Effects of Geographic Range Variation

Lessons from the impact of post-glacial geographic range expansion on the morphology, development and competitive interactions of the American Toad (*Anaxyrus americanus*)

David AJS O'Connor
Department of Biology
McGill University, Montréal
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Abstract

One of the fundamental goals in ecology is to explain the variation in species richness and distribution across the planet, and in the geographic range size of individual species. Within the context of species' geographic ranges, there are two principle aspects: the *potential* and *realized* range, which can be affected by such abiotic and biotic limitations to expansion as geophysical barriers, environmental limitations and competitive exclusion. Herein, I performed a review and analysis of post-glacial range expansions of herpetofaunal species across Canada to ascertain general patterns in the geophysical barriers that these species encountered that may be directing their range expansion. I used a simulation model to explore how differences in behaviour of closely related species may be a driving factor in competition and hybridization where their ranges meet. Furthermore, I examined how a large heterogeneous geographic range affects the evolution of a species. I did a detailed survey of variation in the morphological traits within and between mature adults in populations of the American Toad (*Anaxyrus americanus*). I also performed a common garden experiment with American Toad tadpoles and found evidence of physiological adaptations for local climate between populations separated by vast geographic distances. Overall, my thesis provides empirical evidence of possible local variation within an amphibian species with a very large latitudinal and geographic range and how a single species expresses significant variation in its morphology and physiological response to latitude as a proxy for environmental variation.

Un des objectifs fondamental de l'écologie est d'expliquer la variation des distributions et quantités des espèces au monde, et dans la variation des tailles des

domaines géographiques des espèces. Dans le contexte des domaines géographique des espèces, il y a deux aspects qu'il faut considérer : la domaine *potentielle* et *réalisée*, qui sont affectés par des limitations d'expansion tel que les barrières géophysiques, les limitations environnementales et l'exclusion compétitive. Dans cette thèse, j'ai fait une revue et analyse des herpétofaune du Canada pour trouver des généralisations dans les barrières qui ont dirigé l'expansion des domaines de ces espèces après la retraite des glacières. J'ai aussi utilisé une simulation pour explorer comment les différences de comportement entre deux espèces similaires affectent la compétition et hybridations entre eux là ou leur domaines se croisent. En plus j'ai examiné comment un grand domaine géographique affecte l'évolution d'une espèce. J'ai examiné les variations morphologique dans, et entres, les adultes mature dans les populations de crapaud d'Amérique (*Anaxyrus americanus*). En plus j'ai fait une expérience de jardin commun avec les têtards de crapaud d'Amérique et j'ai trouvé des signes des adaptations pour les climats locaux entre des populations écartées par des longues distances et des climats différents. En tout, ma thèse présente des éléments de preuve de la variation dans une espèce amphibien avec un domaine latitudinale très vaste et comment une seule espèce peut avoir des variations signifiantes dans sa morphologie et réponses physiologiques en utilisant la latitude en tant que proxy pour la variation environnementale.

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This thesis is dedicated to Fergus and Paul, who opened my eyes to the world and to Abigail, may she never stop exploring it.

Preface and Contributions of Authors

For Chapter 2, I collected all the data and species range maps that were used in the analysis; I also performed all of the analyses and prepared the map. Dr. Green revised the document as well as provided essential guidance in the preparation of the discussion, and collation of trends in phylogeographic patterns. This work represents a much-needed update to the COSEWIC Terrestrial Amphibians and Reptiles Faunal Provinces Map that is especially pertinent given the recent increase in available phylogeographies since the previous map was made. This map will be used in conservation legislation for many years to come.

For Chapter 3, I created and coded the simulation model, and performed all analyses. Dr. Green assisted revision. This is a significant simulation model that explores how behaviour can be a driver of hybrid zone dynamics and mitochondrial introgression between two closely related species. The general applicability of this model will allow for new exploration of how individual interactions can affect the long-term development of species.

