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Phylogeographic history and temperature-mediated evolution of the green anole, *Anolis carolinensis*

A dissertation presented

by

Shane Cornell Campbell-Staton

to

The Department of Organismic and Evolutionary Biology

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

In the subject of

Organismic and Evolutionary Biology

Harvard University

Cambridge, Massachusetts

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Phylogeographic history and temperature-mediated evolution of the green anole, *Anolis carolinensis*

ABSTRACT

Temperature plays an important role in shaping the form and function of every species. Ectothermic organisms are particularly sensitive to fluctuations in their thermal environment. Their inability to produce appreciable amounts of heat through physiological mechanisms makes them particularly vulnerable to thermal shifts, and ideal for the study of temperature-mediated evolution. The central goal of this dissertation is to understand how temperature shapes the evolutionary history of terrestrial ectotherms during the colonization of novel environments. Towards this aim, I focus on a single species of lizard, the North American green anole, *Anolis carolinensis*.

In the first chapter of my dissertation I trace the phylogeographic history of *A. carolinensis* in order to identify the geographic distribution of major genetic lineages within the species and its Cuban relatives, date times of divergence between these lineages, and identify geographic barriers to dispersal. In the second chapter, I use an integrated approach to identify aspects of the environment that may have influenced evolutionary adaptation within the species. I combine geo-referenced climate data, environmental niche modeling, thermal physiology, common garden experiments and genomic techniques to understand phenotypic and genomic response of this historically subtropical Cuban lizard to the more temperate regions of the American Southeast. Finally, in the third chapter I use experimental temperature manipulations and physiological testing

to explore the roles of phenotypic plasticity and local adaptation in shaping latitudinal variation in thermal tolerance and identify potential systemic mechanisms involved.

As a result, I have identified a Miocene-Pliocene origin of the initial over-water dispersal event leading to the establishment of the green anole in peninsular Florida, followed by a rapid Pleistocene range expansion of the species northward into higher latitudes. Range expansion on the mainland has led to thermal niche expansion, mediated by a combination of local adaptation of cold tolerance and genetic isolation by environment between populations from different thermal habitats. Phenotypic plasticity and canalized differentiation both shape variation in cold tolerance across latitude and energy conservation via metabolic suppression under acute and chronic cold onset may help to extend the limits of cold tolerance in this species at its northernmost latitudes.

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STATEMENT OF CONTRIBUTIONS AND RELATION TO PREVIOUS WORK

Chapter 1

Contributions: Rachel M. Goodman and Jason J. Kolbe provided genetic sequence data for this study. Niclas Backström aided in principal components analyses. Scott V. Edwards and Jonathan B. Losos provided valuable insight in shaping the ideas and text therein.

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Chapter 2

Contributions: Rachel M. Goodman provided tissue samples for genomic extraction, Thomas J. Sanger provided animal husbandry and aided in lizard care during common garden experiments. Scott V. Edwards and Jonathan B. Losos provided valuable insight in experimental design, and shaping of the ideas and text therein.

Chapter 3

Contributions: Anna Bare aided in all physiological testing during experimentation. Zachary A. Cheviron provided animal care facilities, and aided in experimental design of this chapter. Zachary A. Cheviron and Jonathan B. Losos provided valuable insight in shaping the ideas and text therein.

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INTRODUCTION TO THE DISSERTATION

Temperature plays an important role in shaping the form and function of every species. Therefore, understanding the link between thermal environment and biological function is of great importance. Ectothermic organisms are particularly sensitive to fluctuations in their thermal environment. Their inability to produce appreciable amounts of heat through physiological mechanisms makes them particularly vulnerable to thermal shifts, and ideal for the study of temperature-mediated evolution. Additionally, in a world of human-accelerated ecosystem change it is increasingly important to understand the how organisms interact with their thermal environment and how temperature change influences processes of adaptation and acclimation. The central goal of this dissertation is to understand how temperature shapes the evolutionary history of terrestrial ectotherms during the colonization of novel environments. Towards this aim, I focus on a single species of lizard, the North American green anole, *Anolis carolinensis*.

Anolis carolinensis is an emerging model species and the sole member of its genus native to the continental United States. After overwater dispersal from Cuba, the species has spread from Florida throughout the southeast to occupy a range of environments as far north as Tennessee and North Carolina and west into Texas and Oklahoma, where environments differ dramatically from that of its ancestral range. Considerable morphological and physiological variation has been described in the species, and the recent sequencing of its genome makes it an attractive system for studies of genome variation.

To inform the studies of molecular and phenotypic variation within *A. carolinensis* addressed in Chapters 2 and 3, the first chapter of my dissertation supplies a rigorous account of intraspecific population structure and relatedness within this species. Here, I present the most extensive

phylogeographic study of this species to date. Using mitochondrial genetic markers, I reconstruct the evolutionary relationships within *A. carolinensis*.

In the second chapter, I use an integrated approach to identify aspects of the environment that may have influenced evolutionary adaptation within the species after its invasion of the mainland. I test for niche conservatism of the mainland lineage, a phylogenetic pattern thought to apply widely across closely related species. I utilize niche modeling to estimate changes in niche breadth associated with mainland colonization of the green anole. By measuring variation in thermal tolerances and quantifying patterns of genomic variation across the species range, I identify evolutionary processes that may have acted in this species to overcome the evolutionary factors responsible for producing niche conservatism.

Finally, in the third chapter I explore the roles of phenotypic plasticity and local adaptation in shaping latitudinal variation in thermal tolerance and identify potential systemic mechanisms involved. In this chapter I use experimental temperature manipulation to measure cold tolerance, metabolism, and oxygen carrying capacity in response chronic and acute cold onset. With this dataset, we measure the effects of phenotypic plasticity and local environment on variation in cold tolerance along a latitudinal cline, identify subordinate physiological mechanisms that may contribute to this trait, and gain a clearer understanding of how the timing of cold onset impacts phenotypic response in terrestrial ectotherms.

This dissertation provides valuable insight into how temperature variation shapes phenotypic and genetic evolution within terrestrial ectotherms and how, in turn evolution allows these species to expand their distributions and occupy new environments. These insights are becoming increasingly important in the face world of global warming, which threatens the survival and function of organisms around the planet.

1 CHAPTER 1 – OUT OF FLORIDA: MTDNA REVEALS PATTERNS OF MIGRATION AND PLEISTOCENE RANGE EXPANSION OF THE GREEN ANOLE LIZARD (*ANOLIS CAROLINENSIS*)

1.1 INTRODUCTION

Phylogeographic studies examine the spatial distribution of genetic lineages within species and are critical to understanding organismal variation (Avise 2000). By revealing patterns of intraspecific relatedness, they place observations of natural variation and divergence into an evolutionary context, allowing development of hypotheses about their origin.

Previous phylogeographic studies of *Anolis* lizards have revealed high levels of population divergence within widespread species. On islands of the Greater Antilles, deep mitochondrial divergence has been reported for widespread species from Puerto Rico (Rodriguez-Robles et al. 2007), Hispaniola (Glor et al. 2003), Jamaica (Jackman et al. 2002), and Cuba (Glor et al. 2004, 2005, Kolbe et. al 2004, Knouft et al. 2006). This trend has also been well documented in species across the Lesser Antilles (Malhotra and Thorpe 1994, 2000, Schneider 1996, Ogden and Thorpe 2002, Thorpe and Stenson 2003, Thorpe 2002, Thorpe et al. 2005) and species occupying the Amazon Basin of mainland South America (Glor et al. 2001, d'Angiolella et al. 2001). In several cases, the degree of mitochondrial divergence between populations is comparable to that observed between species, suggesting that some widespread *Anolis* species may be complexes of allopatrically or parapatrically distributed species. Moreover, divergence in mitochondrial loci is not always congruent with historical boundaries, geographic distance or morphological variation, suggesting that mitochondrial clades may delimit reproductively isolated units across a species range (Gibbs et al. 2006). For a review of anole phylogeography, see Losos (2009).

Anolis carolinensis is a model species for laboratory studies in neurobiology and physiology as well as reproductive behavior and morphology (Lovern et al. 2004). With the recent publication of the green anole genome (Alfoldi et al. 2011), its utility as a model system will be extended to include studies of genome architecture and evolution (Fujita et al. in press), links between genotypic and phenotypic variation, and the genomic basis of local adaptation and population differentiation. *Anolis carolinensis* is the sole member of its genus native to the U.S. where fossil bones dating to the Wisconsinan glacial period have been identified from caves in Florida, Georgia, and Alabama (reviewed in Holman 1995). The current species' range spans the vast majority of the southeastern U.S. extending as far north as Tennessee and North Carolina and as far west as eastern Texas. Phylogeographic evidence from other species in this region reveals a substantial influence of glacial history and geographic topology on patterns of relatedness within species (reviewed in Soltis et al. 2006).

Rivers often serve as important genetic boundaries for reptiles and amphibians in the southeastern U.S. including the Apalachicola (e.g. Church et al. 2003, Zamudio and Savage 2003, Liu et al. 2006), Tombigbee (Lawson 1987), and the Mississippi (e.g. Hoffman and Blouin 2004, Austin et al. 2004, Moriarty and Cannatella 2004). The Appalachian Mountains have also been identified as a significant barrier to gene flow in the southeastern herpetofauna (e.g. Austin et al. 2004, Zamudio and Savage 2003, Jones et al. 2006). Evidence from southeastern flora and fauna suggest that Pleistocene refugia in Florida (Scott and Upchurch 1982, Riggs 1984, Hayes and Harrison 1992, Ellsworth et al. 1994), the Gulf Coast and Mississippi Valley (Delcourt and Delcourt 1981, Jackson et al. 2000, Swenson and Howard 2005) and the southern Appalachians (Brant and Orti 2003, Church et al. 2003, Austin et al. 2004) have influenced the genetic structuring of species across this region (reviewed in Soltis et al. 2006).

Previous studies of the extent and distribution of genetic variation within *A. carolinensis* indicate little genetic divergence throughout the species' range, with genetic uniformity between

Florida, Louisiana, and Texas populations (Webster et al. 1972), small genetic distances between Texas and Georgia (Buth et al. 1980), and a high degree of similarity between Alabama, Texas and Florida populations (Wade et al. 1983). However, these studies have not provided a comprehensive picture of population-level relationships due to their limited geographic scope and the discrepancy between sampling localities among these studies. Specifically, both Webster et al. (1972) and Buth et al. (1980) only studied three populations, and although Wade et al.'s (1983) sampling was more extensive, including Tennessee, Louisiana, Texas, Alabama, and Florida, it left much of the Atlantic coast and western portions of the species' range unsampled.

To better inform studies of phenotypic and genomic variation within this species, a comprehensive account of evolutionary history and population structure is needed. Toward this aim, we report the most geographically extensive phylogeographic analysis of *A. carolinensis* to date by using samples from field collections, museum samples and sequences from the NCBI online genetic database (GenBank) representing 37 sites across the species' range and 19 populations of its Cuban progenitor *Anolis porcatus*. Using mitochondrial DNA (mtDNA) sequences, we 1) examine phylogenetic relatedness of populations across the species' range and 2) identify geographic factors and historical events that potentially influenced the evolutionary history of the species.

1.2 MATERIALS AND METHODS

1.2.1 Sample Collection

During May-June of 2006 and 2007, we collected 29-42 male and female *A. carolinensis* from each of 17 populations throughout the southeastern U.S. Collection sites included both natural and human-modified habitats, but did not contain artificial water sources. Due to low population densities, collections were restricted to 10 and 17 lizards from Brownsville, TX and Naples, FL, respectively. We also sampled 3-8 individuals from 10 populations throughout Florida, and obtained 24 samples from museum collections, 1 individual from North Carolina and 23

individuals from 9 populations in Florida (see Figure1A for population sampling). Previously published ND2 mtDNA sequences for *A. porcatus* and *A. carolinensis* (Glor et al. 2004, Kolbe et al. 2007) as well as sequences of four other closely related species (*A. brunneus, A. longiceps, A. maynardi,* and *A. smaragdinus*), were obtained from GenBank.

1.2.2 Molecular Methods

We extracted genomic DNA from liver, tail, or toe tissue using a salt extraction protocol (Sambrook and Russel 2001). We amplified and sequenced a ~1200 bp fragment of mtDNA including the genes ND2, $tRNA^{Trp}$, and $tRNA^{Ala}$. This entire region was amplified using the primers L4437 (Macey et al. 1997) and H5934 (Glor et al. 2004). Our PCR protocol was as follows: denaturation at 95°C for 180 s, 30 cycles of 95°C for 35 s, 53°C for 35 s, and 70°C for 150 s, and a final extension at 70°C for 300 s. Reaction volume of 30 *u*L included 2 *u*L genomic DNA and a mixture of 47% ddH_2O, 10% 10x buffer, 10% BSA, 10% 25 mM $MgCl_2$, 3% dNTPs, 10% 2 pmol of each primer, and 0.5% *Taq* DNA polymerase. PCR products were purified using ExoSAP-IT (USB Corp.). The purified PCR products were used as template for Big Dye® Terminator v.3.1 sequencing reactions (MCLAB), which were cleaned with Sephadex® (Sigma-Aldrich) and visualized on an ABI 3730 (Applied Biosystems).

1.2.3 Phylogenetic Analyses

Sequences were aligned manually using MacClade version 4.0.5 (Maddison and Maddison 2000). We used *Anolis altitudinalis, A. isolepis, A.loysiana, A. oporinus,* and *A. sagrei* as out-groups. Phylogenetic trees were constructed using Bayesian inference in the program BEAST version 1.5.3 (Drummond and Rambaut 2007). The program jModeltest version 0.1.1 (Posada 2008) was used to identify the best-fit model of nucleotide frequencies, substitution model and transition-transversion ratio as evaluated by both Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC). The identified model, HKY+G, was then used to perform three independent runs of 20 million generations each. Analysis of the MCMC run via Tracer

determined the burn-in period for the analyses to be 2.5 million generations. Twenty million generations ensured thorough sampling after the burn-in period.

Divergence times were estimated by applying a relaxed molecular clock model across the trees, using a rate of molecular evolution of 1.3% divergence/million years (0.65% change/ lineage/million years) as in previous studies of reptiles using the ND2, $tRNA^{Trp}$ anf $tRNA^{Ala}$ regions of the mitochondrial genome (Macey et. al. 1998). This rate was used in previous phylogenetic analyses of the *carolinensis* group to date divergence times between species (Glor et al. 2004, 2005) and therefore, in the absence of informative fossil markers within the clade, we used this rate for comparisons with previous results. Maximum Likelihood (ML) analyses were performed using the program RAxML version 7.3.0 (Stamatakis 2006, Stamatakis et al. 2008) implemented on the CIPRES cluster (Miller et al. 2010). We used 1000 iterations of the novel bootstrapping algorithm RAxML to obtain branch supports. The rapid bootstrap analysis and search for best-scoring ML tree was conducted in a single program run. A GTR + Optimization of substitution rates + GAMMA model of rate heterogeneity was used for bootstrap and final analysis. The alpha parameter was estimated by default in this option.

1.2.4 Principal Components Analyisis

To visualize population differentiation across the range of *A. carolinensis*, a principal components analysis (PCA) was conducted using SMARTPCA (Patterson et al. 2006) as implemented in Eigensoft version 3.0 (Price et al. 2006). Scores from the first three principal components were plotted using the program R (R Developmental Core Team 2010).

1.2.5 Molecular Diversity and Gene Flow

We used DNAsp version 5 (Librado and Rozas 2009) to calculate the number of haplotypes, haplotype diversity (Hd, Nei 1987), average number of nucleotide differences (K, Tajima 1983), and nucleotide diversity (π, Nei 1987). Gene flow was calculated by estimating the proportion of genetic diversity explained by allele frequency differences among populations (F_{ST},

Lynch and Crease 1990), and corrected for multiple hits using the Jukes-Cantor distance model (N_{ST}, Nei 1982) considering that the probability of multiple substitutions at a single site increases as two sequences diverge. Additionally, these parameters were measured for each major clade identified from the phylogenetic analyses. A Wilcoxon rank sum test was used to test for significant differences in nucleotide diversity between clades.

