularly if they move up in elevation. Differences in fitness due to changes in temperature along elevation could eventually lead to genetic divergence between populations.

A primary result of our study is that populations of this tropical poison frog can shift their thermal limits in response to the temperature they are exposed to before measuring thermal

limits. Moreover, we found that treatment temperature had a stronger effect than population of origin. The physiological plasticity observed in these populations contradicts the general notion that tropical ectotherms have limited acclimation abilities (Feder 1978; 1982; Tsuji 1988; Patterson 1984; Overgaard et al. 2011; Gutiérrez-Pesquera et al. 2016), as well as the general trend of restricted elevational ranges in the tropics across taxa (Cadena et al. 2012; Duellman 1999; Gill et al. 2016). However, one of the reasons we chose to work with *Epipedobates anthonyi* was because it was one of the few tropical anuran species that shows broad elevational ranges and therefore allowed intraspecific comparisons along an elevational gradient. It remains unclear whether this is a species that shows atypical patterns of thermal physiology, if this species is recently diverging along elevation, or both. There is considerable gene flow along elevation, but the amount of gene flow decreases with increasing elevation, mainly due to differences in temperature (Chapter 2; see also Graham et al. 2004). Our results show that tadpoles from lower elevations can survive the temperatures observed at higher elevation. Nonetheless, the variation observed in CT_{MIN} along elevation suggests that there could be a cost for low elevation frogs that disperse to higher elevations, even with plasticity. Interestingly, the trend of CT_{MIN} decreasing at higher elevations is maintained in all the treatments, reinforcing this idea.

Habitat temperature decreases with elevation, such that each population's thermal limits reflect the environmental temperature gradient. In regards to variation of habitat temperature, the amount of variation in water temperature measured does not increase with elevation, as observed with air temperature. This trend demonstrates the importance of measuring microhabitat temperature as it does not always correlate with air temperature, usually obtain from WorldClim

(Navas et al. 2013; Camacho et al. 2015; but see Livingstone and Lotter 1998). We found that two populations (mid elevation at transect 1, and high elevation at transect 2) occurred in water bodies with very stable temperature regimes during the two months measured, yet they exhibited very similar levels of plasticity as other populations from more variable environments. This suggest that the amount of physiological plasticity in thermal tolerance does not correspond to the amount of temperature variation in their natural habitat, as seen in other studies (Richter-Boix et al. 2015). This mismatch between temperature variation in their habitat and therefore the thermal regimes experienced naturally by the tadpoles, poses the question of why this species retains physiological plasticity, particularly in populations that live in very stable water temperatures. Information on the trade-offs between broad tolerances and performance are relatively scarce (Angilleta et al. 2003; Snyder and Weathers 1975). *Epipedobates anthonyi* could be a "Jack-of-all-temperatures but master of none", a hypothesis that describes species with broader thermal ranges that have relatively low performances at their optimal temperatures (Huey and Hertz, 1984). Other studies have shown that tropical high elevation anurans are able to increase performance at colder temperatures without an evident reduction in absolute performance (John-Alder et al. 1988; Navas 1996). However, to test these hypotheses, we need to characterize thermal performance curves, which in turn, would allow a deeper understanding of the role of temperature as a selective pressure along elevation, and how organismal performance changes in response to environmental temperatures.

Understanding the potential of organisms to respond to novel selective pressures will not only allow us to determine the factors that have driven and currently drive their evolutionary history, but also their ability to respond to climate change (Tewksbury et al. 2008; Williams et al. 2008;

Chown et al. 2010; Duarte et al. 2012). If populations have physiological plasticity, they may be buffered by the effects of climate change to allow persistence (Chown et al. 2010; Gunderson and Stillman 2015). Although physiological plasticity is ubiquitous along elevational (and latitudinal) gradients, ectotherms sometimes experience temperatures outside of their thermal optimum. Therefore, they have to rely on plasticity or behavioral thermoregulation (Sunday et al. 2014). In the case of aquatic ectotherms, behavioral thermoregulation might not suffice to buffer changes in temperature because there is less microhabitat variation in temperature over time (Duarte et al. 2012; Gunderson and Stillman 2015). Some evidence, however, exits that tadpoles aggregate at warmer or colder spots within a pond, depending on their thermal preferences related to temperature variation in their habitat (Jara et al. 2006). We observed this aggregation behavior in tadpoles, but it was most likely associated with anti-predator behavior, or niche partitioning between sympatric species. Additionally, organisms might be able to cope with the effects of climate change by selecting water bodies with different temperatures. For instance, poison frogs exhibit parental care, in which one or both parents transport tadpoles from egg clutches to water bodies after hatching (Lötters et al. 2009). Ringler et al. (2013) found adaptive plasticity in parental behavior in terms of sites where tadpoles are deposited. However, more studies are necessary to reveal if the amount of plasticity observed will buffer the effects of climate change (Gunderson and Stillman 2015).

A variety of selective pressures likely drive adaptive divergence along elevation. Our results suggest that variation in temperature plays a role in shaping the critical thermal minimum temperature limits of populations along elevational gradients. Likewise, populations are able to shift their CT_{MIN} in response to environmental temperatures experienced. Nonetheless, despite

acclimation ability, the trend observed in CT_{MIN} is maintained (*i.e.* lower temperature at higher elevations), reinforcing the idea of temperature acting as a selective force along elevation. Temperature affects all biological processes, from the molecular level (*e.g*. enzymatic reaction rates) to the organismal level (*e.g*. growth rates, developmental rates, and reproduction; Angilleta et al. 2002; Navas 2002; Navas et al. 2007; Pörtner et al. 2006). It can also affect organisms indirectly by influencing ecological factors such as resource availability, predators, competitors, and parasites (Keller and Seehausen 2012). Thermal biology has much to offer in linking phenotype with environment from an evolutionary, physiological, and conservation perspective. Hence, further integration of thermal performance and fitness of populations, along with comparison of these findings among different taxa and ecological groups will advance our understanding of anuran thermal biology, its role in evolutionary processes, and the ability to adapt and persist in changing environments.

Tables and figures

Table 3.1. Locality name, elevation, coordinates, samples size per treatment, CT_{MAX}, CT_{MIN}, and habitat temperature (°C). For the last three columns mean ± SD are presented, and range for habitat temperature. Temperature data logger from Saracay disappeared.

Population	Elevation (m asl)	Latitude	Longitude	20°C (*n*)	24°C (*n*)	28°C (*n*)	Total (*n*)	CT_{MAX}	CT_{MIN}	Habitat T (°C)
Transect 1										
Jubones	200	- 3.317	79.670	60	71	62	193	39.26 ± 0.72	8.32 ± 1.03	23.45 ± 0.51 *(20.60–25.26)*
San Sebastian	800	- 3.302	79.529	48	62	57	167	39.17 ± 0.85	8.71 ± 0.91	22.23 ± 0.03 *(22.18–24.1)*
Zharug	1600	- 3.275	79.543	66	72	67	205	39.44 ± 0.69	7.85 ± 0.89	17.92 ± 0.73 *(16.61–21.84)*
Transect 2										
Saracay	300	- 3.389	79.588	32	37	35	104	40.31 ± 0.48	8.35 ± 0.82	-
Moromoro	900	- 3.662	79.740	45	47	47	139	39.97 ± 0.58	8.12 ± 0.69	20.71 ± 0.36 *(19.94–25.77)*
Paccha	1700	- 3.583	79.672	32	47	47	126	40.32 ± 0.50	7.73 ± 0.66	19.53± 0.02 *(19.46–19.63)*

Table 3.2. Summary of mixed models for thermal critical temperatures, best models bolded. Population = population of origin; treatment = acclimation temperature treatment. Random effects in parentheses: HL = head length; days = number of days in the treatment temperature; year = for transect 1, if experiment was conducted on 2014 or 2015. Full models included Gosner's stage, and mean of habitat temperature (only for transect 1) but those variables did not have any effect on the models.