For Chapter 4, I measured all museum specimens that were used, collected and measured all live-captured individuals and performed all the analyses. Dr. Green provided essential revisions. This chapter is the first large-scale examination of the American Toad, and represents the most comprehensive examination of morphological variation ever performed in the American Toad. This work also represents one of the few studies done on a species with such a large latitudinal range.

For Chapter 5, I performed the common garden experiment. I organized all collections, acquired necessary permits, received the eggs that were shipped to me from Athens, Georgia, USA, I personally collected the eggs from Ste-Anne-de-Bellevue, Québec and the tadpoles from Radisson, Québec and performed all analyses. Dr. Green assisted with revisions. This is among the first common garden experiments testing for adaptations to local climate, and represents novel empirical evidence of such adaptations.

Introduction

Two principle concepts of species range include the *potential* and *realized* range. The potential range is the area wherein a species could theoretically be able to survive, including the total geographic area where environmental conditions are favourable to the species and its biological requirements are met [1]. The realized range though, is the geographic region wherein the species is consistently found, and it may be drastically smaller than the potential range [1, 2]. The difference between the potential and the realized ranges depends on those factors that prevent the species from completely exploiting its potential range [2-4]. Furthermore, by limiting species ranges, these factors play a significant role in determining global patterns of species distribution [5].

Abiotic limitations to range expansion

There are numerous possible factors that can limit a species from occupying its full potential range. The first of which is the presence of geophysical barriers, these are abiotic barriers in the geography of a region that represent significant barriers to dispersal and limit species to certain areas through inhibiting dispersal ability, or terrain that is physically impassable to a given species [6-15]. These barriers vary dramatically in the time-scale on which they operate and also in the extent to which they act as a barrier to the movement of individuals. For example, the world's oceans have acted as barriers to many species since the break up of Pangaea 175 mya. In south eastern North America, the Appalachian Mountains and rivers such as the Mississippi and the Apalachicola River have acted as geographic barriers over multiple millions of years to species including salamanders and toads [16]. Contrastingly, while glaciation in the Pleistocene imposed significant barriers, their retreat in the last 10,000 years has reduced any barrier effect that they may have had.

In addition to preventing movement between regions and limiting species range size, geo-physical barriers may also act to shape species ranges during expansion events as they channel dispersal along specific paths. In western North America during the period of glacial retreat at the end of the Pleistocene glaciation, the Rocky Mountains presented a significant barrier to multiple coastal species whose resultant range expansion was limited to a north-south direction despite the presence of favourable terrain to the east whereas in Eurasia mountains and drainages forced an east-west range expansion for many species [16-18].

The temporal nature of glacial events provides insight into the manner in which species ranges can be limited and then channelled by geophysical boundaries. As glaciers render huge amounts of terrain inhospitable for many species, these species are restricted to specific ice-free refuge areas. Yet, as glaciers retreat, and species disperse and expand their range; dispersal is limited by many barriers including mountains, rivers and proglacial lakes. The congruencies in the timing of dispersal between multiple species can result in similar patterns of dispersal due to the long-term and transient barriers that were present depending on this timing [16].

By comparing and contrasting dispersal patterns between species it is possible to identify specific routes of, and significant barriers to, geographic range expansion. In this context, phylogeographic studies provide invaluable evidence as to the timing and path of post-glacial expansion [16]. This type of work has been performed in multiple species, and for different families including plants, mammals and amphibians [16, 19-22] and has led to the discovery of important cryptic refuge areas and, importantly, areas of post-glacial secondary contact.

Range expansion from established populations also commonly results in decreased genetic diversity that correlates with distance from the origin population due to a series of bottleneck events and founder effects associated with each successive population that is established [23, 24]. This decrease in genetic diversity has been repeatedly observed and is especially remarkable when species traverse the terminal moraine and expand from refugia following glacial retreat [16, 19]. However, the highest genetic diversity of a species may be found in these areas where previously separated clades reconnect, making identifying these areas of post-glacial secondary contact increasingly important [25]. In the case of amphibian species a particularly poignant example is that of the spring peeper (*Pseudacris crucifer*) which has populations with the highest genetic diversity in previously glaciated areas where two separate clades have made secondary contact [25].