1.2.6 Demographic History

We calculated site frequency spectra to infer population size changes for the species and individual clades using the population size changes option in DNAsp version 5 (Librado and Rozas 2009). The observed distributions of allele frequencies were then compared to expected frequencies under a model of constant population size to determine possible shifts in the site frequency spectrum. Tajima's *D* was calculated to assess significance of the observed shifts in the frequency spectrum.

Under a demographic model of constant population size, the distribution of the count of alleles should decrease as the allele frequency increases and Tajima's *D* (Tajima 1989) should approximate zero under this scenario. In contrast, a population expansion event should be represented by an increase in the number of rare alleles, causing Tajima's *D* to become positive. A bottleneck event, however, should increase the proportion of medium to high frequency alleles, resulting in a negative Tajima's *D* statistic.

1.3 RESULTS

Bayesian and maximum likelihood analyses of mtDNA haplotypes recovered nearly identical topologies, including relationships among major clades of the *A. carolinensis*/*A. porcatus* species complex (for full Bayesian and ML trees see Figures S1-1 and S1-2, respectively). A monophyletic *A. carolinensis* was well supported and most closely related to populations of *A. porcatus* from western Cuba (Figure 1-1B). A relaxed molecular clock model places the divergence of *A. carolinensis* from *A. porcatus* pre-Pliocene, 6.8-17.8 MYA. All other *carolinensis* series species were more closely related to East Cuban *A. porcatus*. *Anolis brunneus*, *A. longiceps*, and *A. maynardi* form a monophyletic groups that diverged from East Cuban *A. porcatus* ~11.5-23.9 MYA, while *A. smaragdinus* diverged from East Cuban *A. porcatus* ~4.4-10.9 MYA.

Within the *A. carolinensis* clade, populations from the Central Florida Clade I including Highlands Hammocks were basal. Divergence between central Florida populations and those from the rest of the range was estimated at ~6.8-12.6 MYA. Within the Central Florida Clade I, the Highland Hammocks populations formed a strongly supported monophyletic group, whereas the other populations displayed some degree of paraphyly with respect to one another. The divergence time between Highland Hammocks and the rest of the Central Florida Clade I was estimated at ~ 3.1-8.1MYA. Within the Southern Florida Clade, southeastern populations (Miami metropolitan area) formed a paraphyletic group with respect to the southwestern (Fort Myers) population. These two regions were separated from Central Florida Clade II, North Carolina and Gulf Coast/Inland populations by ~5.1-10 MYA. Populations across the majority of the species range, from Texas to the central Atlantic coast formed a strongly supported, but shallowly divergent clade with a substantial degree of paraphyly among populations. This geographically widespread clade diverged from its most closely related populations (Orlando, FL, Palatka, FL, and North Carolina) ~2.5-6.1MYA. North Carolina samples formed a well-supported, monophyletic clade that was most

closely related to populations from inland Florida (Central Florida Clade II). These populations were estimated to have separated ~1.4-4.1MYA.

The PCA showed five distinct population clusters (Figure 1-2A) with the proximity of clusters closely reflecting the phylogenetic relationships recovered in the Bayesian and the Maximum Likelihood trees. Populations from southeastern and southwestern Florida clustered together. Central Florida populations split into three distinct clusters, one comprised of all individuals from Highland Hammocks, another comprised of all other individuals from Central Florida Clade I, and the last combined Central Florida Clade II with the North Carolina populations. Populations within the Gulf Coast/Inland Clade formed a single cluster, but no discernible clustering can be seen within this clade (Figure 1-2B).

Nucleotide diversity estimates within Florida clades were on average higher than in populations from other parts of the species' range. The diversity in the Gulf Coast/Inland Clade was significantly lower than in the Central Florida Clade II (W=33, p= 0.04) and in southeast Florida (W=64, p= 0.007), and was also lower than in Central Florida Clade I and southwest Florida, but not significantly so (W = 63, p=0.12 and W=16, p= 0.17, respectively). When populations from the Central Florida and Southern Florida Clades were combined, each group displayed significantly higher levels of variation than the Gulf Coast/Inland Clade (W= 23, p= 0.022 and W=5, p=0.003, respectively). Diversity in North Carolina did not differ from other clades; however, sampling in this region was limited. For a full diversity table see Table S1-1.

F_{ST} and N_{ST} statistics showed identical patterns of relationship between major clades of the species and therefore we report only the F_{ST}-values. Within Florida, southeastern and southwestern populations showed the lowest levels of genetic differentiation (F_{ST} = 0.36), whereas the highest level of genetic differentiation was found between Central Florida Clade I and Southwest Florida (F_{ST} = 0.67). Across the entire range of *A. carolinensis*, North Carolina showed the highest levels of genetic differentiation as compared to other populations (see Table S2-1), with F_{ST} values ranging

from 0.79 to 0.85 in all comparisons except with Central Florida Clade II (F_{ST} = 0.41). A similar pattern was found for the Gulf Coast/Inland Clade with a relatively high level of differentiation from all Florida regions (F_{ST} = 0.74-0.81), but lower differentiation with Central Florida Clade II (F_{ST} = 0.46). Within the Gulf Coast/Inland Clade there was no significant difference in genetic diversity among populations. For a full list of F_{ST} values see Table S1-2.

The observed distribution of the frequency spectrum for all *A. carolinensis* populations showed a slightly higher proportion of rare alleles than expected under a model of constant population size (Figure 1-3A). Furthermore, Tajima's *D* for the species was negative, as expected under population expansion. However, the deviation from neutral expectations was not significant (D = -0.8175, P > 0.10), providing weak evidence for population expansion when considering all populations. When analyzed separately, the Gulf Coast/Inland Clade populations showed a greater shift in the spectrum towards rare alleles (Figure 1-3B) and Tajima's *D* for the Gulf Coast/Inland Clade was significantly negative (D = -2.56, P < 0.001), supporting population expansion within this clade. In contrast, the frequency spectrum for populations within Florida displayed an overrepresentation of intermediate frequency alleles when compared to the expected distribution (Figure 1-3C), suggesting a recent population bottleneck. However, Tajima's *D* for these data was not significant (D = 0.064, P > 0.10).

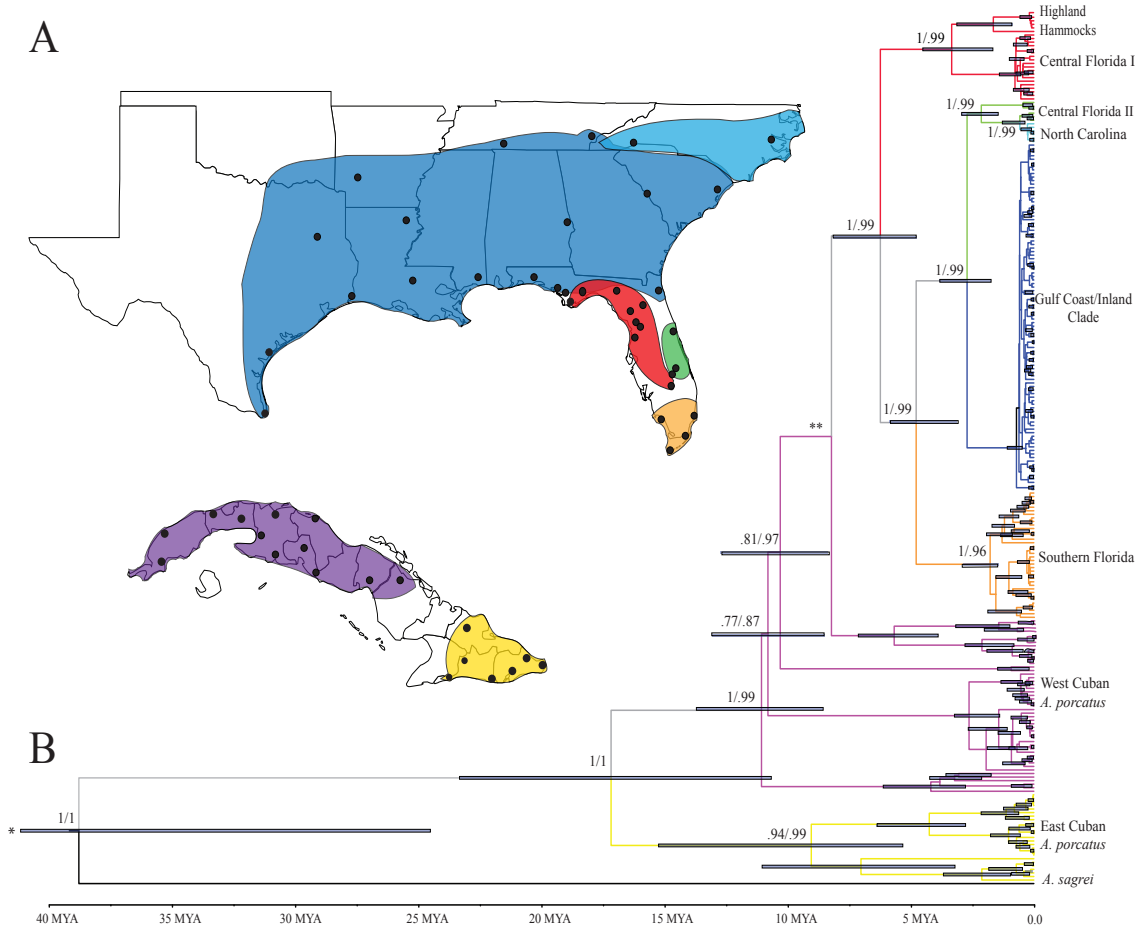


Figure 1-1: A) Map of collection sites throughout the *A. carolinensis* range in the southeastern United States and *A. porcatus* in Cuba. B) Consensus Bayesian tree of *A. carolinensis*, *A. porcatus, A. brunneus, A. longiceps, A. maynardi, and A. smaragdinus* samples from three independent analyses in BEAST with the application of a molecular clock rate of 1.3% pairwise divergence/million years. *Anolis altitudinalis, A. isolepis, A. loysiana, A. oporinus, and A. sagrei are* used as the out-group species. The x-axis shows dates of the divergence events in years before present. Clades are labeled to the right and colors correspond to the map. Support values above nodes are posterior probabilities and bootstrap values (PP/BS). Node error bars represent the 95% confidence intervals on each divergence time calculated using a relaxed molecular clock model, and are not calculated for nodes with posterior probability support less than 0.50. The single asterisk (*) marks a truncated error bar with an upper divergence time of 170.5MYA.

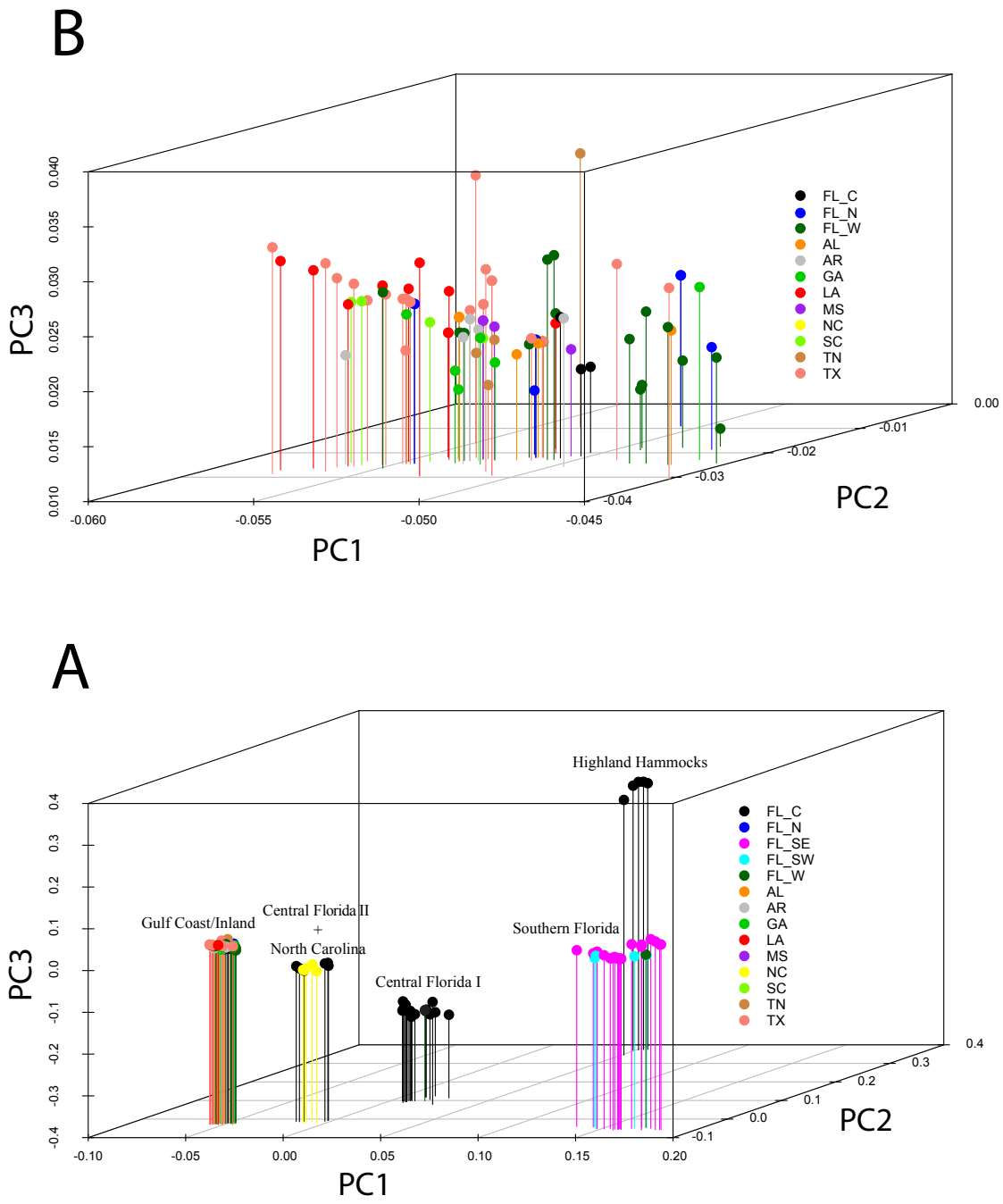


Figure 1-2: Principal components analysis of mitochondrial DNA sequences from populations across the range of *A. carolinensis*. A) Genetic clustering for all samples in the dataset. B) Genetic clustering for populations within the Gulf Coast/Inland Clade only. The x-axis represents principal component axis 1, the z-axis corresponds to principal component axis 2, and the y-axis corresponds to principal component axis 3. Major clades recovered from phylogenetic analyses are labeled above their corresponding clusters in principal component space. Each individual is represented by a pin and color coded by geographic region as shown in the figure legend.