Model	AIC	ΔAIC
a) TRANSECT 1		
- **CT_{MAX} ~ Population * Treatment + (days) + (year)**	**402.38**	**0.00**
- CT_{MAX} ~ Treatment + (days) + (year)	429.25	26.87
- CT_{MAX} ~ Population + Treatment + (days) + (year)	429.79	27.41
- CT_{MAX} ~ Population + (days) + (year)	567.50	165.12
- **CT_{MIN} ~ Population + Treatment + (days)**	**564.30**	**0.00**
- CT_{MIN} ~ Population * Treatment + (days)	566.16	1.86
- CT_{MIN} ~ Treatment + (days)	589.62	25.32
- CT_{MIN} ~ Population + (days)	720.10	155.80
b) TRANSECT 2		
- **CT_{MAX} ~ Population + Treatment + (HL)**	**95.20**	**0.00**
- CT_{MAX} ~ Population * Treatment + (HL)	97.50	2.30
- CT_{MAX} ~ Treatment + (HL)	120.72	25.52
- CT_{MAX} ~ Population + (HL)	277.42	182.22
- **CT_{MIN} ~ Population * Treatment + (days)**	**367.65**	**0.00**
- CT_{MIN} ~ Population + Treatment + (days)	371.95	4.30
- CT_{MIN} ~ Population + (days)	389.14	21.49
- CT_{MIN} ~ Treatment + (days)	403.87	36.22

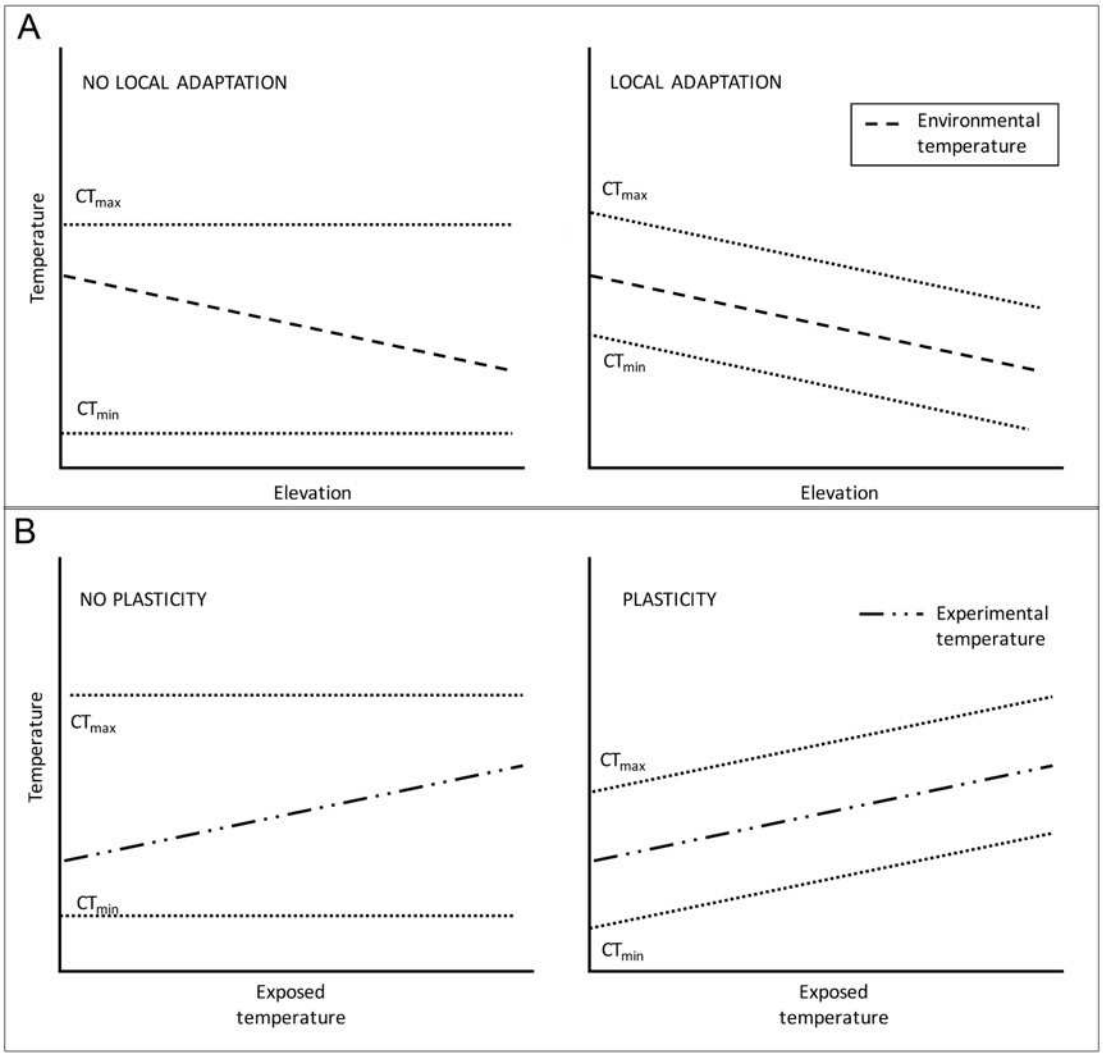

Figure 3.1. Hypotheses tested, with null hypotheses on the left and alternative hypothesis on the right. (A) Temperature is a driving force leading to local adaptation along elevation in thermal tolerance. (B) Organisms are able to shift their thermal tolerance depending on the environmental conditions they are exposed to prior to measuring CT_{MAX} and CT_{MIN} due to plasticity.