Barriers may not be just significant geophysical barriers that inhibit movement or dispersal [26] but may also be habitat or climate that individuals can move into, but where survival is significantly hampered [27]. Whether these factors are due to temperature, precipitation, or other environmental conditions; abiotic factors can present significant barriers when they exceed the physiological tolerances of a species [28, 29]. Darwin's observations of few amphibians on islands as the salinity of oceans prevents amphibians from surviving the voyage from the mainland to islands represent a classic case of such an effect. Variation in climate and abiotic factors are easily explored across altitudinal or latitudinal clines [30, 31]. There are some crucial similarities and contrasts between abiotic factors, including temperature, precipitation, photoperiod, and seasonality, in relation to altitudinal and latitudinal clines [32, 33]. Although species can be well adapted for a particular set of abiotic conditions, true generality is difficult and trade-offs in performance and fitness need to be made [34-36]. As a result species that are well adapted to a particular region may find themselves incredibly maladapted to neighbouring conditions [36]. If abiotic factors are beyond the physiological tolerances of a species, then long-term survival becomes impossible in these areas [36]. These environmental factors can therefore work to limit the ability of a species to disperse into and establish sustainable populations within a region or area.

Given that abiotic environmental factors and geophysical factors both act on seemingly large geographic scales, it should then be possible for generalities to be drawn from these factors with respect to patterns in the distribution of species, regional communities and the composition of species assemblages. Such generalities would allow for predictions to be made in terms of identifying areas of particular conservational, or research interest.

Interspecific limitations on range expansion

While barriers may impede dispersal this is not to suggest that the climate and environmental conditions are unfavourable on the opposing side of the barrier. Distant or even neighbouring conditions may even be more favourable than those where the species is currently found and in some cases, barriers may be absent yet the species range is still limited. Competitive exclusion by neighbouring species may be an additional limiting factor for the range of the species [37-40]. The impact of this competition is well demonstrated when it is removed and a species experience a *competitive release*, as is seen in some invasive species, where population sizes and densities in novel habitats can greatly surpass that in the original native range. Introduced species, for example: Chinese Muntjac (*Muntiacus reevesi*) in England, Fallow deer (*Dama dama*) in Europe, Ring-necked parakeets (*Psittacula krameri*) in Belgium, numerous climbing plant species in Michigan, and American beavers (*Castor canadensis*) in Patagonia, have been shown to reach abundances far beyond those in their native range, and in the case of introduced deer and several climbing plants, the effect of competitive release in invasive populations meant that these populations were only reduced through stringent hunting and control operations [41-44].

One of the reasons why invasive species may be so proficient at expanding their range in novel habitats is that they are no longer under the same competitive pressures from co-evolved species as they are in their native range [45]. This has been found to be especially true in the case of plant sepecies, where enemy release from herbivory pressure is likely a driving force behind invasive species success in novel environments [46]. In the case of introduced deer, where there may be species that already inhabit a similar niche space as the invasive species, competitive release provides a significant advantage to the invasive over the native species [43]. The effect of competitive release, can also be estimated by the impact that reintroduction of a native predator can have on populations of invasive species. In the case of invasive populations of American Bullfrog (*Lithobates catesbeianus*), tadpole populations declined drastically as a result of predation from reintroduced native predatory pike [47]. Thus, inter-specific competition may prevent a species from expanding its range into that of other species. In this way, a species' range is limited to the geographic area wherein it is able to out-compete, or at least maintain a stable population, in the presence of other directly and indirectly competitive species. Where a species is unable to do so, competitive inhibition, a lack of necessary symbiotes, or presence of predation will prevent it from expanding and achieving its potential range, even if neighbouring habitat is more favourable [40, 48, 49].