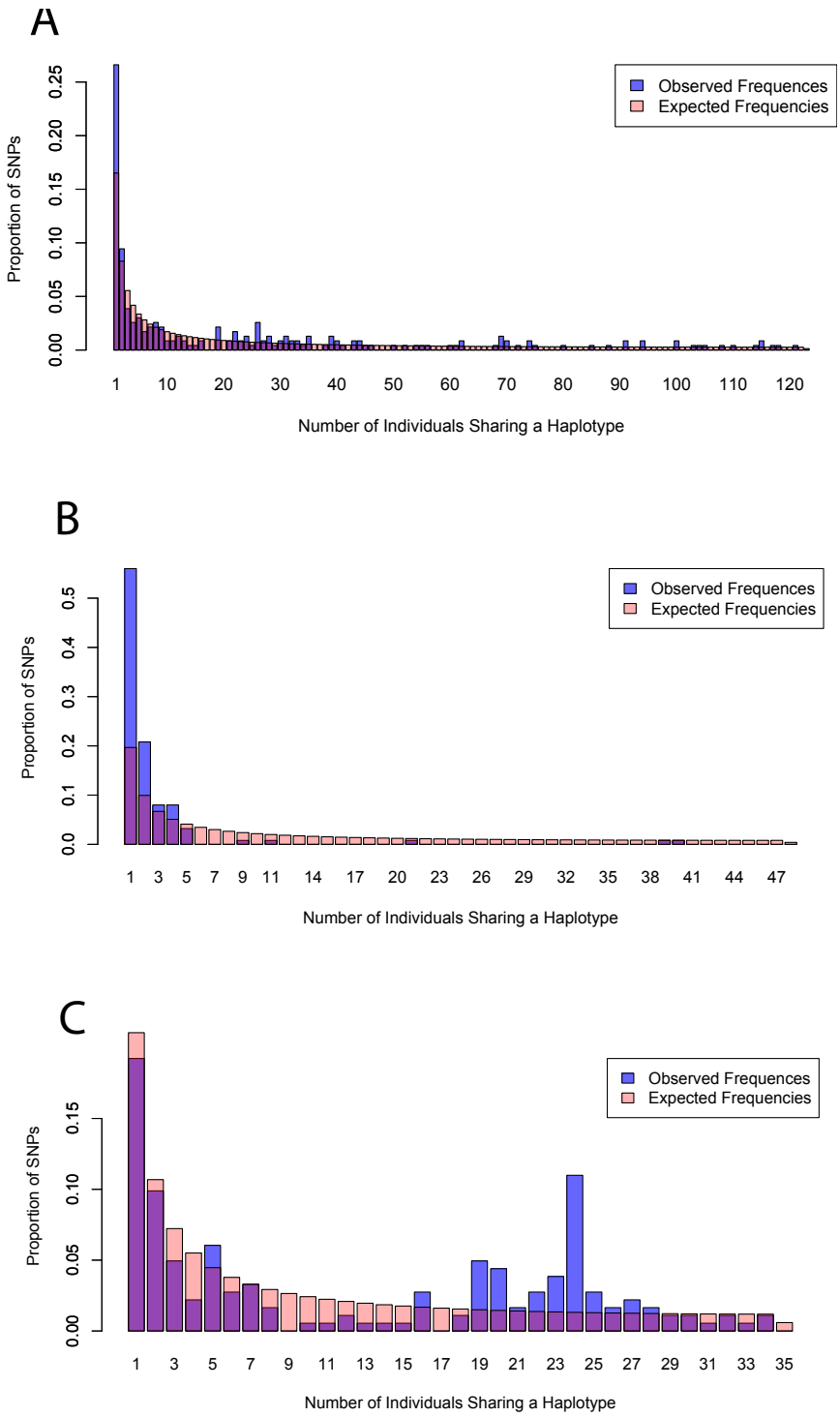


Figure 1-3: Site frequency spectra of haplotype alleles for geographic groups of *A. carolinensis*. A) All populations collected across the range of the species. B) Populations from Gulf Coast/Inland Clade identified in phylogenetic analyses. C) Populations from Florida only. The x-axis of each graph represents the number of individuals that share a given haplotype and the y-axis represents the proportion of variable sites represented by each category. Observed spectra are colored blue and the expected distribution of alleles under a model of constant population size is colored translucent red. Therefore, areas of overlap between the observed and expected distributions appear purple.

1.4 DISCUSSION

Phylogeographic analyses reveal patterns of divergence within *A. carolinensis* similar to those observed in many other species of widespread anoles. There are five highly differentiated clades within the species; three of these clades occur within Florida, one in North Carolina, and one encompasses populations across the Gulf Coast, Atlantic Coast, and inland southeastern U.S. The geographic boundaries between these clades do not correspond to major river boundaries of the region described in previous phylogeographic studies (reviewed in Soltis et al. 2006). However, the Appalachian Mountains may serve as a barrier to migration between the Gulf Coast/Inland Clade and North Carolina Clade at the northern edge of the species' range.

Phylogenetic relationships support previous hypotheses for the geographic origin of *A. carolinensis*. Williams (1969, 1989) identified Cuba as the likely point of origin of *A. carolinensis* and other West Indian members of the *carolinensis* subgroup, and Buth et al. (1980) suggested a Pliocene migration of *A. carolinensis* to the U.S. Glor et al. (2005) corroborated both of these hypotheses with a phylogeographic analysis, dating diversification of the *carolinensis* subgroup out of Cuba in the late Miocene or early Pliocene. The monophyly of *A. carolinensis* with respect to its ancestral congener supports the results of Glor et al. (2005) suggesting a single colonization event to southeastern U.S. from western Cuba, however divergence times of other species from Eastern Cuba populations suggest that diversification of the *carolinensis* subgroup may have occurred much earlier than reported in Glor et al.(2005). While *A. smaragdinus* diverged from Eastern Cuban *A. porcatus* sometime within the Miocene to Pliocene (3.5-16.2 MYA) based on our estimates, the divergence event that separates the clade containing *A. brunneus*, *A. longiceps* and *A. maynardi* is estimated at ~9.3-32.6 MYA, as early as the early Oligocene. However, because our dataset only represents a portion of the mitochondrial region used by Macey et al. (1998), contains a higher proportion of protein coding sequence with respect to tRNAs, and does not contain the slower

evolving COI gene, the rate of evolution within this region may be faster, and divergence times may be younger than our estimates.

Based on Bayesian and maximum likelihood tree topologies, central Florida, a previously described refugial area (Scott and Upchurch 1982, Riggs 1984, Hayes and Harrison 1992, Ellsworth et al. 1994), holds the ancestral populations of the species. Branching times and geographic positioning of the Gulf Coast/Inland, North Carolina, and Southern Florida Clades suggests multiple migrations out of Florida into the species current range. Divergence times place these migration events between the mid-Miocene to the early Pleistocene.

North Carolina populations may represent a Pleistocene refugial population. Divergence times place the separation of North Carolina and Central Florida Clade II populations within the Gelasian and Calabrian stages the Pleistocene, ~0.78-2.5 MYA. This region has also been identified as a possible refugium for other species (Brant and Orti 2003, Church et al. 2003, Austin et al. 2004), and displays a high degree of genetic differentiation from geographically proximate populations in our analysis. Alternatively, the geographic separation of this haplogroup from central Florida could signify mitochondrial introgression of the expanding Gulf Coast/Inland Clade populations into the Atlantic Coast.

Most of the species' range shows little phylogeographic structure (i.e., the Gulf Coast/Inland Clade), indicating a possible recent range expansion across this region. This hypothesis is further supported by the observed shift in the site frequency spectrum toward rare alleles and a significantly negative Tajima's *D* statistic. Earliest divergence times within this clade place the start of this expansion within the Pleistocene, ~0.96-2.0 MYA In addition, the short branch lengths and extensive paraphyly that characterize the Gulf Coast/Inland Clade may explain previous observations of population relatedness across this region.

Webster et al. (1972) compared the genetic distance between populations of *A. carolinensis* from Florida, Louisiana, and Texas, reporting a high degree of genetic uniformity among these

populations using allozymes, with Florida and Louisiana populations more closely related to each other than to Texas. These results contrast with the findings of this study, which show that Florida populations are highly diverged from Louisiana and Texas. The Silver Springs, Florida population used by Webster et al. (1972) lays near the border of the Gulf Coast/Inland Clade and Central Florida Clade I haplogroups identified in this study. The observed similarity described may be the results of nuclear gene flow between these two groups.

Buth et al. (1980) used electrophoretic comparisons at 35 allozyme loci to estimate genetic differences between Texas and Georgia populations of *A. carolinensis* and western Cuba populations of *A. porcatus*. They found small genetic distances separated Texas and Georgia, a result consistent with our findings.

Wade et al. (1983) found that populations from the Gulf Coast (Birmingham and Auburn, AL, and Tellico, TN) and Gainesville, FL were genetically similar based on allozyme data. This again, may reflect nuclear genetic admixture between Gulf Coast/Inland Clade and Central Florida Clade haplogroups. San Marcos, TX displayed intermediate genetic distance between this group and New Orleans, LA. Populations from Naples, FL, which have been suggested as a subspecies based on morphological (Christman 1980) and physiological (Wilson and Echternacht 1990) evidence, are the most genetically distinct group. This agrees with our findings that southern Florida populations represent a genetically distinct lineage within the species, although the geographic range described by these studies is much smaller than that occupied by the Southern Florida haplogroup.

A recent expansion of populations within the Gulf Coast/Inland Clade explains the small genetic distances reported in earlier studies. The Lower Mississippi Valley, a hypothesized refugial area (Davis 1981, Delcourt and Delcourt 1981) lies in the center of the Gulf Coast/Inland Clade haplotype distribution. The expansion of this clade may have occurred out of this region, however, further data are needed to test this hypothesis.

The observed shifts in the site frequency spectrum of the ND2 region of *A. carolinensis* show signatures of population expansion by the species. However, the drastic shift seen in the Gulf Coast/Inland populations suggests that the species-level signature of a shift towards rare alleles is being driven by this clade. In the absence of this clade, *A. carolinensis* populations display shifts, though statistically non-significant, in site frequency spectra expected from a bottleneck event. Given the topology of the tree, these data may highlight molecular signatures of both the bottleneck associated with the initial colonization of the species from Cuba into mainland Florida and the recent expansion of populations out of Florida into its current range. To better understand the genetic effects of this demographic history, a multilocus study including nuclear markers is needed.

Clustering patterns identified in the PCA largely mirror the topological structure recovered in the Bayesian and maximum likelihood trees. The tight clustering pattern of most major clades reflects a high degree of genetic structure. Conversely, the lack of clustering of populations within the Gulf Coast/Inland Clade further supports the hypothesis of low genetic differentiation between populations occupying most of the species' range, likely due to a recent expansion event.

1.5 CONCLUSION

Pleistocene glaciation has influenced the current distribution and population relatedness within and between species of *Anolis* lizards and many other species, and has been proposed as a major factor for Caribbean *Anolis* diversification (Glor et al. 2004). The timing and pattern of divergence events suggests that cyclical variation in temperature throughout southeastern U. S. may have had a significant effect on population expansion across the majority of the *A. carolinensis* range. Molecular analyses suggest there are at least five highly diverged lineages within *A. carolinensis*. However, despite the Miocene-Pliocene arrival of the species to the southeastern U.S., three of these haplogroups represent limited geographic distributions within Florida, the likely region of colonization. It is not until the periodic warming cycles of the Pleistocene that significant

changes in the geographic distribution occur, leading to migrations north along the Atlantic Coast and west across the Gulf coast and establishment of populations in newly habitable areas.

Our work sets the stage for future studies of phenotypic and genetic variation, demographic history, and adaptation within *A. carolinensis*. Future studies of the nuclear genome of this species will elucidate the importance of demographic history in shaping patterns of variation and adaptation. We are aware of the limitations of phylogeographic studies based solely on mitochondrial loci (Edwards and Bensch 2009). Therefore, we encourage additional study of phylogeographic patterns using nuclear markers.

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2 CHAPTER 2 – LOCAL ADAPTATION AND ISOLATION-BY-ENVIRONMENT ENABLE NICHE EXPANSION AFTER MAINLAND COLONIZATION OF THE CUBAN GREEN ANOLE

2.1 INTRODUCTION

Understanding the ability of native environments to constrain the range of invasive species has important implications for understanding the effects of human-mediated climate change on biodiversity. The human-mediated spread of non-native species combined with shifting global climates sets the stage for drastic ecosystem changes in the coming decades. Invasive species are amongst the most severe threats to native biodiversity worldwide. The introduction of invasive predators, competitors and deadly prey species has had severe impacts on biomes around the globe. In the wake of this change an important question emerges: will species be able to adapt to conditions to which they are not previously adapted?

Niche conservatism is the tendency of species to retain aspects of their ancestral fundamental niche over time (Wiens and Graham 2005). The extent to which niche conservatism exists has been strongly debated, with some studies finding evidence for niche similarity among closely related species (Ricklefs and Latham 1992, Peterson et al. 1999, Prinzig et al. 2001), while others have documented niche lability (Losos et al. 2003, Bohning-Gaese et al. 2003, Graham et al. 2004). If niche conservatism prevails, invasive species should be limited to environments similar to their native ranges. Wiens and Graham (2005) lend support to this theory, showing a significant correlation between maximum latitudes of invasive and native ranges of 35 species of reptiles and amphibians and other studies have garnered complimentary evidence (Huntley et al. 1989, Wen 1999, Peterson et al. 1999, Webb et al. 2002, Ackerly 2003 and 2004, Qian and Ricklefs 2004). However, several studies have reported species that have extended their invasive range beyond that predicted by their native climate envelope (Broennimann et al. 2007, Fitzpatrick et al. 2007,

Roedder et al. 2009, Medley et al. 2010, Kolbe et al. 2012, Parravicini et al. 2015). These seemingly contradictory observations bring to bear a key question: Can evolutionary mechanisms such as local adaptation allow species to overcome niche constraint and occupy novel climates?

Though many species have been established in introduced ranges via human-mediated dispersal for several decades, understanding the roles of local adaptation and the constraining effects that produce niche conservatism in shaping the evolutionary history of these lineages may require longer time periods of inhabitance than have yet been realized. Natural invasion events provide longer timescales with which to study processes of niche evolution and local adaptation associated with introduction into novel habitats. Studies of natural invasions can thus provide valuable insight into the long-term implications of invasive species and shifting climate currently beyond the view of human-mediated dispersal events.

The green anole, *Anolis carolinensis*, provides an intriguing example of natural invasion. *Anolis carolinensis* is nested within the Cuban green anole (*A. porcatus*) clade, and is the result of overwater dispersal leading to establishment in peninsular Florida as late as the Pliocene (Glor et al. 2005, Campbell-Staton et al. 2012, Tollis et al. 2012). The only anole native to the continental United States, *A. carolinensis* has spread from Florida throughout the southeast to occupy a range of environments as far north as Tennessee and North Carolina and west into Texas and Oklahoma. Winter temperatures may limit the northern edge of the species' distribution (Williams 1969). Northern populations do not hibernate as is common for most reptiles at these latitudes. Despite regular ambient temperatures below freezing, northern populations of this species endure winter by retreating to sheltered sites and basking on rock faces during periods of sun exposure (Bishop and Echternacht 2004). Populations also display geographic variation in acute cold tolerance (CT_{min}) (Wilson and Echternacht 1987, 1990).

The recently published genome of the species (Alfoldi et al. 2011) provides an invaluable resource for understanding the influence of climatic variation on population structure and natural

selection at the molecular level. In this study, we combine niche modeling, thermal physiology, and genomic techniques to explore climate-mediated evolution associated with the natural invasion of this ancestrally subtropical Cuban lizard species into more temperate habitats in mainland North America. Towards this aim we test three hypotheses:

1) Colonization of mainland North America has resulted in exposure novel climate conditions and a concomitant shift in the climate niche of *Anolis carolinensis*

2) Northern range expansion on mainland has led to local adaptation of thermal tolerance

3) Local environment influences patterns of local adaptation and populations dynamics at the genomic level in *A. carolinensis*

2.2 MATERIALS AND METHODS

2.2.1 Climatic Differentiation between Mainland and Cuban Green Anole Lineages

Climate is known to be an important contributor to intraspecific divergence via local adaptation (Morgenstern 1996, Savolainen 2007**,** Olson et al. 2013**)** as well as interspecific divergence via ecological speciation (Rundle and Nosil 2005, Schluter 2009**,** Schluter and Conti 2009, Keller and Seehausen 2012, Nosil 2012). Identifying climatic differences that distinguish populations and/or species is integral to developing hypotheses concerning climate-mediated evolution. Environmental factors that differ most between divergent lineages are most likely to be causal agents of selection shaping local adaptation and population dynamics. Once identified, these abiotic features may then be used to develop hypotheses regarding niche shift, phenotypic selection, and patterns of genetic structure across geographic space.