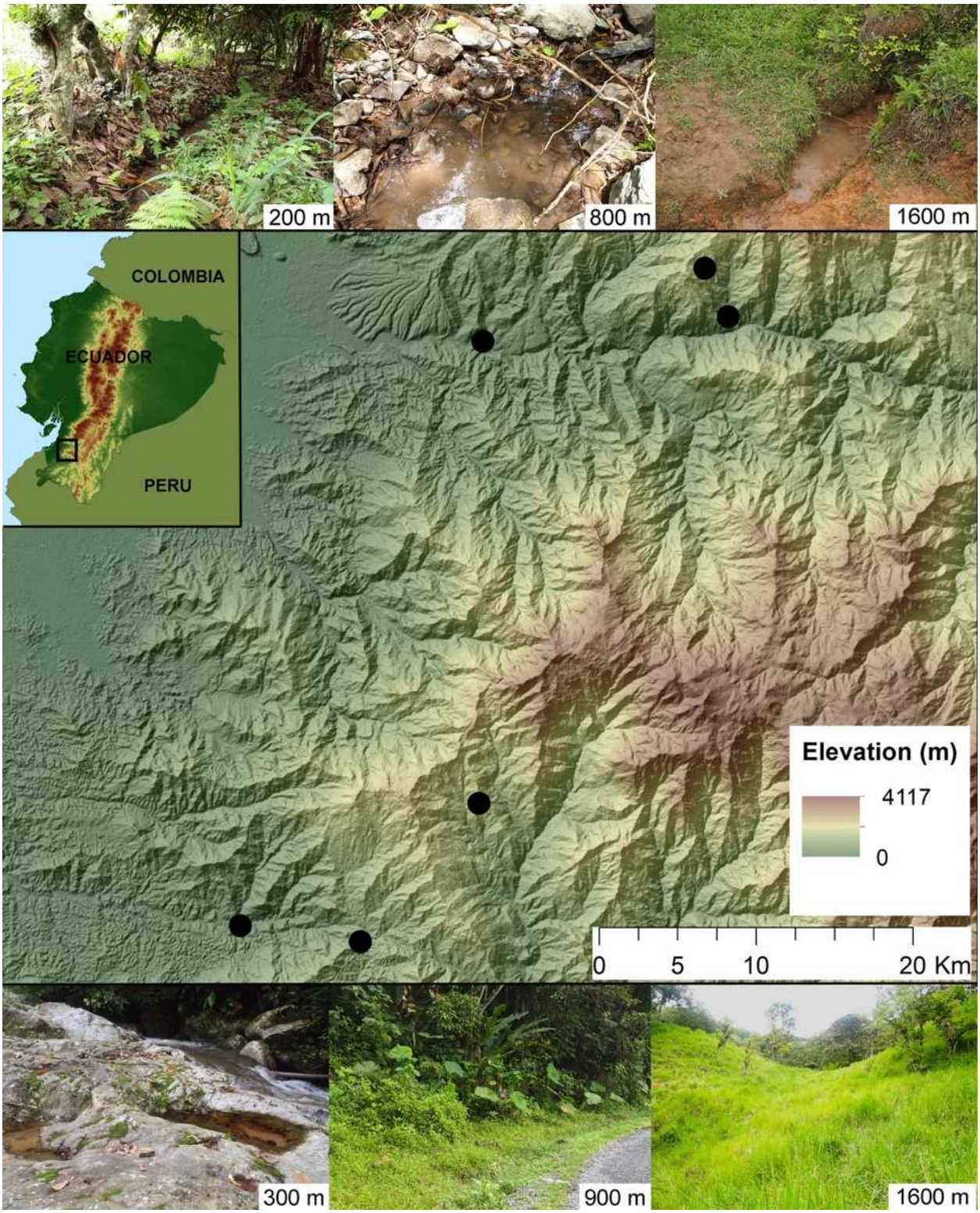

Figure 3.2. Studied localities along two elevational transects in Southern Ecuador. Top panels correspond to the habitats of the northern transect 1; bottom panels to southern transect 2.

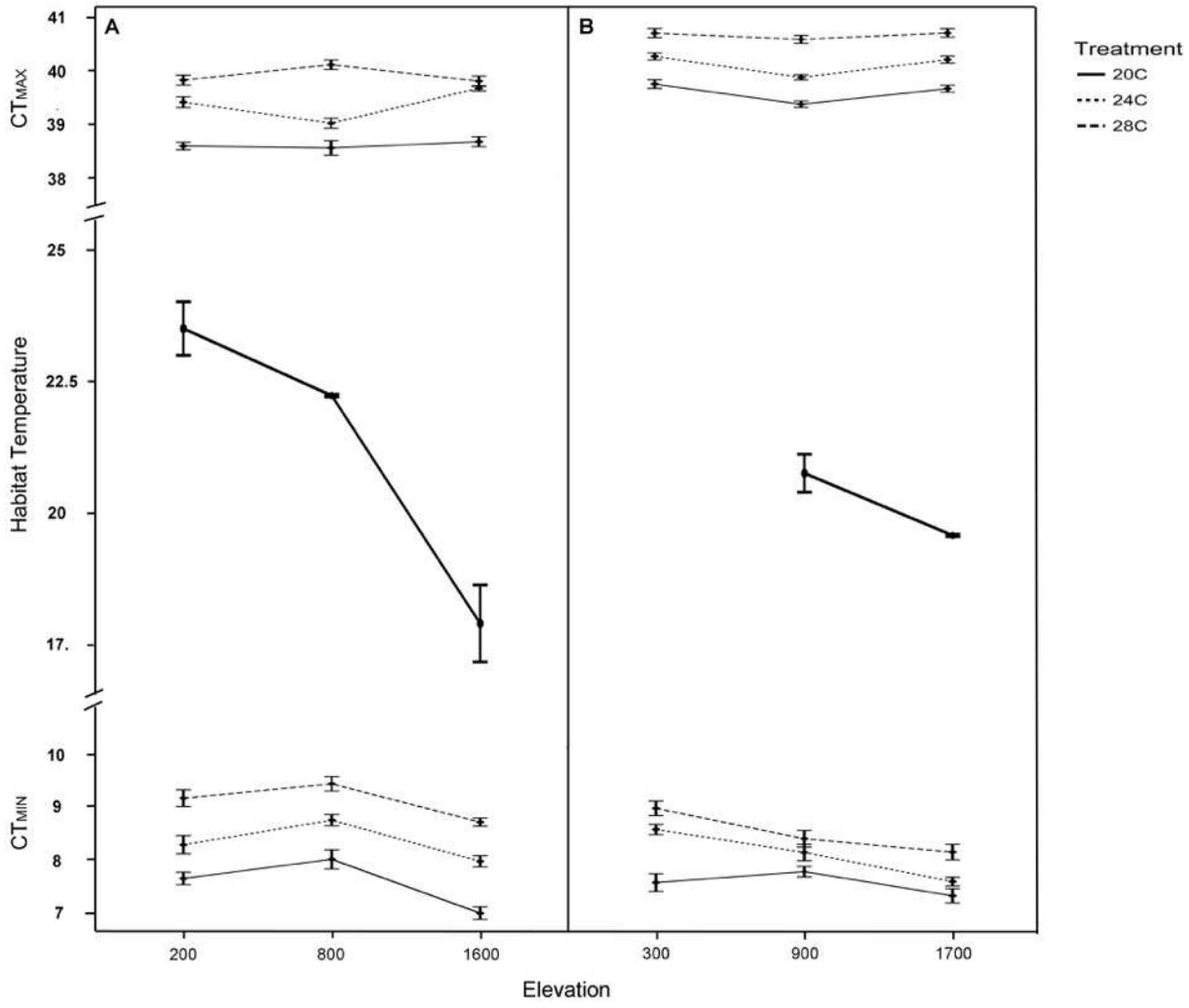

Figure 3.3. CT_{MAX}, habitat temperature, and CT_{MIN} along elevation (m). (A) Transect 1 and (B) transect 2. Data logger from 300 m in Transect 2 was lost during the experiment.