Impact of a large heterogeneous range

However, even in the presence of geophysical barriers and competitive conspecifics there are species with large ranges that do not appear to be constrained by these same factors. The coconut, for example, has adapted to use oceanic currents as a mode of dispersal and in this way it has taken a potential significant geographic barrier and turned it into a mechanism to colonize almost all tropical islands [50]. While the coconut has overcome barriers through dispersal many species, birds in particular, use high motility to overcome barriers [51, 52]. In these highly motile or very efficiently dispersing species, geographic barriers can be easily overcome and favourable environmental and habitat conditions can be inhabited regardless of geographic separation, or the quality of habitat between favourable patches [50, 53, 54]. Therefore, while they may have a very large geographic range, these species might still only inhabit specific regions that are relatively homogeneous to one another compared to the surrounding landscape as a whole.

Classic models of range dynamics often paid little attention to the role that local adaptation could play in determining a species' response to climate change [55]. The assumption is often made that the environmental conditions that each population encounters will be similar for all populations, and in this way, the climate niche of a single population will be representative of the niche of the entire species [56]. This may be the case among those highly motile species previously mentioned, those with good dispersal, or narrow latitudinal or geographic ranges where climate conditions may not vary drastically between subpopulations and each may be representative of the climate niche of the entire species [57]. This is especially important in newer models that commonly depend on the climate within the current geographic range of a species to create a 'climate space,' a climatic niche that the species occupies [58-61]. Thusly, the species' range is predicted to track the geographic location of its currently habitable climate space through time [58-61].

However, by handling a species' climate space as a uniform and homogeneous entity rather than as a collection of the climate requirements of each locally adapted population these models are likely ill equipped to predict future ranges [58]. This can be exacerbated for those species with very large ranges as the local climate may vary drastically between populations [57]. This variation in the selective pressures experienced by different populations can have important impacts on the evolution of the species as a whole.

Populations may not be representative of the species, especially if there has been local adaptation within each population, which has given rise to intraspecific variation in physiological tolerances or plasticity [56]. A simulation model of virtual tree species found that if populations had significantly different phenology due to local climatic variation, then models that incorporated this local adaptation had significantly different predictions of climate change impacts than those that used homogeneously high plasticity in phenology across the entire species range [56]. These local adaptations can manifest in a multitude of ways, including morphologically or physiologically, in response to different ecological or environmental conditions. Along an altitudinal gradient, a study of Spanish Sandwort (*Arenaria tetraquetra*) found that the environmental changes with elevation resulted in significant variation in the morphological appearance, and the physiological status in terms of leaf size and water and nutrient content [62]. These findings are a clear example of how the morphological variation of the species can show distinct and consist trends across geographic distance, or environmental gradients. Moreover, these trends should be especially evident in species known to have specific traits that are strongly influenced by the environment, or the behaviour of individuals [63].

Local adaptations across a large heterogeneous range

Species must deal with a high degree of environmental heterogeneity and variability in selective pressures that could affect the evolution of the species. It may then be unlikely that these species are composed of uniformly distributed identical individuals, rather, they are more likely to be composed of clusters of individuals, or sub-populations, that make up the species as a whole [64]. These ‘populations of populations’ can be considered under the context of being a ‘metapopulation”.

Metapopulation theory was initially coined by Levins in 1969 to describe populations of pests in an agricultural setting [65] and was expanded upon with metaphorical “island” and “mainland” populations of the same species and how isolation, or contrarily, interconnectivity between them affects the survival and evolution of each [66, 67]. Populations may be found in similar environmental conditions, or they may be under drastically different environmental or selective pressures [68-70]. As a result, different populations may express significantly different life-history traits due to strongly varying local selective pressures between the regions where each population is found.

Ring species provide a rather extreme example of how a species, with continuous gene flow throughout the range, may still have significant morphological or life history variation between populations [71, 72]. A case study of Song Sparrows (*Melospiza melodia*) in North America examined both geographic isolation and ecological factors, including varying selective pressure, and how these can give rise to a ring species[73]. It also highlighted the role that local adaptation plays in driving ring species patterns when ecotones where the species is found have different selective pressures [73]. Interestingly, and in contrast to classic ring species theory, Song Sparrow populations that had secondary contact following geographic separation were still able to interbreed despite having significant differences between the two populations [73]. The salamander *Ensatina escscholtzii* also demonstrates a ring species pattern with the species spread along the mountain ranges of California around a central valley [74]. There is such extensive variation in *E. escscholtzii* that seven subspecies have been identified [75]. However unlike the Song Sparrow, sympatric subspecies of *E. escscholtzii* have limited or no hybridization and are at the species level of divergence [74].