To identify novel climatic conditions associated with mainland colonization of the *A. carolinensis* subgroup, we gathered georeferenced locality data for *A. carolinensis*, and its Cuban relatives, *A. allisoni* and *A. porcatus*. We combined data from Herpnet (accessed April 2013) and our own collections. Because *A. porcatus* contains two genetically distinct clades (Glor et al. 2004), we treated localities from the eastern and western sides of the island separately. Duplicate sites and

those not corresponding to land areas were removed from the dataset. We downloaded climate layers for 19 bioclimatic variables from the Worldclim database (Hijmans 2005) and extracted climate data for each variable at each occurrence point using the raster package in R (version 3.1.2, R Core Team 2014).

We quantified environmental variation within and between each species with a principal components analysis of bioclimatic variables from all sample localities using prcomp function in R. The first two principal components were retained for further analyses. We used variable loadings of principal components to determine the relative importance of temperature and precipitation in determining climatic differentiation between clades.

2.2.2 Environmental Niche Expansion Associated with Mainland Colonization

Biological constraint may result in ecological niche conservatism in invasive populations. Recent literature has highlighted the prevalence of niche conservatism among closely related species and its importance in generating biodiversity (Wiens 2004, Kozak and Wiens 2006, Wiens et al. 2007, Kozak and Wiens 2010). However, populations exposed to novel climate may adapt to take advantage of novel resources or survive novel challenges, resulting in niche expansion. Several studies have highlighted the importance of environmental heterogeneity in the adaptive divergence of lineages (Graham et al. 2004).

We estimated the degree of niche shift associated with the mainland colonization of *A. carolinensis* by comparing the breadth of its fundamental niche with that of its closet Cuban relatives. We performed an additional principal components analysis on 19 bioclimatic variables across all cells of a geographic range including Cuba and southeastern North America using Arcmap software (version 10.1). The first principal component was transformed into a climate raster for environmental niche modeling. Environmental niche models for each clade were made using maximum entropy modeling implemented in Maxent (version 3.3.3). We generated each model using 10,000 random background points from the native range of each lineage. Each model

was run ten times, each time using independently generated background points. The ten models were then averaged to generate the final model using the logistic output option of the program. To avoid potential biases in niche breadth estimation due to the physical isolation of the Cuban clades, we projected each model onto a common environmental range including both southeastern North America and Cuba for comparisons of environmental suitability between clades.

To identify geographic regions that may have been limiting during range expansion of *A. carolinensis*, we used the niche projection of the western clade of *A. porcatus*, the sister lineage of *A. carolinensis*, to generate a multivariate similarity surface (MESS) map in Maxent. MESS maps highlight the similarity of environmental variation within a given geographic space to that experienced during model training. Since *A. carolinensis* falls within the *A. porcatus* clade, we used the western lineage as a proxy for the ancestral state of *A. carolinensis*. This map was used to determine where geographically the *A. carolinensis* range is most dissimilar to the western *A. porcatus* model, under the assumption that these regions would also have been difficult to colonize during the spread of the mainland lineage into its current range.

We compared niche breadths between *A. carolinensis* and its Cuban relatives, using the ENMtools package (Warren et al. 2010). Habitat suitability rasters from projected models were standardized and imported into the niche breadth calculator within the ENMtools toolkit. The program was used to generate a measure of niche breadth, inverse concentration (Levins 1968), for comparison across each green anole lineage.

2.2.3 Genomic Data Collection and Variant Calling

Liver, tail or toe tissue samples were collected for 186 individuals across 22 populations within the native range of *A. carolinensis*. Tissues were flash frozen in liquid nitrogen or preserved in ethanol or RNAlater, then stored at -80°C before tissue extraction. Genomic DNA was extracted using Qiagen DNA extraction kits. Double digests were performed using EcoR1 and Sbf1 restriction enzymes to fragment DNA. Genomic libraries were prepared using a modified version

of the protocol described in Peterson et al. (2012). Sequencing was performed using 100bp single-end and 150 bp paired-end sequencing on the Illumina Hiseq platform.

We used Geneious (version 6.1.8) to separate the resultant sequence fragments by individual barcode. Trimmomatic (Bolger et al. 2014) was used to quality filter reads for each individual. The quality of each read was examined in a 4-bp sliding window, and regions of sequence were discarded once the average read quality in this window fell below a Phredd33 score of 15. An end-to-end alignment to the *A. carolinensis* genome was performed on single-end and forward reads of each individual using bowtie2 (Langmead and Salzberg 2012). Variants were called using the Stacks 1.06 pipeline (Catchen 2011, 2013). Using the populations pipeline contained therein, we filtered the resulting variants for use in two downstream analyses. We first filtered for variants with at least 5x coverage and with a minor allele frequency of 0.05 of higher, shared between at least two populations in the dataset and present in at least 40 percent of individuals within each population. This dataset was used to calculate a pair-wise fixation index (Fst) matrix between populations with the STACKS software for use in mixed model and regression analyses. Second, we selected a subset of populations with at least 4 individuals with ≥ 500k representative reads. Allele counts from this dataset were then used to identify regions of the genome associated with geographic variation in environment.

2.2.4 Evidence for Climate-Mediated Phenotypic Adaptation within Mainland Anole Populations

Phenotype-environment correlations are among the oldest and most widely used evidence for local adaptation in wild populations (Endler 1986). Under a hypothesis of climate-mediated local adaptation, geographic variation in a selective force is expected to give rise to variation in phenotypes under selection (Endler 1986). However, the non-independence of phenotypes due to shared ancestry and ongoing gene flow between populations can confound patterns of phenotypic variation due to local selection pressures (Stone et al. 2011). To account for this, it is necessary to

sample phenotypes across a wide distribution (Endler 1986) and control for phenotypic variation due to shared ancestry and migration between populations (Stone et al. 2011).

We tested for associations between thermal tolerance and temperature extremes across the entire native range of *A. carolinensis* using the critical thermal method (Cowles and Bogert 1944). We collected critical thermal maximum (CT_{max}) and minimum (CT_{min}) data for 202 lizards from 16 sites across the entire native range of the species during the summer of 2012. These two measurements commonly are used to test the thermal tolerance limits of non-avian reptiles (Hertz and Huey citations). Each lizard was acclimated to 10°C for 24 hours. After acclimation, we placed a thermocouple into the cloaca of each animal to monitor internal body temperature during testing. Each lizard was then placed in a cooling chamber, cooled at a rate of ~1°C /min and periodically flipped onto its back and stimulated with forceps. The body temperature at which an animal could no longer right itself after 30 seconds was recorded as its CT_{min}. After CT_{min} trials, we acclimated each lizard to room temperature (~25°C) for 24 hours. After the second acclimation period each animal was then set up as above and heated by ~1°C /min and tested for righting response. The temperature at which an animal could not right itself after 30 seconds was recorded as its CT_{max}. Rates of temperature change during each trial were recorded for use in statistical analyses.

To identify correlations between thermal tolerance and thermal extremes, we performed linear regressions using R. The first linear regression analysis was performed testing the significance of association between mean population CT_{max} and BIO10, using rate of heating as a covariate. The second linear regression was performed testing the significance of association between CT_{min} and BIO11, using rate of cooling as a covariate. BIO10 and BIO11 were chosen to represent temperatures experienced during the warmest and coldest parts of the year at each site, respectively.

To account for phenotypic similarity between populations due to ancestry and ongoing gene flow, we fit a generalized linear mixed model (glmm) using Markov chain Monte Carlo techniques in the mcmcglmm package in R (Hadfield 2009) as described by Stone et al. (2011). Using 12 populations for which we had both genetic and phenotypic data, we structured glmms to search for the statistical significance of the relationship between thermal tolerance and extremes of local temperature. We used standardized genetic distance between populations, as estimated with the fixation index (Fst), as a random variable in these analyses.

2.2.5 Evidence that Cold Tolerance is Heritable

While phenotypic correlations with environmental variation are suggestive of local adaptation, this pattern may also be the result of non-evolutionary phenotypic response to different post-hatching environments (phenotypic plasticity). Therefore, we performed a common garden experiment to test for population differences in cold tolerance under identical environmental conditions. Gravid females from three populations were brought into a common laboratory setting. Females were kept under a 12 hour light:dark cycle at ~30°C and fed three times/week. Eggs laid in the laboratory were incubated until hatching and the offspring were raised under identical laboratory conditions for seven months. CT_{min} trials were performed as described in the previous section. We performed a linear regression using CT_{min} as the dependent variable, population of origin and generation (parent or offspring) as independent categorical variables, and mean rate of cooling per group as a covariate.

2.2.6 Genetic Isolation by Environment

Patterns of gene flow between populations can have a direct impact on the ability of a species to expand its range. Genetic isolation by environment (IBE) can limit gene flow between climatically differentiated populations, enabling local adaptation to novel environments in marginal habitats and allowing range expansion into novel climate. Under IBE, climate acts as a barrier to

gene flow, leading to associations between genetic distance and environmental dissimilarity across a species' range (Nosil 2004, Nosil et al. 2005, Wang et al. 2013a, Wang et al. 2013b).

To estimate the effects of isolation by environment and geographic distance we performed a multiple matrix regression with randomization analysis (MMRR, Wang 2013). A matrix of pair-wise genetic distance was calculated using global Fst values estimated in Stacks (Catchen 2011, 2013). A geographic distance matrix was calculated using geographic coordinates in ArcMap. Linear geographic distances were calculated between each pair of coordinates. When the linear distances traversed a major body of water, the shortest overland distance was calculated using least cost path analyses.

To create environmental distance matrices, we focused on three environmental variables that together describe variation in low temperature extremes and variability. We used Bio 6 (Minimum temperature of the coldest month of the year) as a measure of winter severity at each site. Bio 2 (mean diurnal range of temperatures) and Bio4 (temperature seasonality) were used as metrics of daily and annual fluctuations in temperature, respectively. We ran principal components analysis of these three variables and calculated environmental distance between populations as the euclidean distance in the first principal component, using the prcomp function in R. To test for isolation by distance and environment we ran a MMRR analysis using genetic distance as a dependent variable. Geographic distance and environmental distance were used as predictor variables.

2.2.7 Genome Scans for Climate-Mediated Selection

As a result of natural selection, allele frequencies at loci important for climate adaptation may track spatial variation in climate despite ongoing gene flow between populations (Haldane 1948, Slatkin 1973, Nagylaki 1975, Lenormand 2002, Coop et al. 2010). Allelic associations with environmental variation have been used to identify putatively adaptive loci in several species

(Dobzhansky 1948, Berry and Kreitman 1993, Hoekstra et al. 2004, Storz et al. 2007, Schmidt et al. 2008, McCracken et al. 2009).

To search for regions of the *A. carolinensis* genome associated with climatic variation, we calculated Bayes factors (BF) for associations between population allele counts and geographic variation using bayenv2 (Gunther and Coop 2013). The program was run in two steps. First, we estimated a population covariance matrix over 200,000 MCMC iterations to control for the effects of population structure using all genomic variants. Next, we detected environmental correlations between each allele and estimates of the upper and lower extremes of temperature (BIO10 and BIO11) and precipitation (BIO16 and BIO17). Because BF estimates can be sensitive to outliers, we also calculated non-parametric Spearman's rank correlation coefficients (ρ). Previous work has shown that the results of this analysis can be variable between runs (Blair et al. 2014). Therefore we ran the program independently five times for 500,000 MCMC iterations and averaged estimates across all runs before identifying candidates. Candidate variants were identified as those lying within the top 99% of both BF and ρ distributions. The VariantAnnotation package in BioConductor was used to identify genes containing candidate SNPs or within a 20kb window around intergenic candidates. The bioMart package was used to identify names and ontology descriptions for all candidate genes.

2.3 RESULTS

2.3.1 Climatic Differentiation between Mainland and Cuban Green Anole Lineages

We collected climate data for 56 localities for *A. allisoni*, 146 for *A. carolinensis*, 56 for western *A. porcatus* and 72 for eastern *A. porcatus*. Principal components analysis reveals divergent patterns of climate availability across the *A. carolinensis* range when compared to its Cuban relatives (Figure 2-1A).

The first two principal components explain 73.9% of variation within the dataset. The top five variables contributing to the first principal component (53.18% of variation) all represent aspects of environmental temperature (BIO1 = 0.3055, BIO4 = -0.3031, BIO6 = 0.3053, BIO7 = -0.2975, and BIO11 = 0.3084. *A. carolinensis* localities show high variation across this axis, while *A. porcatus* locations cluster in principal component space indicative of warmer temperature with low annual thermal variability.

In contrast, the second principal component (20.74% of variation) loads most heavily on precipitation related variables. Four of the top five contributing variables to this component are associated with aspects of environmental precipitation (BIO5 = 0.295, BIO12 = 0.4491, BIO13 = 0.4336, BIO16 = 0.4664, and BIO18 = 0.4249). *A. carolinensis* localities show less differentiation along this axis when compared to Cuban lineages, clustering in principal component space characterized by relatively dry climate with low precipitation seasonality.

2.3.2 Environmental Niche Expansion Associated with Mainland Colonization

Inverse concentration measurements for each clade show a large increase in niche breadth associated with mainland colonization of the green anole (*A. carolinensis* = 0.406) when compared to its island counterparts (western *A. porcatus* = 0.057, *A. allisoni* = 0.074, eastern *A. porcatus* = 0.039) (Figure 2-1B).

Projection of the western *A. porcatus* niche onto the *A. carolinensis* range reveals increasingly negative similarity values with increasing latitude (Figure 2-1C). This result suggests that higher latitudes within the *A. carolinensis* range contain environmental variation outside that currently experienced in the species' ancestral range on Cuba. A map of all niche models can be found in Figure S2-1.

2.3.3 Phenotypic signatures of climate-mediated local adaptation

Multiple linear modeling reveals no increase in CT_{max} in relation to mean temperature of the warmest quarter of the year in either the linear regression (adjusted $R^2 = 0.016$, $p = 0.383$, $\beta_{Bio10} = -0.024$, $p = 0.583$) or MCMCglmm (pMCMC = 0.530) (Figure 2-2B). Conversely, CT_{min} shows a significant association with the mean temperature of the coldest quarter of the year at site of capture in linear regression (adjusted $R^2 = 0.157$, $p = 0.022$, $\beta_{Bio11} = 0.141$, $p = 0.025$) (Figure 2-2A). This association remains significant after accounting for genetic relatedness between populations in MCMCglmm (pMCMC = 0.016) This result suggests that geographic variation in cold tolerance is being driven by differences in local environment across the species' mainland range.

2.3.4 Evidence for heritability of putatively adaptive phenotypes

Analysis of variance shows that parent/offspring distinction has no significant influence on variation in CT_{min} ($df = 1$, $F = 0.123$, $p = 0.727$), suggesting that lizards born in the laboratory retain population-specific signatures of cold tolerance. Population of origin has a significant influence on variation in cold tolerance ($df = 2$, $F = 10.551$, $p < 0.001$) (Figure 2-2C). This result suggests that the observed variation in cold tolerance has a genetic basis and is not simply the result of plastic response to climate during pre- or post-hatching development.

2.3.5 Evidence for genetic isolation by environment

STACKS filtering resulted in 53,486 single nucleotide variants. The linear model including geographic and environmental distance between populations ($R^2 = 0.116$, $F = 8.69$, $p = 0.034$) significantly explains the observed patterns of genetic variation within *A. carolinensis* (Figure 2-3). Within this model there is no significant effect of genetic isolation by distance ($\beta_{geographic\ distance} = 0.07$, $t = 0.84$, $p = 0.53$). Conversely, differences in cold variation and extremes between collection localities contribute significantly to the model ($\beta_{environment} = 0.32$, $t = 3.86$, $p = 0.033$). Association

between environmental variables and latitude as well as loadings of each variable on PC1 used in the environmental dissimilarity analysis can be found in Figure S2-3.