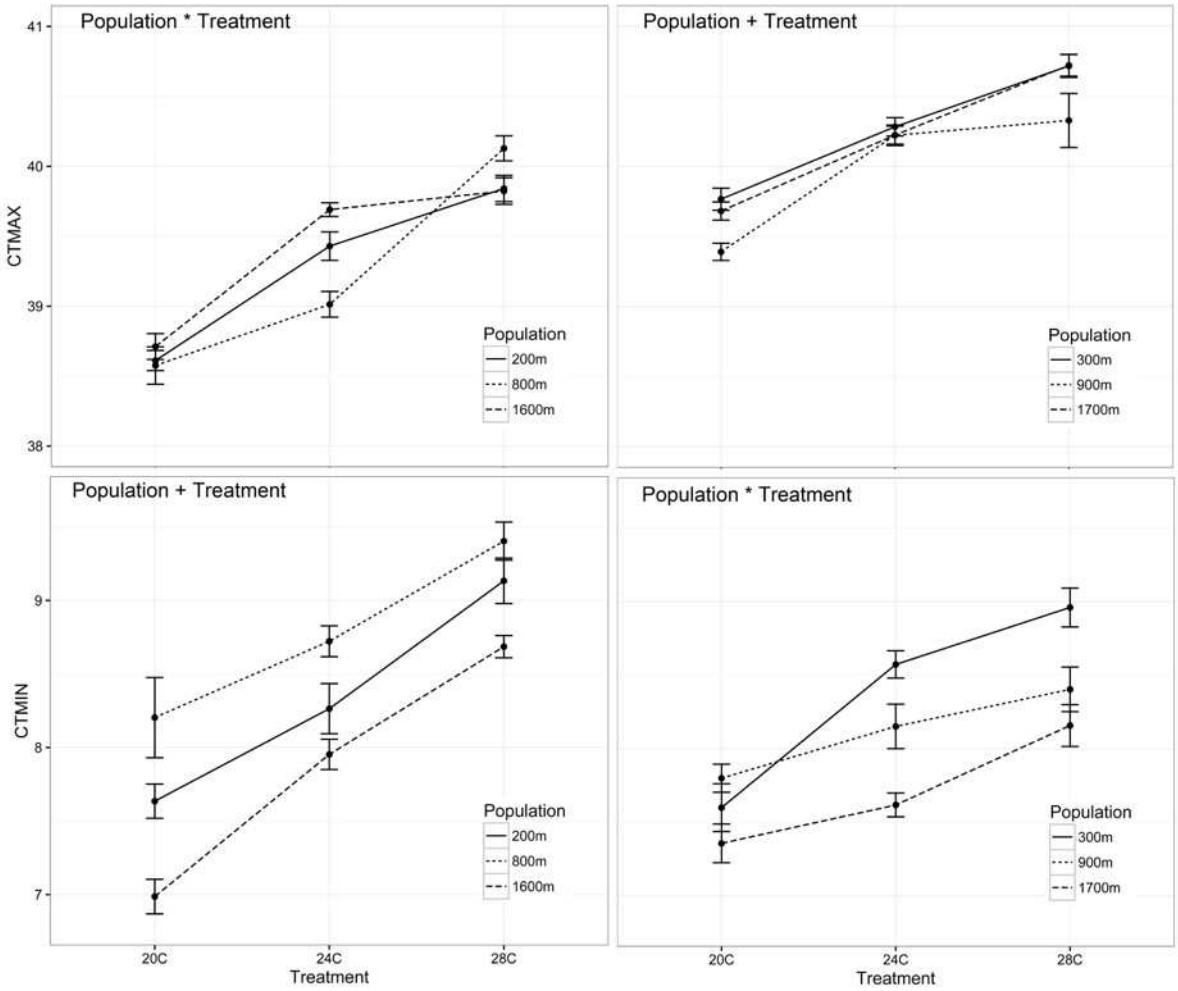

Figure 3.4. CT_{MAX} and CT_{MIN} as a function of treatment temperature along Transect 1 (left), and Transect 2 (right).

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4. LOCAL ADAPTATION IN LIFE HISTORY TRAITS ALONG ELEVATIONAL GRADIENTS?

Summary

Environmental variation can be a selective force causing phenotypic change. Elevational gradients are an excellent system to understand environmental effects on phenotypes, such as life history traits. We conducted a reciprocal transplant experiment to investigate if populations of the poison frog *Epipedobates anthonyi* are locally adapted to the environmental conditions at their local elevations. We evaluated life history traits of populations from high and low elevations from two elevational transects for seven months. Overall, populations had higher reproductive rates at lower elevation, and low elevation populations had higher reproductive rates at high elevation. Populations from each transect revealed different patterns of variation in life history traits. At low elevation, the lower elevation population of one transect had higher reproductive rates than the high elevation one, consistent with a pattern of local adaptation. In contrast, in the other transect, the high elevation population had higher reproductive rates than the low elevation one, suggesting countergradient selection. These results suggest that environmental variation along elevational gradients has an important effect on life history traits, and that its influence varies at the intraspecific level.

Introduction

Understanding the effect of environmental variation on phenotypes is a major question in evolutionary ecology. If environmental variation causes divergent selection, local populations can evolve traits that provide an advantage under their local environmental conditions in the absences of other forces or constraints, a process known as local adaptation (Kawecki and Ebert

2004). Local adaptation in populations connected by gene flow is indicative of natural selection imposed by particular environmental factors (Lenormand 2002; Räsänen and Hendry 2008) and might have a crucial role in initiating the divergence of incipient species (Schluter 2001). Therefore, the study of local adaptation is particularly interesting in the context of speciation.

With local adaptation, we would expect that individuals from the local population have higher fitness than foreign individuals. Likewise, we would expect that an individual has a higher fitness at its local conditions than at an unfamiliar environment (Kawecki and Ebert 2004). However, new environmental conditions can also reveal hidden variation. Countergradient variation occurs when selection produces phenotypes that oppose or compensate for environmental effects (Levins 1968; Conover and Schultz 1995). Countergradient variation has been typically described in the context of temperature and growth, where organisms' exposed to different temperature show the same growth, despite underlying differences at the genetic level (Levins 1968; Conover and Schultz 1995; Ghalambor et al. 2007).

Elevational gradients provide a great opportunity to study how environmental gradients affect phenotypic variation. Elevational gradients occur at a relatively small spatial scale, which limits confounding effects at larger scales due to evolutionary trajectories, and populations are putatively experiencing gene flow (Keller et al. 2013). Moreover, montane regions are hotspots of diversity and endemism, and also among the most vulnerable regions on earth (Myers et al. 2000; Tewksbury et al. 2008). Therefore, understanding how divergent environmental selection along elevation affects phenotypes is particularly interesting in speciation (Schluter 2001). It can

also aid conservation by improving our ability to assess how populations will respond to rapidly changing environments (Bridle et al. 2009; Keller et al. 2013).

Amphibians are exceptionally diverse and are at the forefront of the current biodiversity crisis in montane regions (Stuart et al. 2004; Morrison and Hero 2003). According to phenotypic variation previously documented, amphibian populations occurring at higher elevations are expected to have larger body size, fewer clutches, larger clutch size, larger egg size, and slower growth and development (Bull and Shine 1979; Morrison and Hero 2003). However, when controlling for female body size, some of these trends are lost (Koslowzka 1971; Berven 1982a). Moreover, the review by Morrison and Hero (2003) found that intraspecific variation occurs in these life-history traits, and that the number of studies addressing these issues are relatively few. Likewise, some studies have found countergradient variation along elevation in growth and developmental rate (Berven et al. 1979; 1982a; Riha and Berven 1995). Therefore, there are still gaps of knowledge on the effect of environmental gradients along elevation on phenotypic variation in amphibians, and no clear patterns emerge from the relatively scarce studies.

The poison frog *Epipedobates anthonyi* is an abundant species distributed along the western slopes of the southern Ecuadorian Andes from 80 to 1800 m above sea level (m asl, Fig. 4.1). It is ideal for experimental investigations due to their ability to breed successfully in captivity (L. Coloma, *pers. comm.* Centro Jambatu for Amphibian Research and Conservation, Quito, Ecuador). Previous results (Chapter 2) indicate that this frog species varies in size, coloration, and male advertisement calls, and that this variation can be associated with differences in elevation. Northern population are larger and redder, and become larger and brighter with

elevation, which might be indicative of adaptive divergence. On the other hand, southern populations are browner and smaller and do not show this trend along elevation.