As shown in both Song Sparrows and mathematical models, the partial geographic isolation between distant populations may not be sufficient to cause differentiation without additional ecological drivers [73, 76]. Furthermore, two of the main metapopulation theory models have contrasting approaches to gene flow between populations [77-81]. The first model stipulates that populations may be isolated, experiencing little exchange of genetic information with the populations around them and therefore genetic variation or allelic frequencies within each population may differ significantly. However, the second model has populations as highly connected, with a steady and uninterrupted gene flow between them resulting in significant and maintained genetic exchange, and greater similarity, between populations [77-80]. In the case of those species that are more separated, the differences between populations can be driven either by differences in selective pressure due to the heterogeneous nature of the environment in which different populations are found or through genetic drift or founder effects [82]. In addition to genetic connectivity, populations can also act either as sinks or sources for the overall population of the species, where a sink population is one where a net influx of individuals supports the population and a source population is one that is self sustained and has a net out flux of individuals; populations may change between either state over time [83].

Geneflow and selection at the core and periphery

A species range can be thought of using a model in which central core populations are found near the centre of the range and are surrounded by peripheral populations. Movement of individuals within the species as a whole can therefore be modelled as a unidirectional source-sink dispersal pattern. Individuals move from source populations outwards to the periphery until a point where geographic barriers or selective pressures, either through environmental extremes or interspecific competition, are too antagonistic for individuals to survive and limit the species' range [83-85].

A slightly more complex alternative model to this posits that there is an omnidirectional flux of individuals both from the core to the periphery and also back from the periphery to the core, with range limited again by selective pressures or geographic barriers that prevent establishment and range expansion beyond a certain limit [83-85]. These two models have profoundly different implications for the evolutionary history of a species. In the case of a central core acting as a constant source, gene flow is uniformly outward to the habitat of peripheral populations. These peripheral populations are likely to be subject to a variety of novel selective pressures yet novel alleles are swamped by an influx of alleles from the core source population and peripheral populations are therefore unable to adapt [84, 86, 87].

However, despite constant influx of genes from the same core, and with each peripheral population being genetically similar to the central core, peripheral populations may still be genetically distinct from each other if each has only received a subset of the genetic diversity from the central origin [84]. A recent study of European alpine plants found that in the majority of cases, larger central populations were the source of the majority of gene flow, and even of novel alleles, that were then carried out to the peripheral populations [88].

Under the second model of gene flow, where there is an omnidirectional flow between the core and the populations at the periphery of the range, adaptation gradients can occur where novel alleles can be maintained, and even exchanged back into central populations. This kind of omnidirectional gene flow has been observed between geographically disperse populations of Rocky Mountain Elk (*Cervus elaphus*) in North America [89]. Although there had not been sufficient time to observe the rise or effect of novel alleles, omnidirectional gene flow and genetic exchange was still observed between central and periphery populations, and also surprisingly between peripheral populations as well [89].

The novel environment encountered by peripheral populations can also result in rapid evolutionary responses due to accelerated selection processes affecting allelic frequencies, or novel alleles through mutation [90-93]. This rapid evolutionary response can act as an evolutionary rescue where populations that were rapidly declining due to environmental stress rebounded following mutation of adaptive novel alleles, as demonstrated in a yeast model in both single and metapopulations [93, 94]. Furthermore, a study of Trinidadian guppies found that gene flow from peripheral populations which had novel alleles could act as an evolutionary rescue for central populations [95]. The extent of the adaptive gradient that can arise depends on both the degree of variation in selective pressures, and the adaptive responses within populations themselves.