2.3.6 Genome Scans for Climate-Mediated Selection

Filtering for high coverage individuals resulted in 20,282 SNPs representing 28 individuals from six populations across the *A. carolinensis* range. Bayesian analyses of genotype-environment associations resulted in 81 total candidate SNPs associated with BIO 10 (44 unique to this environmental variable). Seventy-two candidates SNPs (58 unique) were identified for BIO 11, 61 SNPs (41 unique) for BIO 16, and 24 SNPs (16 unique) for BIO17. Temperature related variables (BIO 10 and BIO 11) shared 12 candidate SNPS, while precipitation related variables shared two. A full diagram of chromosomal positions of SNP outliers and their overlap can found in Figure 3-4, bayes factor association plots can be found in Figure S2-2.

Candidate genes associated with geographic variation in cold (BIO11) included 19 previously recognized genes and six uncharacterized proteins. Previously characterized genes within this dataset are associated with several biological processes that may be important for colonization of temperate environments. Several of these genes have been linked to aspects of neuromuscular development. AGRN is involved in the formation of neuromuscular junctions during embryogenesis (Rupp et al. 1991) and ELAV3 has been suggested as a contributor to neurogenesis (Sakai et al. 1994). Yet another candidate, TCF4, has been associated with involuntary neuromuscular function, and mutations in this gene have been linked to developmental and respiratory dysfunction (Zweier et al. 2007). Two genes related to DNA repair also show correlations with geographic variation in cold temperature within *A. carolinensis*. MMS21 has been linked to recovery from DNA damage (Payne et al. 2014) and DM5B is a regulator of genomic stability (Li et al. 2014).

Three candidate genes are also involved in aspects of physiology and metabolic efficiency. Polymorphisms in one candidate, TIMP3 (an inhibitor of extracellular matrix degradation), are

associated with high altitude adaptation in some human populations (Kobayashi et al. 2013). ROCK2 is associated with variation in blood pressure and cardiovascular physiology (Riento and Ridley 2003, Noma et al. 2006, Seaholtz et al. 2006, Rankinen et al. 2008) and MOGAT2 plays an important role in metabolic efficiency and regulation of energy metabolism (Gao et al. 2013). Lastly, two genes within this dataset have been associated with immune response. MASP1 is a component of innate and adaptive immune response pathways (Sato et al. 1994) and USP13 is a regulator of the interferon pathway, which mounts host response to pathogen infection (Yeh et al. 2013).

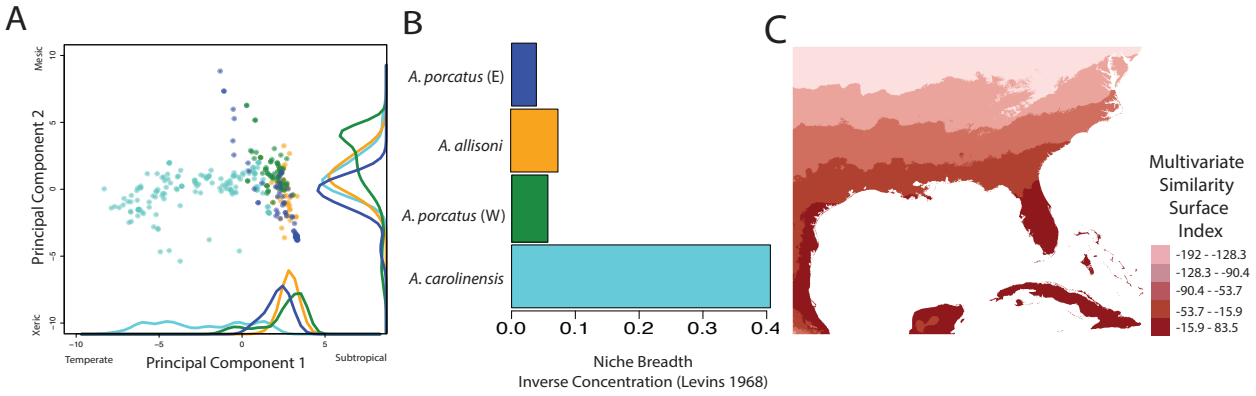


Figure 2-1: A) Principal components analysis of environmental variation across the range of *A. carolinensis* (light blue), *A. allisoni* (yellow), and the western (green) and eastern (blue) clase of *A. porcatus*. B) Measure ments of niche breadth for each clade based on the inverse concentration metrics of Levins (1968) C) Multivariate similarity surface map depicting differences between the envionmental niche of the western clade of *A. porcatus* and local environmental variation acorss geographic space of Cuba and southeastern North America.

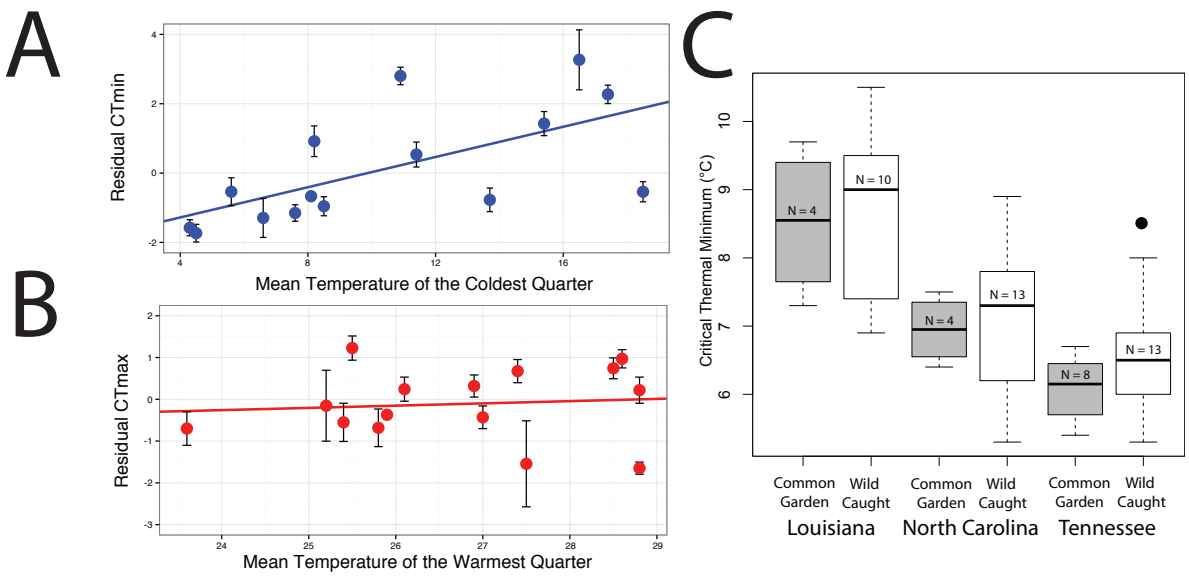


Figure 2-2: A) Association between CTmin and the mean temperature of the coldest quarter of the years at site of capture. B) Association between CTmax and the warmest quarter of the year at site of capture. C) CTmin of three populations collected from the native range of A. carolinensis. Grey boxes denote the distribution of animals hatched and raised under common conditions; white boxes denote animals collected in the wild.

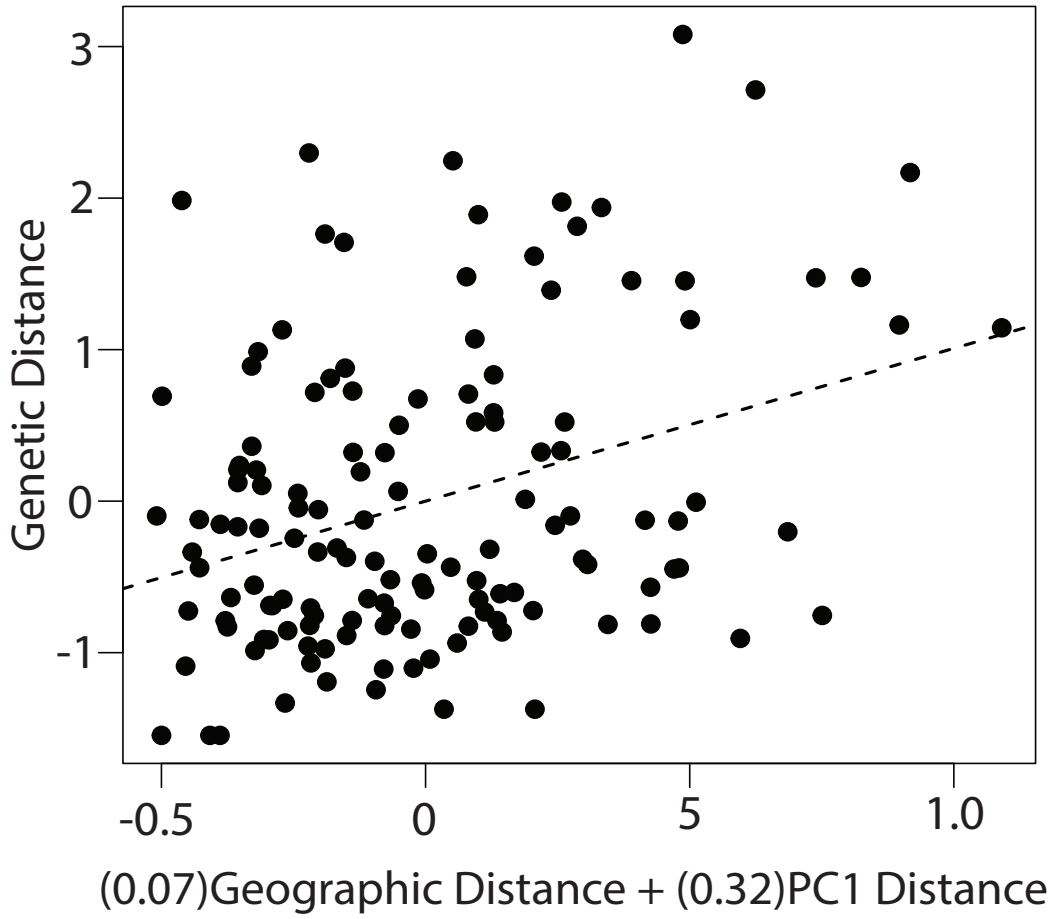
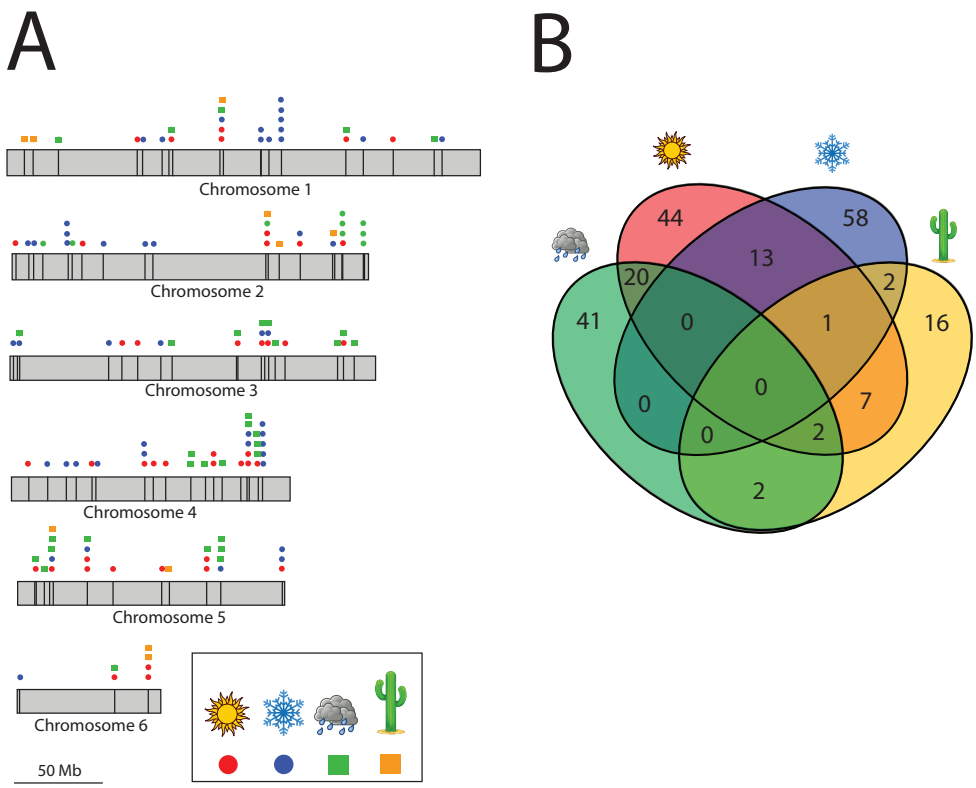


Figure 2-3: Linear relationship between pair-wise genetic distance between populations and a linear model including pair-wise geographic distance and environmental distance between populations. Within the linear model only environmental distance is a significant contributor.



Figre 2-4: Results of outlier analysis of allelic association with the mean temperature of the coldest quarter of the year (snowflake), mean temperature of the warmest quarter of the year (sun), mean precipitation during the driest quarter of the year (cactus), and mean precipitation during the wettest quarter of the year (raincloud) across 6 populations of *A. carolinensis* A) Distribution of environmental outliers across the six annotated macrochromosomes of the *A. carolinensis* genome. B) Overlap of outliers between the four environmental variables surveyed.

2.4 DISCUSSION

The major goal of this project is to understand the degree to which the evolutionary processes that produce niche conservatism constrain invasive species to their native environmental envelopes. Towards this goal, we have identified novel climatic features associated with the migration of a subtropical island lizard into higher latitude environments in mainland North America and explored evolutionary response to novel climate at the niche, phenotypic, and genomic levels. Comparisons of climate availability between Cuba and the southeastern U.S highlight the climatic shifts associated with the mainland invasion of the Cuban green anoles. Significant differences in extreme cold and temperature variability between the two ranges provide the potential for climate-mediated selection in allopatry. Niche modeling results support this finding, revealing niche expansion (increased niche breadth) of *A. carolinensis* beyond that of its Cuban relatives.

Projection of the western *A. porcatus* niche onto the *A. carolinensis* range further supports this hypothesis. The increasing dissimilarity of the western *A. porcatus* model to the *A. carolinensis* range with increasing latitude suggests that decreasing temperatures and increased thermal variability at higher latitudes would limit the distribution of the Cuban green anole within the known range of *A. carolinensis*. Therefore, adaptive evolutionary response to cold-mediated selection may have been necessary for *A. carolinensis* to obtain its current distribution on the mainland. These results suggest that novel thermal environments across the *A. carolinensis* range may have played a significant role in adaptive divergence from its Cuban ancestors and may have a significant ongoing influence on population dynamics within the species.

If geographic variation in winter temperature across the *A. carolinensis* range has played a significant role in shaping evolutionary trajectories within the mainland lineages the *carolinensis* subclade, then populations across the species' range should show concomitant shifts in temperature-related phenotypes to match local environment. Furthermore, if the pattern of

variation in these phenotypes is the result of natural selection, then such differences should persist between climatically differentiated lineages across generations, independent of climate.

Analyses of geographic variation in thermal tolerance support the first of these hypotheses. Animals occurring in colder winter environments at higher latitudes are able to maintain function at lower temperatures than their southern counterparts, even after controlling for phenotypic variation due to shared history and ongoing migration between populations. Additionally, common garden experiments give support to the second hypothesis. Lizards from climatically differentiated populations maintain population specific signatures of cold tolerance, despite being raised in identical thermal environments. However, trans-generational plasticity cannot be ruled out as a potential factor influencing the observed patterns of variation in cold tolerance.

Finally, under the hypothesis of climate-mediated selection, we expect the effects of the putative selective pressure to be apparent at the genomic level. This effect is expected in two potential ways. First, differences in local environment may act as a barrier to dispersal, selecting against migrants from climatically differentiated source populations and resulting in climate-mediated genetic isolation. Landscape genetics analyses reveal support for climatic influence on the genome. MMRR results show geographic variation in low temperature extremes and variability are a significant predictor of gene flow across the *A. carolinensis* range. Conversely, isolation by distance is not a significant contributor to genetic differentiation within *A. carolinensis*.