The aim of this study was to test whether ecological differences along elevational gradients promote local adaptation by performing reciprocal transplant experiments. Reciprocal transplant experiments are the most powerful way to test for the effect of environmental differences on phenotypes and local adaptation (Sibly 1996; Kawecki and Ebert 2004). We used a pair of populations from the northern range that have phenotypic variation in size and color along elevation, and a pair of populations from the south that do not vary along elevation. We chose these populations because they exhibit differences in the amount and patterns of phenotypic variation along elevation; consequently, we wanted to know if they exhibit either similar or different patterns of local adaptation. We focused on life history traits, such as reproductive and developmental rates. By using this approach, we were able to test the following hypotheses: (i) populations are locally adapted to their native elevation range. Under this hypothesis, we predicted that individuals would exhibit higher fitness (or proxies for fitness) when transplanted to an elevation similar to their origin and/or exhibit higher fitness at their own elevation than foreign individuals, suggesting local adaptation to elevation due to divergent natural selection (Fig. 4.2A). (ii) Populations exhibit countergradient variation in life history traits. Under this hypothesis, we predicted that individuals would have higher fitness (or proxies) when transplanted to a different elevation from their origin, or that foreign individuals will have higher fitness, although this might not happen both ways (Fig. 4.2B). (iii) Given that populations studied show differences in the patterns of variation along elevation (Chapters 2 and 3), we tested if the patterns of phenotypic variation in life-history traits are also different at the

intraspecific level (Fig. 4.2C). Under this hypothesis, we predicted that populations from the northern transect would show more evidence of local adaptation in life history traits, given that they show more phenotypic divergence along elevation (e.g. size, color, and calls; Chapter 2).

Methods

Study populations

For this experiment, we studied four populations of *Epipedobates anthonyi* that correspond to a high (~ 1500 m asl) and a low (~ 500 m asl) elevation population from a northern elevation transect (High1 and Low1, respectively), and a high (~1500 m asl) and a low (~500 m asl) elevation population from a southern elevation transect (High2 and Low2, respectively; note that these southern transect correspond to Transect 3 of Chapter 2). We studied two different elevational gradients, to identify if observed differences are due to elevation or other local features. We collected 40 individuals from each population (24 females, 16 males) on 11–15 December 2013, which were carefully transported in individual containers to the study sites. Local frogs were also transported to our hotel after collection, and brought back the next day to the study site to control for any effects of transportation. These frogs were photographed, measured, and provided a unique code to create identification cards.

Study site and experimental design

The reciprocal transplant experiment was conducted from mid-December until mid-July 2014 (208 days, ~ 7 months). We set up four enclosures at each of two focal elevation sites located in Transect 1 at 500 and 1600 m asl, respectively (total number of enclosures = 8; Fig. 4.1). The enclosures used in this study were built based on the enclosures successfully used for amphibian

breeding in the facilities of the Centro Jambatu for Amphibian Research and Conservation in Quito, Ecuador.

Our enclosures were 3x3x2 m and were divided in four subdivisions (sub-cages), one subdivision for each of the four study populations (Fig. 4.1B). Enclosures were built using mesh (~1.5 mm mesh size) to minimize environmental differences with the outer environment and to allow small insects in and out. The enclosures had a closed mesh roof that let light in (Fig. 4.1C), and the lateral mesh was buried ~50 cm underground for the enclosures to be sealed to the frogs and possible predators. The ground of the cages was the natural soil with leaf litter to emulate the habitat conditions at each site. We included bark, bromeliads, and other natural elements where these frogs naturally perch and use as cover. Finally, every subdivision had an artificial pond with detritus to allow tadpole deposition and development. After reassuring that the cages did not contain any other creatures (*e.g.* other frog species, lizards, tarantulas or spiders), three females and two males from each population were assigned randomly to each division within the enclosures. Thus, each enclosure had 20 adult frogs, with 5 from the same populations in each subdivision. With this design, we included a local population in each enclosure. Frogs were placed in their enclosure between 1-5 days from their collection date.

During the course of the experiment, we supplemented the enclosures with bananas infested with *Drosophila* weekly, and with leaf litter (and associated microfauna, such as small arthropods) monthly to ensure that food availability was not a limitation. We did a census in the enclosures every two weeks to record adult survival and life-history traits, namely number of clutches, number of tadpoles, tadpole size and stage, number of metamorphs, size of metamorphs, and

days to complete metamorphosis. At completion of the experiments, frogs and tadpoles were fixed and preserved following standard museum collection protocols and deposited in the scientific collection of Centro Jambatu for Research and Conservation of Amphibians (CJ, Quito-Ecuador). Preserved females were dissected and five eggs from each were measured using a 0.01 mm Digital Caliper. All procedures were approved by the Colorado State University Institutional Animal Care and Use Committee.

Data analyses

Reciprocal transplant experiments can be examined following two criteria. Comparisons can contrast the fitness of different populations at each elevation, the 'local vs. foreign' criterion (Kawecki and Ebert 2004). Individuals from the local populations are expected to have higher fitness than individuals from foreign populations. In contrast, the fitness of a particular population can be compared at different elevations, the 'home vs. away' criterion (Kawecki and Ebert 2004). Under this criterion, individuals are expected to have higher fitness at their own environment than at any other environment.

We first compared reproduction of different populations at each elevation (*i.e.* 'local vs. foreign' criterion; Kawecki and Ebert 2004). To compare the proportion of sub-cages that produced tadpoles and metamorphs per population at a given elevation, we used a Fisher Exact Test. We then compared number of clutches, tadpoles, metamorphs for each population using a Kruskal-Wallis test in R (R Development Core Team). Because not all enclosures maintained the same number of adults throughout the experiment, we corrected the final reproduction results for the days per adult in each enclosure. If a sub-cage lost all the individuals from the same sex at some

point of the experiment, we did not include that period of time, as reproduction could not take place. We used linear mixed models to compare egg size between populations (fixed effect), and females nested within sub-cage as a random effect. For tadpole size between populations, we used sub-cage and Gosner stage as random effects. For developmental rate of populations at either low or high elevation sites, we used linear mixed models. Models included population as a fixed effect and cohort nested within sub-cage as random effects. The response variables were size at metamorphosis and days to complete metamorphosis. Models were run using the *lmer* function in the lme4 package in R (R Development Core Team).

We then examined if populations had higher reproduction at their local elevation (*i.e.* 'home vs. away' criterion; Kawecki and Ebert 2004). As above, we used a Fisher Exact Test for proportions of sub-cages. We then compared numbers of clutches, tadpoles, and metamorphs for each population using a Two Sample Wilcoxon test for count data in R (R Development Core Team), also corrected by adult/days. Finally, we used linear mixed models to compare egg size, tadpole size, metamorphs size, and days to complete metamorphosis, explained by population (fixed effect), using the same random effects and package in R detailed above.

Results

We found significant differences in reproduction between the two sites at high and low elevations (Table 4.1). All populations showed more reproduction events (*i.e.* more clutches and tadpoles per population) at the low elevation site (Table, Fig. 4.3). At the high elevation site, surprisingly, low elevation populations had higher reproductive rates than the high elevation populations; whereas high elevation populations had very low or no reproduction at all. At the

low elevation sites, each transect showed a different trend. For populations from transect 1, the low elevation population had more reproduction that the high elevation one (Table 4.1, Fig. 4.3). However, both populations produced similar number of metamorphs (Fig. 4.3). For populations from transect 2, on the other hand, the high elevation population had more reproduction that the low elevation one. As in transect 1, the only exception to this trend was the number of metamorphs. In this transect, the low elevation population produced more metamorphs than the high elevation one (Fig. 4.3). Although eggs, tadpoles and metamorphs had similar size in high and low elevation sites for all populations, developmental rate at high elevation was significantly slower (Table 4.1; Fig. 4.4).

Discussion

We found that there were dramatic differences in life history traits between sites. All populations had higher reproduction rates at the low elevation site compared to the high elevation site. These results are not consistent with the general expectation that populations would have better performance in their local environment. Apparently, the environmental conditions at low elevations are more suitable for every population. The average temperature difference between high and low elevations is 6 °C (annual mean temperature at high elevation is 20 °C, at low elevation, 26 °C). Higher environmental temperatures at lower elevations, along with higher body temperatures, influence physiological rates (Pörtner et al. 2006). Thus, an ectotherm's behavioral and ecological performance can be influenced by body temperature (Huey and Kingsolver 1989). The general hypothesis that hotter is better predicts a positive correlation between the organisms' optimal temperature and its maximum performance (Bennet 1987; Kingsolver and Huey 2008; Knies et al. 2009). The strongest support for this hypothesis comes

from evidence of comparative studies between species, and such studies are still scarce (*e.g.* Frazier et al. 2006; Kingsolver and Huey 2008; but see Knies et al. 2009). The finding that all four of our study population perform better at the lower elevation, suggests that, in the absence of other pressures (*e.g.* predators, food limitation), hotter is better for this species.