Selection at the range edge

It is currently unclear whether selection at the range edge is so strongly negative that it causes edge populations to become "sinks" sustained only by immigration from more central populations, if it drives diversification as peripheral populations adapt to novel environments, or possibly both [5]. Should the periphery be a selective sink then adaptation along the periphery is highly unlikely [5]. In the case of gene outflux from the core to the periphery, local adaptation of the periphery is reduced through gene swamping and thus the periphery is unable to expand outwards into decreasingly hospitable habitat [5]. In several species of *Anopheles* mosquito, the inability of the species to expand is thought to have been one of the major factors in limiting their range [96].

However, if there is adaptation along the periphery, then populations along the periphery may be a source of novel adaptations, and could play a role in adaptive rescue of the species [5, 95]. *Anopheles* mosquitoes also provide an example of how a mutation arising in a peripheral area, can be brought back into the core population, and then dispersed throughout the population, and even enhance geographic range [96, 97]. A recent study of the malaria mosquito *Anopheles gambiae* found that a chromosomal inversion had been brought back into the core population, and was adaptive for increased resilience to arid environments, allowing several populations to then expand into previously inhospitable terrain [97].

Although the status of a population as a source or sink can be determined indirectly through Fixation Index (F_{ST}) measures of genetic differentiation between populations [98-100], it is by examining the fitness response of individuals in a range of environments that possible adaptations can be identified [101, 102]. These examinations require examining multiple individuals from different populations through large dataset analysis or raising individuals in different environmental conditions in a common garden experiment. A large-scale examination of the spawning dates of the Common Frog (*Rana temporaria*) in Great Britain used more than 50,000 observations to demonstrate significant differences between the spawning dates of individual populations as a result of adaptation to local environmental conditions [101]. Additionally, a common garden experiment with the perennial plant *Arabidopsis lyrata* determined that most evidence for local adaptation was found among those populations from the range margins [102]. An examination of the selection pressures along range edges and within the core of the species range, and the impact this has on the morphological, physiological and genetic variation of populations in recently colonized habitat is crucial in improving our ability to understand and predict future dynamics of species ranges and geographic distributions [103].

Furthermore, controversy remains regarding which of the five current methods of determining the limit of a species' range is most appropriate [104, 105]. These are: 1) marginal occurrence, which uses species observations at the margins to delineate range limit; 2) habitat distributions, which assume that species range correlates with the geographic area of preferred habitat or environmental conditions; 3) range-wide occurrence, where the species range is extrapolated from the varying density of occurrence observations; 4) statistical modelling, which uses statistical probability models based on observation data to predict species distributions and 5) process-based modelling, which is rooted in the ecophysiological traits of a species that, in combination with environmental data, are used to predict the species' range.

However, these models fail to account for variation that can occur within a species across its range [60]. By treating a species as a homogenous unit rather than a mosaic of locally adapted populations, these models might not represent the full range of adaptations in the species [60, 105, 106]. Furthermore, and perhaps more alarming they may provide false predictions where cryptic biodiversity allows for a species to respond in a previously unsuspected manner [60, 105, 106]. An improved understanding of the extent of variation within a species across its geographic range, and differences between populations at the core and the periphery of the range, should improve the ability of these models to predict species' response to changes in climate, which is of especial importance in light of global climate change [107, 108].

Long term impacts of a large range

It has previously been suggested that species with large ranges should be less susceptible to climatic changes due to their geographic range encompassing a wide range of climatic conditions [109]. However, recent theoretical models would suggest that adaptions of populations to their local climate may make them more susceptible to extinction as a result of climatic change [58]. If populations are highly adapted to their local climate, and if the rate of change of local climate exceeds the ability of each population to track or adapt then populations may quickly find themselves in inhospitable climates, possibly resulting in extirpation of the species from an area, or even extinction of the species as a whole in extreme cases [58].