Second, natural selection should act on regions of the genome responsible for producing phenotypic adaptation to local climate. As a result, the frequency of adaptive alleles should display a higher than expected association with its putative selective pressure across a climatically heterogeneous range. Using RADseq data we have identified genes within the *A. carolinensis* genome that show high support as candidates for climate adaptation, based on the strength of their association with climatic variation across the range of the species. Candidate genes associated with local winter temperatures show a diversity of functions. However, the relatively low SNP density

and high proportions of intergenic regions represented in this dataset limit our ability to detect causal variants involved in adaptation, and highlight the limitation of RADseq data towards this aim. These results highlight the possibility that genomic adaptation to climate may involve multigenic contributions of small effect spanning many biological pathways and provide a foundation for further genomic studies utilizing higher density RAD sampling, whole genome resequencing or functional genomics approaches, such as RNAseq.

2.5 CONCLUSION

Invasive species pose a serious threat to biodiversity worldwide. In the U.S. alone there are an estimated 50,000 non-native species contributed in an estimated $120 billion/year in environmental damage and loss (Pimentel et al. 2005). Due to its subtropical climate and status as a major travel and import center, southern Florida has become a hub for invasive reptile and amphibian species, an in some groups such as lizards, invasive species now outnumber natives (Krysko 2011). The potential spread of these invasive species to into higher latitudes has become a major focus of conservation, ecology and evolutionary biology. However the relative roles of the constraining factors that cause niche conservatism, local adaptation and gene flow in influencing range expansion of invasive species are still relatively understudied, partly due to the limited time since the introduction of many species. Natural invasion events provide a longer time frame over which to observe the effects of novel climate on range expansion and may provide valuable insight into the evolutionary mechanisms associated with migration into foreign environments.

The results of this study show that niche conservatism has broken down over evolutionary time in *Anolis carolinenis*. Climatic niche expansion has occured in concert with local adaptation to thermal environments and reduced gene flow between climatically differentiated populations within the species. Since its overwater dispersal, the mainland lineage of green anoles has been exposed to a broader range of thermal environments than its Cuban relatives. In response to novel climate, this lineage has expanded its environmental niche beyond that predicted by its ancestral

climatic envelope. Cold tolerance within the species varies in concert with increasing cold and variable temperatures at higher latitudes, suggesting local adaptation. Additionally, genetic distance between populations correlates with geographic differences in thermal profiles, suggest isolation by environment. This combination of local adaptation of thermal tolerance and reduced gene flow between thermally distinct environments has likely aided in population expansion of the mainland lineage of the green anole.

These results provide valuable insights into potential spread of invasive species. Human-mediated dispersal is likely to increase phenotypic and genetic diversity of invasive populations through multiple invasion events. The establishment of these species in foreign regions with environmental profiles similar to their native ranges has resulted in growing populations and wider distributions of these species. This combination of events is likely to increase to efficiency of natural selection in marginal habitats and allow local adaptation. Decreased gene flow in between climatically differentiated populations may reinforce local adaptation across an environmental gradient and allow for continued range expansion beyond native climates.

Recent years have seen staggering advances in data availability and collection. Freely available climate and geo-referencing archives have made it possible to gather massive amounts of high quality data on species distributions and climatic variability on varying time scales. The progression of next generation sequencing technology is making it cheaper and easier to explore the genetic underpinnings of complex phenotypic response to environmental fluctuations in non-model species. These data will play a valuable role in our continued exploration of the impacts invasive species will make around the world in the coming years.

3 CHAPTER 3 – LOCAL ENVIRONMENT AND PHENOTYPIC PLASTICITY SHAPE VARIATION IN THERMAL PHYSIOLOGY OF THE NORTH AMERICAN GREEN ANOLE ALONG A LATITUDINAL CLINE

ABSTRACT

3.1 INTRODUCTION

Temperature plays a key role in biological function. Changes in temperature extremes and variability can have ramifications at all levels of biological organization and consequently seed evolutionary change through natural selection. All organisms experience a range of temperatures over which they can maintain optimal performance. Thermal fluctuations outside this range can have consequences for individual fitness (Sibley and Calow 1986, Portner and Farrell 2008, Bradshaw and Holzapfel 2010). Natural selection may act on phenotypes to minimize the deleterious effects of a shifting thermal environment through two mechanisms. First, phenotypic plasticity can allow a single genotype to produce optimal phenotypes across a range of environments (DeWitt and Wilson 1998, Pigliucci 2001).

Second, local adaptation can result in multiple genotypes that each code for the optimal phenotype in one environment and that change across geographic space depending on local conditions (Kawecki and Ebert 2004, Hoffmann and Willi 2008, Somero 2010, Barrett et al. 2011, Thomas et al. 2012). Local adaptation and plasticity can evolve in tandem (Calosi et al. 2008, Jensen et al. 2008, Cottin et al. 2012, Dam 2012, Kelly et al. 2012) and phenotypic plasticity may adapt to local conditions, making it difficult to assess the relative contributions of each mechanism to observed patterns of thermal tolerance (Jensen et al. 2008, Pulgar et al. 2005, Bedulina 2012). Through controlled experimental manipulations of organisms sampled across a climatically

heterogeneous range we can disentangle these effects and interactions to better understand how environmental variation generates phenotypic diversity.

The interaction of plasticity and local adaptation may play a key role in the evolutionary trajectories of species in the face of changing climate (Somero 2010). Anthropogenic changes to ecosystems are expected to result in greater exposure to stressful thermal environments for species around the globe through several means. First, temperature increases due to global warming are expected to place many reptiles and amphibians in jeopardy, particularly in tropical environments where species are already exposed to temperatures near their thermal maxima (Huey et al. 2012). Additionally, recent research suggests the possibility of increased frequency and magnitude of extreme weather events (Walsh et al. 2014), including severity of winter cold at mid-latitudes (Francis and Vavrus 2012, Liu et al. 2012, Kim et al. 2014, Cohen at al 2014, Francis and Vavrus 2015). As a result, species at these latitudes may be exposed to radical fluctuations in high and low temperatures. Finally, the recent influx of invasive reptiles and amphibians into foreign environments has resulted in exposure to environmental conditions outside those experienced in native ranges (Gallagher et al. 2010, Angetter et al. 2011, Kolbe et al. 2012). This brings into question the role of local adaptation and plasticity in determining the pace and extent of range expansion of these invaders.

The green anole, *Anolis carolinensis* provides an ideal system for the study of phenotypic plasticity and local adaptation of temperature-related phenotypes. The only anole native to the continental United States, its range extends as far north as Tennessee, North Carolina, and Oklahoma, the highest known latitude of any member this species rich (~ 400 species) genus. Winter temperatures are hypothesized to limit the northern edge of the species' distribution (Williams 1969). *Anolis carolinensis* does not hibernate, even in the northernmost reaches of its range, as is common for most reptiles. By basking in sheltered sites, northern populations are able to survive through the winter months (Bishop and Echternacht 2004) despite regular ambient

temperatures below freezing. Populations from different climates display differences in acute cold tolerance (CT_{min}) (Wilson and Echternacht 1987). However, the relative roles of plasticity and canalized differentiation in shaping geographic variation in thermal tolerance are unclear. Furthermore, the physiological mechanisms underpinning thermal tolerance in terrestrial ectotherms and the time scales over which they act remain poorly understood.

In this study we use experimental temperature manipulation to measure cold tolerance, metabolism, and oxygen carrying capacity in response chronic and acute cold onset. With this dataset, we measure the effects of phenotypic plasticity and local environment on variation in cold tolerance along a latitudinal cline, identify subordinate physiological mechanisms that may contribute to this trait, and gain a clearer understanding of how the timing of cold onset impacts phenotypic response in terrestrial ectotherms**.**

3.2 MATERIALS AND METHODS

3.2.1 Lizard Collecting and Housing

Lizards were collected from five localities along a latitudinal transect during June and July of 2013 (Figure 3-1A). All animals were transported to animal care facilities at the University of Illinois. Animals were housed in individual clear plastic containers (350in^2) at 30°C under a 12-hour light cycle for 18-26 days. Each cage was lined with artificial turf and moss to maintain humidity. A wooden dowel and artificial foliage were provided for perching and shelter. All individuals were fed ad libitum with ½ inch, 4-week old *Acheta domestica*. Each cage was misted with water twice a day. Lizards were then randomly split into two 14-day acclimation conditions. Prior to critical thermal minimum (CT_{min}) testing, one group was kept at 30°C (control treatment), while the other was transferred to 20°C (Chronic cold treatment) for 14 days. All animal husbandry and experimental protocols were approved by the University of Illinois IACUC (protocol # 14049) and Harvard University (protocol #26-11).

3.2.2 Critical Thermal Minimum

To assess the lower thermal limits of each of the experimental animals, we employed a common metric for ectotherms, critical thermal minimum (CT_{min}, Cowles and Bogart 1944). During CT_{min} testing, internal body temperature (T_b) was measured continuously via a 20-gauge digital thermocouple inserted approximately 5mm inside the cloaca and secured with tape. Beginning from room temperature, we cooled the T_b of each lizard 1°C per minute until 13°C. We then flipped the lizard onto its back and stimulated it with forceps, allowing it 30 seconds to right itself. We repeated this protocol each time the internal body temperature dropped one degree until it was unable to right itself within the given window of time. The body temperature at which an animal could no longer right itself after 30 seconds was recorded as its CT_{min}.

To identify factors that may contribute to variation in CT_{min} across the latitudinal transect, we used a linear mixed effects model to describe the response of CT_{min} to experimental treatments. A full model of effects on CT_{min} was built, using sex, acclimation condition and latitude as fixed effects. Cooling rate during each trial was added to the model as a random effect. Next, we built a set of subordinate models, iteratively removing each fixed effect. We then compared each subordinate model to the full model using a likelihood ratio test.

To obtain estimates of population-level plasticity of cold tolerance, mean CT_{min} values were calculated for each population at both acclimation temperatures. Population response to acclimation was then calculated for each population, using the formula: $\frac{(CT_{min_{30}} - CT_{min_{20}})}{30°C - 20°C}$. We then tested for significant association between plasticity of cold tolerance and latitude using a linear regression.

3.2.3 Oxygen Consumption Rates

After CT_{min} testing, lizards were returned to acclimation temperature for 24 hours. Following re-acclimation, half of the lizards from the control and chronic acclimation treatments

were weighed to the nearest 0.01g and sacrificed. A schematic of experimental procedure can be found in Figure 4-1B. Oxygen consumption trials were performed during acute cold onset on the remaining lizards. Mass was measured for each lizard to the nearest 0.01g before being placed into an airtight chamber. Lizards from the 30°C acclimation group were held at 30°C for 45 minutes and oxygen consumption readings were taken continuously. The temperature was then dropped to 20°C and held constant for 45 minutes while oxygen consumption was continuously measured. This procedure was then repeated at 10°C. Lizards from the 20°C acclimation group were held at 20°C for 45 minutes during oxygen consumption readings then subsequently dropped to 15°C and 10°C for 45 minutes each. This procedure ensured that animals from different acclimation groups were tested at the same number of temperatures, exposed to acute cold onset for the same amount of time during testing and that total time of testing during each trial remained constant. The first 15 minutes of each trial were used as an acclimation period, during which baseline oxygen consumption measurements were taken. Oxygen consumption measurements were then averaged for the final 30 minutes at each temperature trial for further analysis. Body mass measurements taken prior to each trial were used to calculate mass-specific rates of oxygen consumption.

A linear mixed effects model was fit to the mass-specific O_2 consumption data, with sex, acclimation group and temperature and latitude during testing designated as fixed effects. Random intercepts were modeled for each individual. Next, we built a set of subordinate models, iteratively removing each fixed effect. We then compared each subordinate model to the full model using a likelihood ratio test.

Metabolic plasticity was measured for each individual by calculating temperature coefficients. For every pair of temperatures at which an individual's metabolic rate was measured the following formula was applied to obtain a metric of metabolic plasticity (Q): $Q = \left(\frac{R_1}{R_2}\right)^{10/(T_1 - T_2)}$

T_1 and T_2 represent the upper and lower temperatures at which metabolic rate was measured, respectively. R_1 and R_2 represent mass-specific metabolic rate at the upper and lower temperatures, respectively.

Linear mixed models were then used to estimate the significance of experimental factors in contributing to metabolic plasticity. First, a full model was assembled specifying Q as the dependent variable. Sex, change in temperature and acclimation temperature were modeled as fixed effects. Random intercepts were modeled for each individual. Subordinate linear mixed models were then used as described above to estimate the significance of contribution of each fixed effect.

3.2.4 Heart Mass and Hematological Measurements

Lizards were anesthetized immediately upon removal from the respirometry chamber using 5% vaporized isoflourane and euthanized via cervical dislocation. Blood hemoglobin and lactate concentrations were measured immediately upon death using Hemocue® and Lactate Pro (Arkray) meters, respectively. Following these hematological measures, heart mass was measured to the nearest 0.001g. Heart mass was then divided by total body mass to obtain relative heart mass. A multiple linear model was run to estimate the effects of experimental conditions on each of the measurements above, using acclimation, temperature treatment and latitude as independent variables.

3.2.5 Associations between Metabolic Rate and Cold Tolerance

To estimate the power of metabolic rate to directly predict variation in cold tolerance, a mixed effects model was performed using CT_{min} as the response variable. We used oxygen consumption measurements taken at 10°C as an estimate of aerobic metabolism at the body temperature nearest critical thermal limits. Lactate concentration was used as a proxy for anaerobic metabolism nearest critical thermal limits. These variables, along with acclimation temperature

were modeled as fixed effects. Cooling rate during each CT_{min} trial was added to the model as a random effect.

3.3 RESULTS

3.3.1 Critical Thermal Minimum

Two-week acclimation temperature had a significant effect on CT_{min} (Chi d.f. = 1, p = 1.742e-11) (Figure 3-2). Exposure to a 10°C decrease in temperature over 14 days lowered CT_{min} by 1.99 C. Latitude of site of capture also contributed significantly to the observed variation in cold tolerance among individuals (Chi d.f. = 1, p = 2.207e-07). An increase in latitude of 1 decimal degree lowered CT_{min} by 0.253 C. There were no significant differences in cold tolerance between males and females (X d.f. = 1, p = 0.7633). These results suggest that both phenotypic plasticity and local environment play significant roles shaping biological response to cold. Plasticity of cold tolerance did not show a significant linear association with latitude ($F(1,3) = 0.1129$, $R^2 = -0.285$, p = 0.759).

3.3.2 Oxygen Consumption Rates

The linear mixed model reveals a significant effect of temperature during acute cold trial on variation in aerobic metabolism (Figure 3-3). As temperature decreased by 1°C during acute cold challenge, rates of O2 consumption were suppressed by ~ 0.019 ml O2/h/g (d.f. = 6, $X^2 = 20.837$, p = 5e-06). Neither sex (d.f. = 6, $X^2 = 0$, p = 1.00) nor acclimation (d.f. = 6, X2 = 0.0229, p = 0.8798) had a significant effect on variation in mass specific oxygen consumption. These results suggest a plastic response of oxygen consumption to acute, but not chronic cold onset. Latitude showed a suggestive, but insignificant, effect on mass-specific oxygen consumption (d.f. = 7, $X^2 = 3.4022$, p = 0.06511).

Because the results of this analysis show that oxygen consumption responds specifically to acute cold fluctuations, we ran an additional analysis, replacing latitude with a specific measure of

acute temperature fluctuation at site of capture. We obtained estimates of mean diurnal range of temperature from the Worldclim database (Bio2, (Hijmans 2005)). A subsequent linear mixed model analysis revealed a significant contribution of this variable to observed patterns of oxygen consumption (d.f. = 7, X^2 = 3.935, p = 0.047). Metabolic plasticity of oxygen consumption did not show a significant response to any experimental treatment (Sex: p = 0.1917, Change in temperature: p = 0.5094, Acclimation temperature: p = 0.3682).