Comparison of population performances at the low elevation site showed that populations from each transect had different patterns of variation. Populations from transect 1 showed a pattern that resembles local adaptation, *i.e.* the low elevation (Low1) population had higher reproductive rates than the high elevation population (High1). In populations from transect 2, on the other hand, the high elevation population (High2) outperformed all the other populations in reproductive rates. These results resemble a pattern of countergradient variation (Conover and Schultz 1995). The high elevation population (High2), in its natural habitat, might be compensating for slower biological rates at low temperatures. Therefore, once that constraint is removed, individuals achieved higher reproductive rates (Huey and Kingsolver 1989; Frazier et al. 2006). Interestingly, the populations from transect 1 that varied in adult size and color along elevational gradient (Chapter 2) showed patterns of local adaptation, whereas populations that did not show variation in size and color along elevation (Chapter 2) did not show local adaptation. This might suggest that populations along the first transect have attained some level of adaptive divergence, but populations from the second transect have not.

In terms of number of metamorphs at the low elevation site, populations from transect 1 had about the same number of metamorphs. In populations from transect 2, the low elevation population (Low2) had more metamorphs at the end of the experiment, despite the fact that the

high elevation population had produce more tadpoles. At the termination of our study, there was a large proportion of tadpoles that had not metamorphosed yet, therefore, we cannot be certain if these patterns would be maintained given enough time for all the tadpoles to complete metamorphosis. However, we suspect that, at least in the case of the high elevation population (High2) that produced hundreds of tadpoles, lower number of metamorphs could be due to lower developmental rates, and perhaps density and competition (Berven 1982b). The observation of cannibalism in the most populated ponds could be indicative of the latter.

At the high elevation site, we found that both low elevation populations performed better than both high elevation populations, particularly one population (High2) that did not reproduce at all at high elevation. Unfortunately, due to the low reproduction success overall at this site we cannot make further home vs. away comparisons. We are not certain why this low reproductive success at high elevation occurred, especially in high elevation populations. Perhaps reproductive rates at high elevation were too slow to be captured by the duration of our experiment. Populations from transect 2 occur in regions with higher precipitation than transect 1 (Chapter 2), so perhaps the population that did not reproduce at all (High2) was very sensitive to different precipitation regimes found in the focal sites (see Chapter 2). Low elevation populations seemed to have higher fecundity compared to high elevation populations. This higher fecundity could be related to faster developmental rates due to higher temperatures, or due to higher mortality rates. We have observed that frogs at different elevations exhibit dramatic difference in behavior, with frogs from lower elevations being stealthier, suggesting higher predation rates.

Life-history traits are expected to vary along elevational gradients (Morrison and Hero 2003; Keller et al. 2013). Our findings corroborate the expected patterns in which populations reproducing at higher elevations tend to have larger size and lower growth and developmental rates (Morrison and Hero 2003). Two populations (High1 and Low2) showed larger eggs at higher elevation; however, those differences were not statistically significant. Moreover, we did not find differences between sites in tadpole and metamorphs size. Pettus and Angleton (1967) suggested that larger sizes of highland tadpoles, due to larger egg sizes, would reduce developmental time, which is expected to be longer due to lower physiological rates at lower temperatures (Smith-Gill and Berven 1979; Berven 1982a; Howard and Wallace 1995). Nonetheless, our results suggest that in *E. anthonyi*, populations at higher elevation are indeed taking more time to develop, likely compensating for the fact that egg sizes are not significantly larger at higher elevations (Berven 1982a; Howard and Wallace 1985, Morrison and Hero 2003). In general, studies have found contradictory results in terms of egg sizes and clutches along elevation (e.g. Gollman and Gollman 1996; Berven 1982a; Howard and Wallace 1985; Morrison and Hero 2002). These studies have also found a positive correlation between egg and clutch size with female body size (Morrison and Hero 2003). Interestingly, in our study the only high elevation clutch produced at high elevation had the largest number of tadpoles (20 tadpoles). This female was from transect 1, in which body sizes increase with elevation. Females that produce larger clutches are expected to produce smaller eggs to compensate the amount of energy devoted to reproduction (Duellman 1992), which could explain why these differences were not significant even if eggs are larger.

Mechanisms that promote speciation are most likely to be discovered by studying the early stages of divergence, while incipient species are still connected by gene flow. In this study, we investigated how divergent selection along elevational gradients would promote local adaptation of natural populations in a poison frog. Our results suggest that indeed, environmental variation along elevation has a significant effect on life history traits. Conditions at lower elevation seem to increase the reproductive rates. Moreover, populations adapted to low elevation environmental conditions exhibit higher reproductive rates, suggesting higher fecundity in these populations. Populations adapted to high elevation conditions, on the other hand, can thrive at low elevation either at lower densities than the local populations, or by outperforming the low elevation counterparts.

These findings highlight the role of environmental variation as a driver of phenotypic divergence in natural populations. Population sensitivity to environmental changes along elevation, variation at the intraspecific level, lower reproduction rates and slower developmental rates at high elevations, all have important implications for conservation of montane organisms. Further research is needed to elucidate if these findings are maintained across populations and species and to further predict their ability to overcome the current threats of montane amphibians.

Tables and Figures

Table 4.1. Results of statistical analyses (*p*-values). In local vs. foreign comparison, we compared all population at a given site. For home vs. away, we compared each population between sites. For mixed models, *p*-value corresponds to Ho = estimate value is equal to zero.

	Local vs. foreign		Home vs. away			
	HIGH[a]	LOW	HIGH1	HIGH2	LOW1	LOW2
No. of clutches[b]	0.05182	0.01991*	0.1241	-	0.0294*	0.02857*
No. tadpoles hatched[b]	0.1211	0.02545*	0.1241	-	0.0294*	0.02857*
No. tadpoles survival[b]	0.04162*	0.03823*	0.1241	-	0.0294*	0.1465
No. metamorphs[b]	0.01317*	0.8301	-	-	0.1081	0.4857
Clutch size[b]	0.2646	0.440	0.5	-	0.2403	0.6857
Egg size[c]	Low1 $p<0.001$	*NS*	*NS*	-	*NS*	*NS*
Tadpole size[c]	*NS*	*NS*	*NS*	-	*NS*	*NS*
Metamorphs size[c]	*NS*	*NS*	-	-	*NS*	*NS*
Days to metamorphosis[c]	*NS*	*NS*	-	-	$p < 0.001$	$p = 0.0226$*

[a]Comparisons were only between populations that had reproduction and/or metamorphs.
[b]Kruskal-Wallis for local vs. foreign comparisons. Wilcoxon for home vs. away comparisons.
[c]Mixed models