3.3.3 Heart Mass and Hematological Measurements

Multiple linear regression (F(4,94) = 20.22, multiple R^2 = 0.46, adjusted R^2= 0.44, p = 4.832e-12) revealed that blood lactate concentration was significantly affected by sex (β =-1.78781 **,** t = -3.411, p = 0.001), acute cold exposure (β =-4.214, t = -8.001, p = 3.18e-12), acclimation temperature (β =1.053, t = 2.014, p = 0.047**),** and latitude (β =-0.176, t = -2.046, p = 0.0436) (Figure 3-4). These results suggest that phenotypic plasticity (acute and chronic) and local environment both effect variation in blood lactate significantly.

Neither acute nor chronic cold onset had a significant affect on blood hemoglobin concentration (chronic: β = .002, t = 0.007, p = 0.99, acute: β = -0.17, t = -0.65, p = 0.52**)** and relative heart mass (chronic: β = -0.0003, t = -0.313, p = 0.76, acute: β = 0.001, t = 1.23, p = 0.22) show no significant acute or chronic cold onset. Latitude had no effect on either phenotype ([Hb]: β = -0.03, t = -0.68, p = 0.50, relative heart mass: β = -0.0001, t = -0.88, p = 0.38). Sex did significantly affect hemoglobin concentration, with males displaying higher concentrations than females (β = 0.72, t = 2.76, p = 0.007).

3.3.4 Associations between Metabolic Rate and Cold Tolerance

Linear mixed effect reveals a significant effect of mass specific oxygen consumption (X d.f. = 1, p = 1.152e-13**)** and blood lactate **(**X d.f. = 1, p = 0.0026) on variation in cold tolerance. Lowering lactate by 1 decreased CT_{min} by 0.069°C. Similarly, as oxygen consumption decreased by

1ml/O^2/hr CT_{min} decreased 4.27°C. These results suggest that depression of aerobic and anaerobic metabolism during cold onset may contribute to cold tolerance in the green anole.

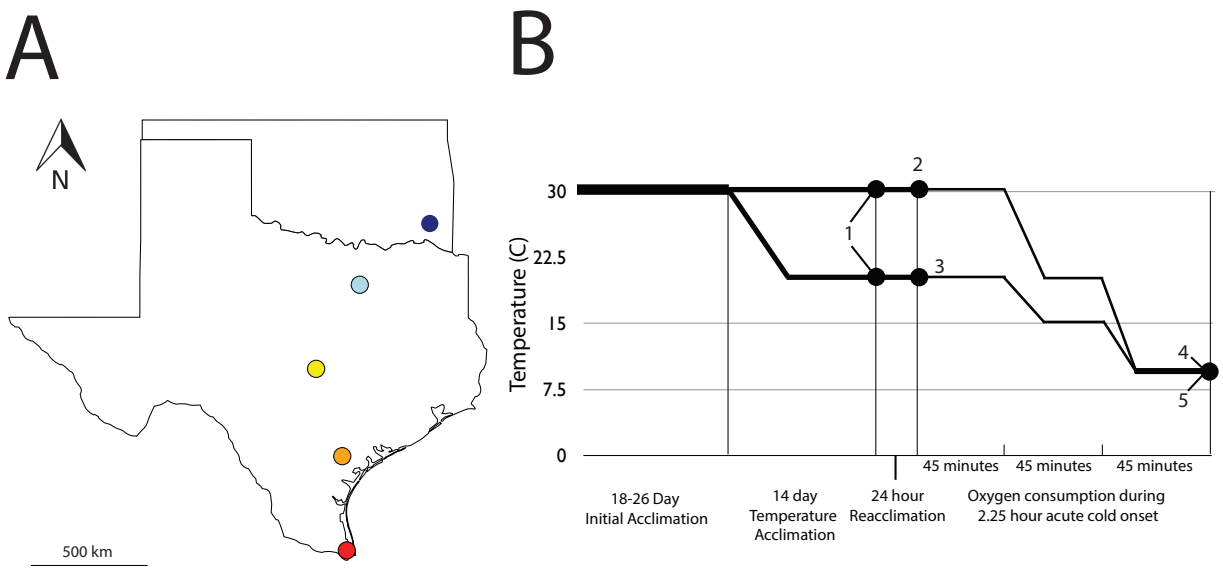


Figure 3-1:

A) Map each collection site across the latitudinal transect studied in Chapter 3

B) Experimental design for collection of physiological data.

B1: CT_{min} trials performed

B2: lizards in the control treatment sacrificed

B3: lizards in the chronic cold treatment sacrificed

B4: lizards in the acute cold treatment sacrificed

B5: lizards in the acute + chronic cold treatment sacrificed

Tissue samples, heart mass, lactate concentration, hemoglobin concentration were taken at the time of sacrifice for each group.

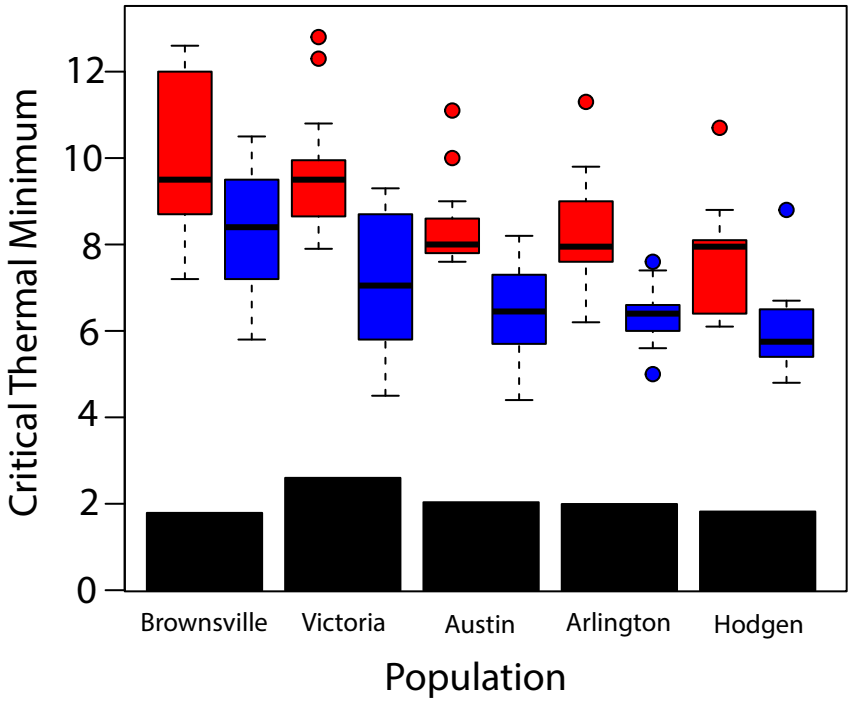


Figure 3-2: Measurements of critical thermal minimum (CT_{min}, in °C) across the latitudinal transect of study. Red boxes denote warm acclimation treatments (two week 30°C acclimation). Blue boxes denote cold acclimation treatments (two week 20°C acclimation). Black bars at the bottom of the graph denote the change in CT_{min} between acclimation conditions for each population.

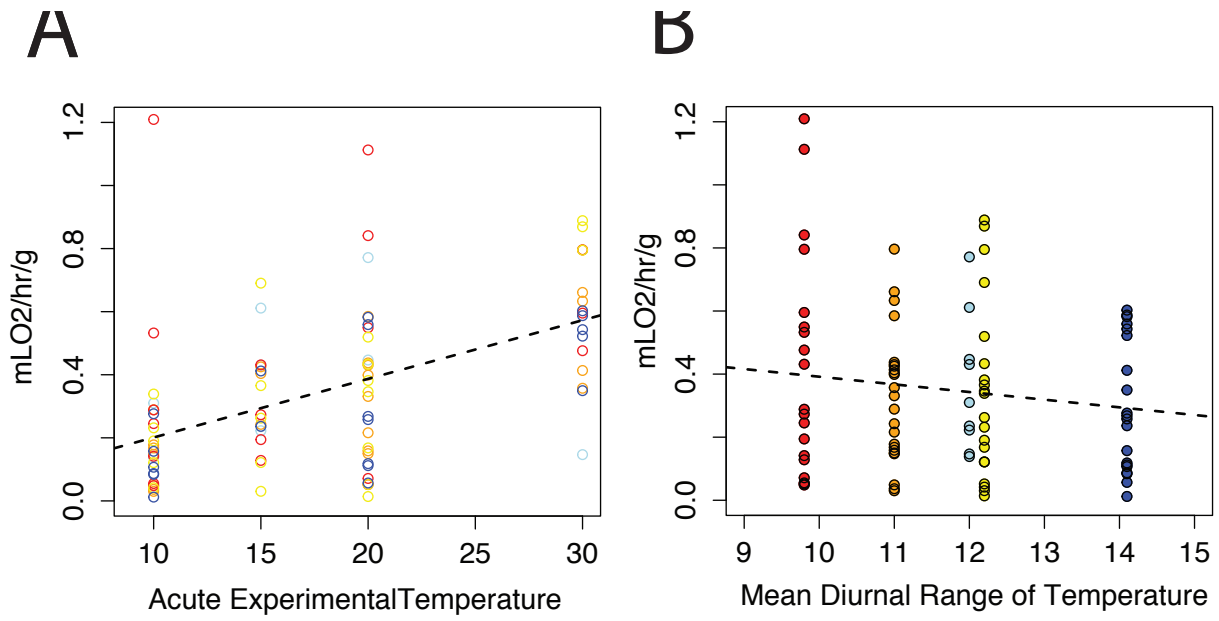


Figure 3-3: Linear relationships between mass-specific oxygen consumption and A) testing temperature during experimental acute cold onset and B) the mean diurnal range of temperature at the site of capture.

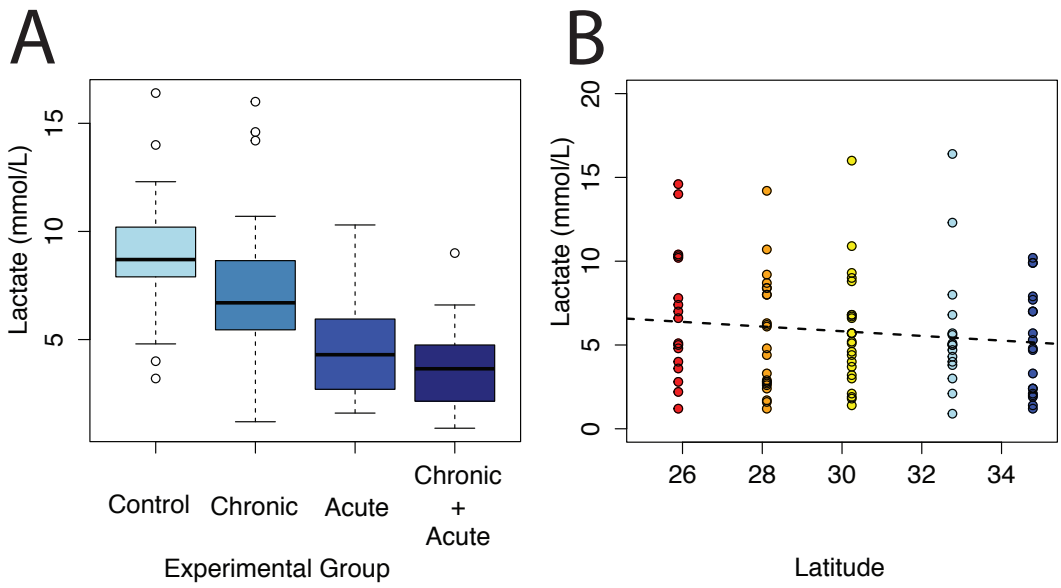


Figure 3-4: A) Boxplot of lactate concentration for each experimental group B) Linear association between lactate concentration and latitude at site of capture

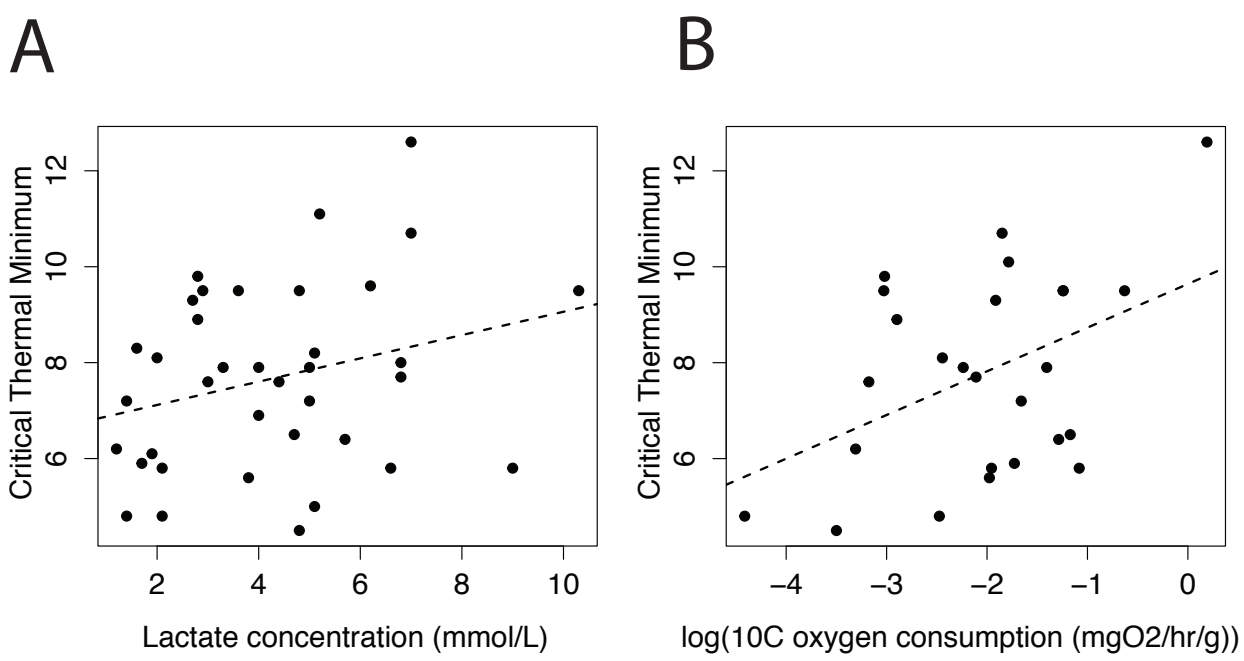


Figure 3-5: Linear relationship between Critical thermal minimum and A) Lacate concentration of the blood and B) Oxygen consumption rates at 10°C

3.4 DISCUSSION

The results of this study show that cold tolerance within the green anole responds to both chronic and acute cold onset. The increased cold tolerance at high latitudes found here supports the findings of Wilson and Echternacht (1987), based on populations from central Florida, southern Georgia and Eastern Tennessee. Wilson and Echternacht (1987) reported a lower CT_{min} in eastern Tennessee anoles when compared to their southern counterparts after a common acclimation period for all populations, suggesting that geographic variation in cold tolerance has a genetic basis in green anoles. This result is supported by similar findings in other anole species (Leal and Gunderson 2012). However, other studies have found that phenotypic plasticity also contributes significantly to variation in cold tolerance of anole species (Kolbe et al. 2012, Rubio-Rocha et al. unpublished).

Our results show that while there is indeed a significant effect of phenotypic plasticity, latitude (i.e. local environment) also plays a significant role in determining geographic variation in cold tolerance, with individuals from colder climates displaying lower CT_{min} regardless of acclimation condition. The correlation between cold tolerance and local environment along a latitudinal cline support a hypothesis of local adaptation of cold tolerance in the green anole. However, experimental validation is needed to explicitly test that these phenotypes provide enhanced fitness in local environments.

Furthermore, our results suggest an important role of the timing and magnitude of cold onset in determining physiological response in this species. Cold tolerance and blood lactate concentration both show a significant plastic response to both acute and chronic cold onset, but plasticity of oxygen consumption appears to be a function of acute, but not chronic, cold onset. This result suggests that adjustments of aerobic metabolism may play a role in rapid cold onset (such as daily fluctuations in temperature or sudden cold snaps), but may be less important for seasonal acclimation during the colder months of the year.