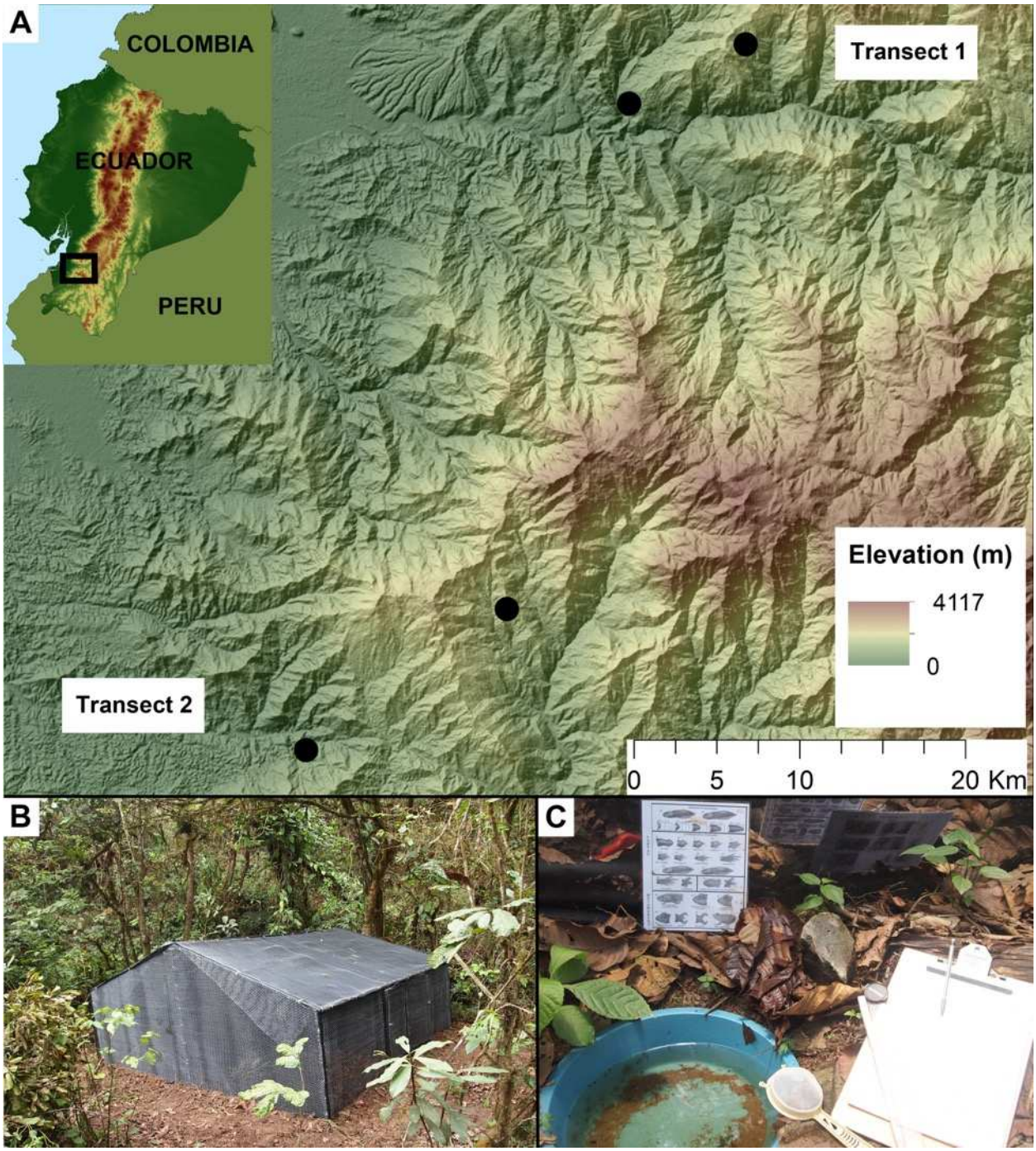

Figure 4.1. (A) Populations studied from transects 1 and 2. Focal sites were at transect 1. (B) Enclosures used for the reciprocal transplant experiment. (C) Enclosure from the inside during biweekly census.

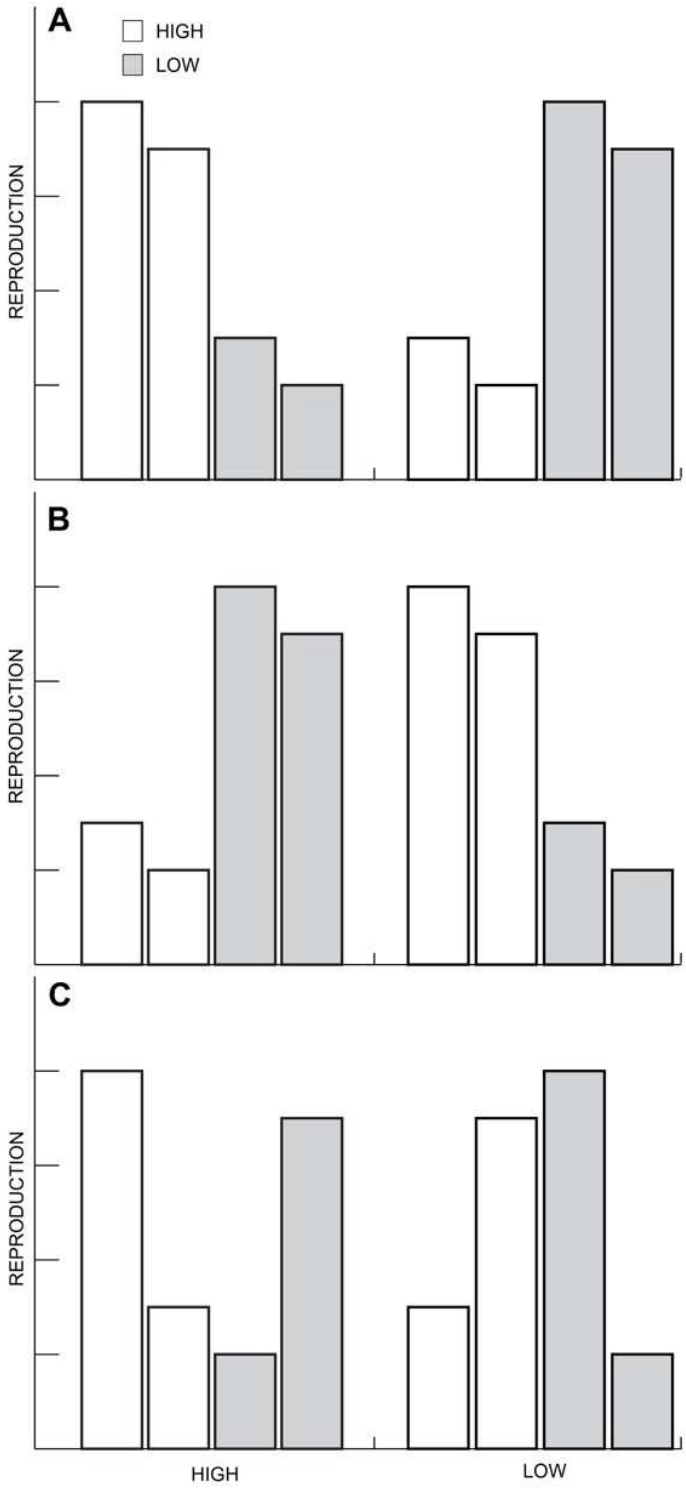

Figure 4.2. Possible outcomes from the reciprocal transplant experiments. Reproduction on the two study sites (high and low) in the case of (A) local adaptation along the elevational gradient, (B) countergradient variation, or (C) intraspecific variation. Populations from high elevation are in white, and populations from low elevation, in gray.

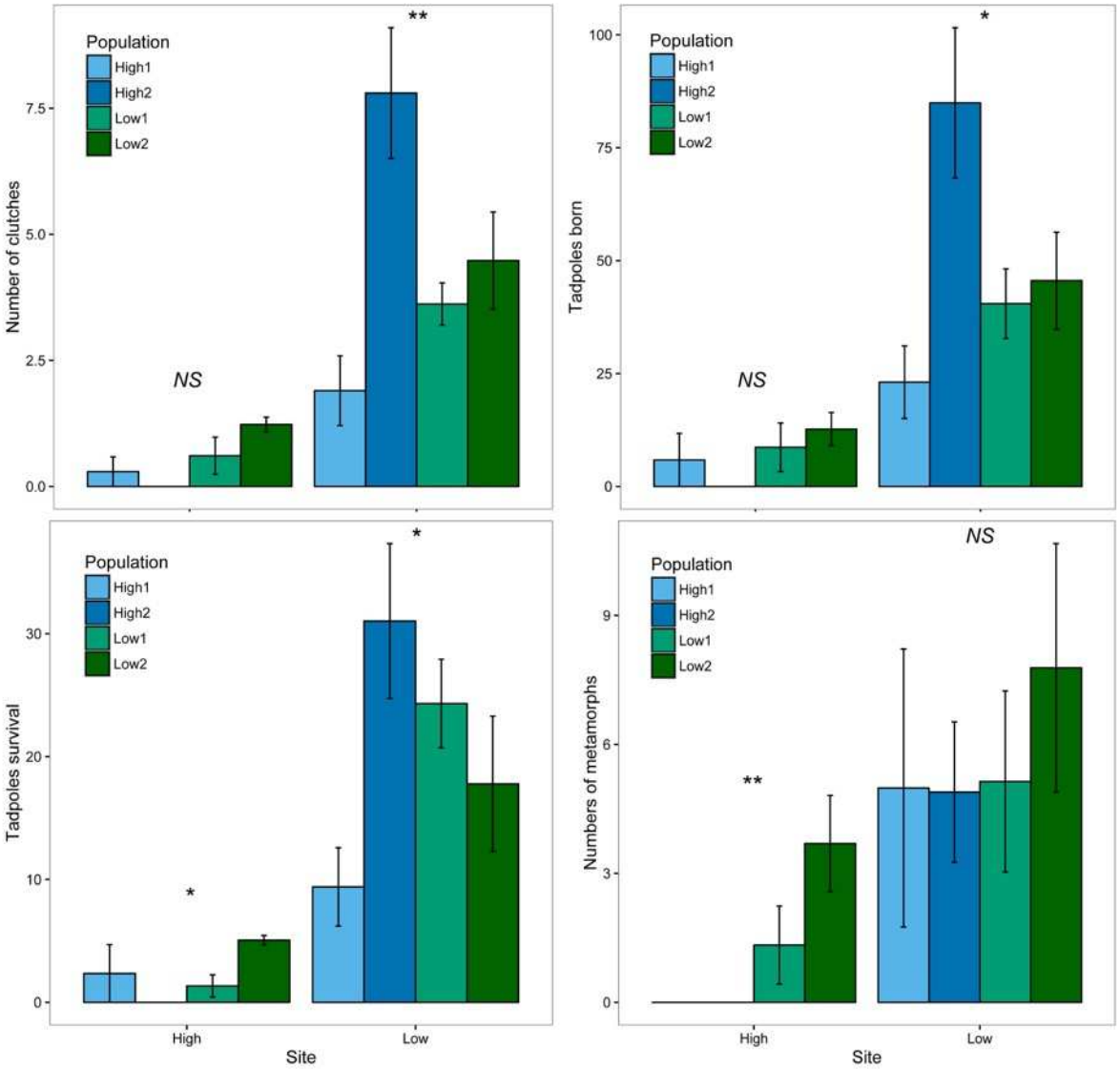

Figure 4.3. Results of life history traits related to reproductive success. Stars denote statistical significance (* $p < 0.05$, ** $p < 0.001$) for comparisons between populations at a given site (*i.e.* local vs. foreign criterion).

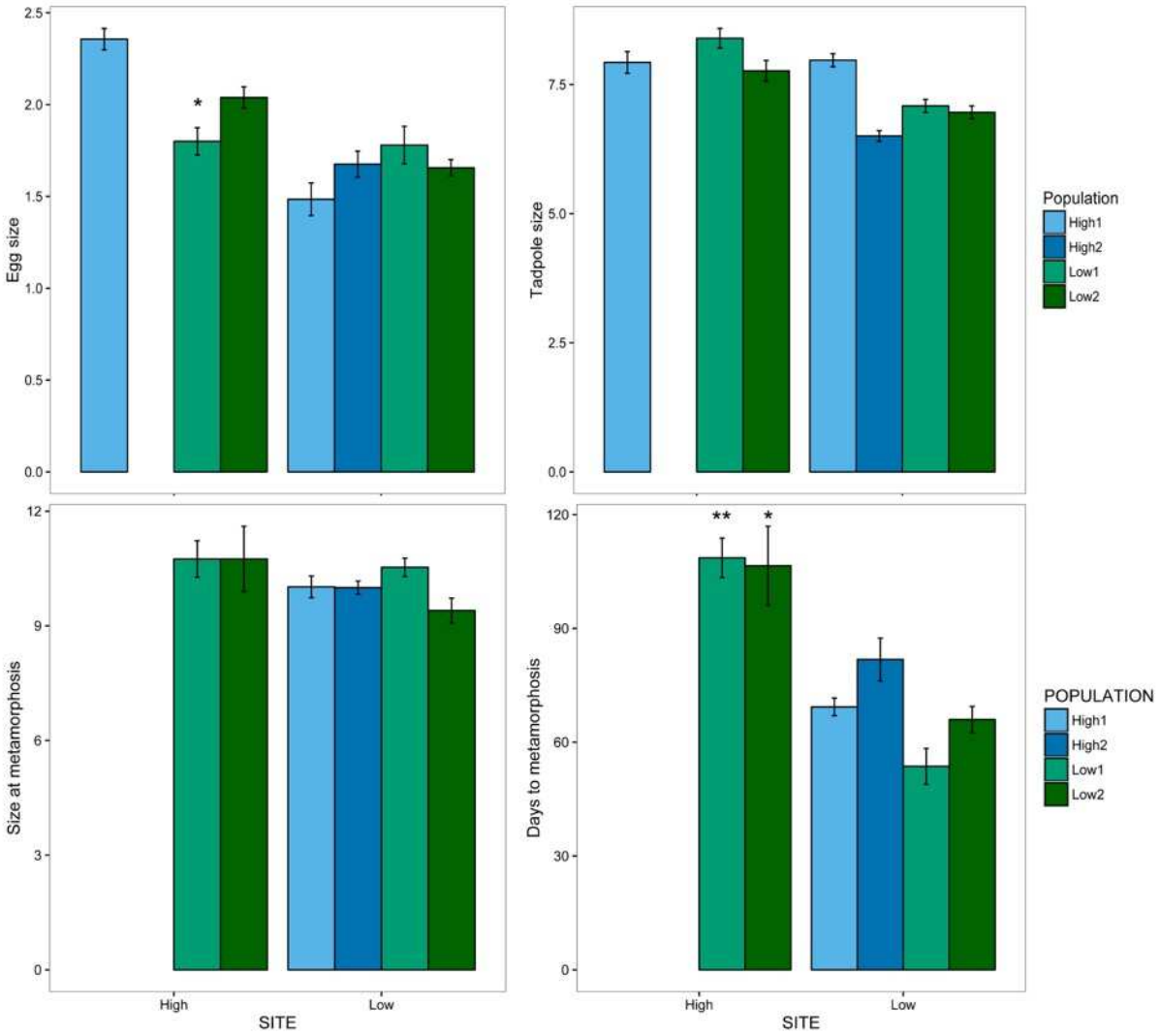

Figure 4.4. Results of life history traits related to developmental rate and size. Stars denotes statistical significance for comparisons of each population between sites (*i.e.* home vs. away criterion), except for egg size (*i.e.* local vs. foreign).

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