The lack of response in relative heart mass and blood hemoglobin to either chronic or acute cold onset suggests cardiac output and oxygen carrying capacity are unaffected by cold challenge in this species, which may necessitate the metabolic depression strategy observed. Maintenance of aerobic scope has been proposed as a major limitation to biological function in ectothermic species (Portner et al. 2006). Under this hypothesis, stressful thermal environments diminish aerobic scope due to inefficiency of oxygen uptake and transportation. Therefore, continued function in thermally stressful conditions necessitates physiological adjustment to maintain aerobic scope. This may be achieved by increasing metabolic rate to counteract the effects of temperature, or by decreasing metabolic demand as a means of metabolic conservation. Lack of plastic response of cardiac output or oxygen carrying capacity in to cold onset *A. carolinensis* suggests the latter strategy is used by this species.

The results of this study also reveal a significant contribution of local environment to variation in metabolic rate across the latitudinal cline. Animals sampled from higher latitudes exhibit lower energetic expenditure than their southern counterparts, regardless of acclimation condition. This result suggests climate-mediated local adaptation may play a significant role in shaping geographic patterns in metabolic rate, with populations from higher latitudes exhibiting a greater degree of metabolic depression than those from more southern localities.

For cold tolerant ectotherms, depression of basal metabolic rate may greatly improve long-term survival (Storey and Storey 1988, Voituron et al. 2000). Decreasing basal metabolic demands allows greater conservation of energetic resources and allocation of energy to specific biological functions such as growth (Weiser 1991 and 1994, Koehn 1991, Sears 2005) under periods of extreme cold and diminished food supply. However, it is unclear whether metabolic suppression can result in increased cold tolerance. The results of this study provide evidence that suppression of aerobic and anaerobic metabolism may aid in increasing cold tolerance within *Anolis carolinensis*.

3.5 CONCLUSION

Understanding the mechanisms that allow animals to adapt to variation in their thermal environment is a central goal of evolutionary biology. However disentangling the effects of phenotypic plasticity and local adaptation in shaping variation in thermal tolerance can be difficult. The results of this study shed valuable light on the environmental and physiological factors that shape geographic variation in cold tolerance in reptiles.

Experimental and geographic effects on cold tolerance and metabolic rate suggest a role of both phenotypic plasticity and local adaptation in shaping these traits. Anaerobic metabolism responds to both acute and chronic cold onset, suggesting a role in acclimation to both seasonal and daily fluctuations in temperature. Aerobic metabolism is responsive to acute temperature fluctuation alone, pointing to a potentially specialized role in physiological acclimation to rapid shifts in the thermal environment.

Correlations between metabolic rate (both aerobic and anaerobic) and cold tolerance suggest metabolic depression may aid in the extending functional limits of this species under chronic and acute cold challenge. Plasticity of these phenotypes show no signature of local adaptation across latitude, suggesting it is not the primary target of selection in more seasonal environments in this species' range. Although we have low power to detect local adaptation of plasticity with this dataset, our results support the finding of a recent literature review of thermal plasticity (Seebacher 2015). That analysis of 202 ectothermic species indicates that costs of plasticity in thermally variable environments may limit its benefits, and therefore favor the evolution of locally adapted, fixed phenotypes. Future studies in this system should aim to estimate the fitness of phenotypes under local conditions along and latitudinal cline and aim to calculate phenotypic plasticity of individuals rather than at the population level to gain greater resolution of geographic variation.

4 CONCLUSION OF THE DISSERTATION

The abiotic environment provides a profusion of potential selective pressures that can apply themselves inescapably across the tree of life. Temperature is universally important to biological function. Geographic and temporal shifts in climatic variability and extremes can have ramifications at all levels of biological organization and consequently seed evolutionary change through natural selection. However, identifying specific environmental factors acting as selective agents and disentangling their effects on evolutionary processes from those of other climatic and demographic factors can prove challenging. In this dissertation I have taken a broad approach to understanding how temperature shapes the evolutionary history of species, using a single species of terrestrial ectotherm, *Anolis carolinensis*.

In the first chapter, I trace the evolutionary history of the species, using phylogenetic techniques. A migration event from Cuba to the United States resulted in the establishment of *A. carolinensis* in mainland North America during the Miocene-Pliocene. The species then rapidly expanded its range northward in two waves during the periodic glaciations of the Pleistocene to occupy the vast majority of the southeast. Mitochondrial data suggest these two northern migrations remain genetically distinct. Ongoing research is using genome scale data to gain a better understanding of the genetic connectedness of populations outside of Florida, identify patterns of ancient and contemporary admixture, and find regions of the genome that may be resistant to introgression between lineages.

In the second chapter I explore phenotypic and genomic mechanisms that may erode the phylogenetic pattern of niche conservatism after migration into novel thermal environments. I find that the mainland lineage of green anole has greatly increased its niche breadth when compared to its closest island relatives. Variation in thermal tolerance and gene flow across the species current

range suggest local adaptation and isolation by thermal environment have aided in this niche expansion. I conclude this chapter with the first selection scans yet conducted on the species' genome to identify chromosomal regions that may be under climatic selection.

In the third chapter I measure physiological response of cold tolerance to acute and chronic variation in cold across a latitudinal transect. I find that both phenotypic plasticity and local environment shape variation in cold tolerance within this species. Further more, metabolic response to cold onset and correlations with cold tolerance suggest a role of metabolic suppression in extending functional limits of this species under cold challenge.

Ongoing research is using tissues from this dataset to look at patterns of gene expression across the muscle transcriptome to identify genetic modules involved in physiological acclimation and adaptation to cold. The sequence data from these analyses will also allow me to further explore the genomic regions highlighted as potential targets of cold-mediated selection from Chapter 2. Finally, I am using the findings of this dissertation to investigate rapid evolutionary response at the phenotypic and genomic level to sudden and extreme cold events in natural populations of the green anole.

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6 SUPPLEMENTAL FIGURES AND TABLES

All Supplemental Material for Chapter 1 can be found at:
http://onlinelibrary.wiley.com/doi/10.1002/ece3.324/suppinfo

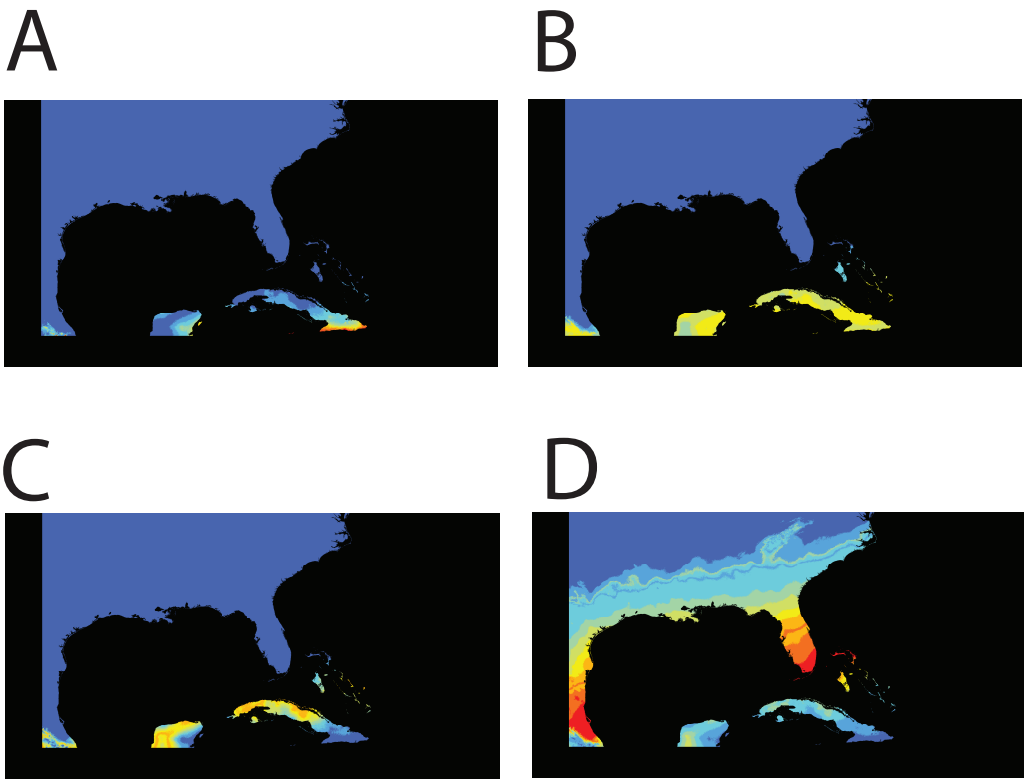


Figure S2-1: Visualization of Projected niche models for the eastern (A) and western (C) clades of *Anolis porcatus*, *A. allisoni* (B), and *A. carolinensis* (D)

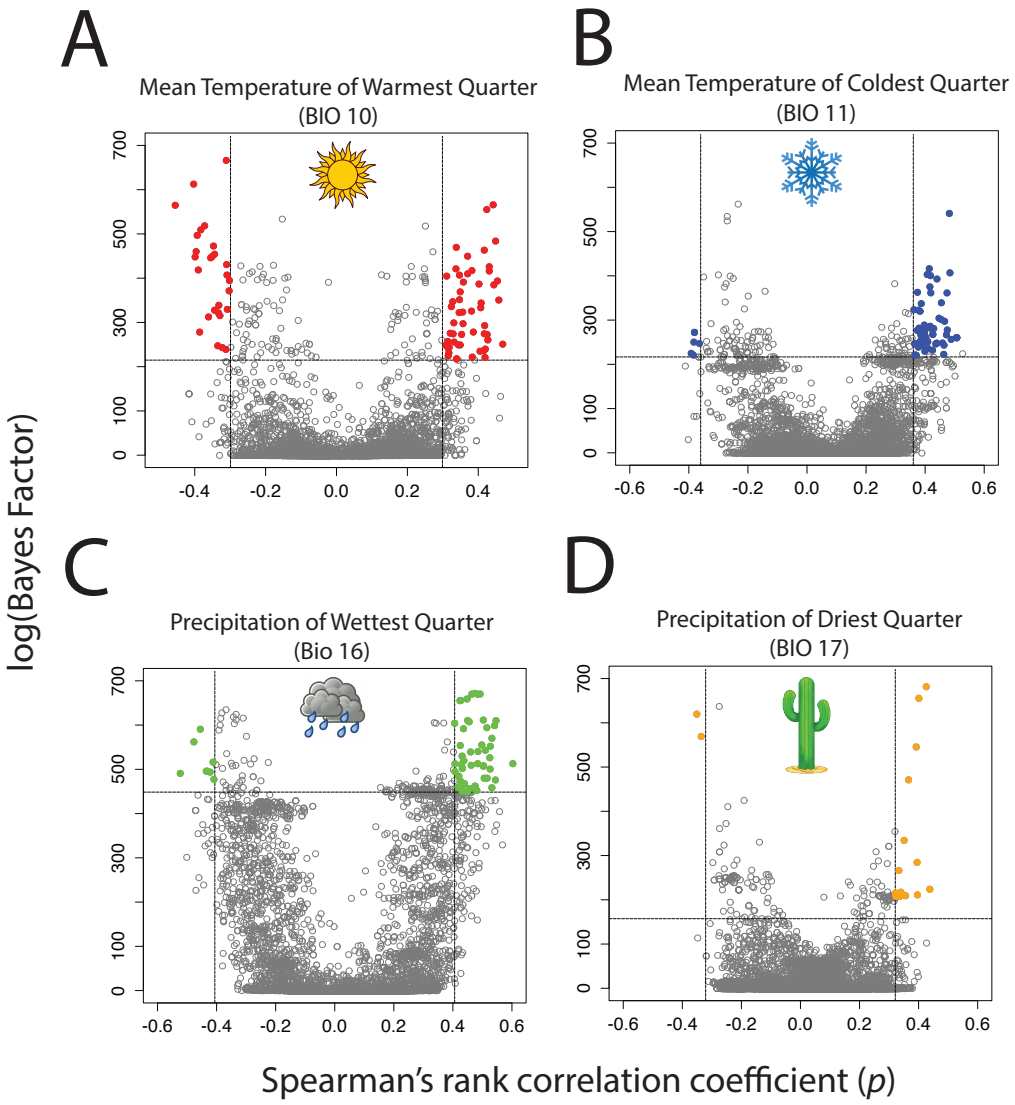


Figure S2-2: Volcano plots of associations between *Anolis carolinensis* genomic variants with A) the mean temperature of the warmest quarter of the year B) Mean temperature of the coldest quarter of the year C) Mean precipitation of the wettest quarter of the year D) Mean precipitation during the driest quarter of the year at the site of capture. The x axis of each plot shows Spearman rank correlation coefficients. The y axis shows bayes factor associations. Colored dots in each plot indicated outliers that fall within the to 99% of both axes.

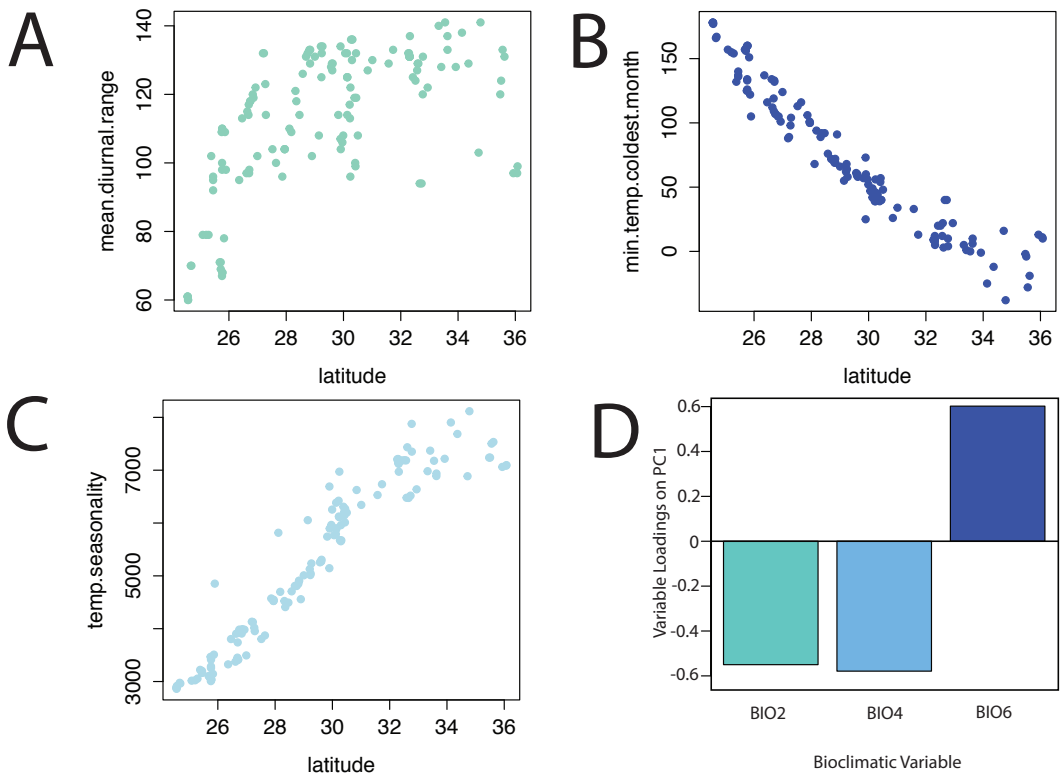


Figure S2-3: Associations between latitude mean diurnal range of temperature (A), temperature seasonality (B), and minimum temperature of the coldest quarter of the year (C) for 147 known presence localities of *Anolis carilinensis*. These three variables were used to calculate environmental dissimilarity used for multiple matrix regression with randomization in Chapter 2, based on Euclidean distance between localities in the first axis of principal components analysis (D)