Several recent studies of anuran development have shown that geographically distant populations may have drastically different responses climatic or environmental factors including food availability and temperature [110, 111]. However, empirically determined levels of local adaptation across geographic distance within large-ranged species generally remain unknown [58]. This presents a significant weakness in current climate response models as local adaptation is likely to play a crucial role in predicting how species will respond to climatic change and track their climate space. This problem is exacerbated when considering that local adaptation may also be the result of both abiotic and biotic interactions. A study of the freshwater mussel *Unio pictorum* in the River Thames found that there were significant differences in morphology between different populations, despite a lack of genetic differences between these populations [112]. Across distance there were marked changes in shell elongation and the shape of the dorso-posterior margin, possibly as a result of differences between populations in the host species upon which larvae are obligate parasites [112].

Strong differences in the climate experienced by geographically distant populations may result in changes to intraspecific interactions[113]. Furthermore changes in the species community, composition and richness of different regions across the range may alter inter-specific interactions as well [113]. As a result, the varying selective pressures of these interactions may drive intraspecific variations, for example in sexual dimorphism and colouration, and have been observed in numerous species [114-117]. A large-scale study of bird species found that there were often significant changes in clutch size with both latitude and species richness for multiple species and across several taxa [114]. It has been suggested that these changes may be especially important in wide-ranging species where there is an increased importance of species recognition [118].

Much remains unknown with regards to how heterogeneity in biotic and abiotic factors across a large geographic range may drive selection within a species, and influence physiological, and morphological variation within and between populations. There is a demonstrated need for studies of species with large ranges, especially of species that show a high proclivity to be strongly dependent on environmental conditions [58]. If local adaptation is occurring in such a species then there should be trends in its morphology, or populations should show physiological adaptations to their respective local climate. Improving our understanding of how the impacts of heterogeneity in the environmental conditions encountered by populations across a species' range may affect selection at the local level is especially important in light of global climate change [58, 108] and in understanding the dynamics of invasive species [86, 90]. By comparing and contrasting the morphology and physiological responses to environmental conditions of populations in a species with an expansive geographic range, it should be possible to assess whether local adaptation has been occurring, and to what extent each population is truly representative of the entire species.

Model organism choice

There are several key aspects of amphibians that make them a valuable model system for examining the influence of the abiotic and biotic factors that underlie species range [103]. Amphibians are ectotherms, completely dependent on external temperature for thermoregulation and therefore should be highly sensitive to climate variation [103]. Furthermore, amphibian species in North America have undergone repeated events of range contraction and expansion over the last million years. During glacial expansion across most of northern North America, species were constrained primarily to southern refugia and it was only following subsequent retreat at the end of the Pleistocene that species ranges expanded northwards [16, 119]. Due to their relatively slow dispersal rate [120], it is even possible that some North American amphibian species have not yet reached the northern limit of their range [119]. This provides the opportunity to study expansion, possibly even as it is occurring. Furthermore, amphibian species can also have very large ranges. The American Toad (*Anaxyrus americanus*) has one of the largest latitudinal ranges of any North American species, with a range covering nearly half of North America, from Mississippi, north to the Hudson Bay in Northern Québec and Ontario and from the Eastern seaboard west across to Manitoba.

It is for these reasons that I selected the American Toad as my study species. The American Toad is a primarily terrestrial, insectivorous amphibian [121]. Furthermore, it has a biphasic lifecycle, with rapid development of tadpoles into metamorphosed adult forms. Adult American Toads range in size from 50-90mm on average, with females historically reported as being larger than males and maturity occurring normally after the first or second year of life [122]. Breeding occurs in the spring as soon as the ice has completely thawed, in semi-permanent water bodies, most commonly ponds [122]. As a result, toad reproductive timing is highly dependent on the external temperature and spring climate in which they are found [122].

Males exploit two methods of breeding behaviour, firstly by calling where they remain stationary and use vocal cues to attract females [123, 124]. The second method is through a scrambling and grappling behaviour where males will actively patrol the periphery of the pond, or swim around calling males [123, 125]. As females approach the pond, drawn by environmental or calling cues, they must first pass through this gauntlet of scrambling males if they are to reach one of the calling males [123, 125]. These two breeding methods should select for different traits, although individual males have been observed switching between both [125]. Whether drawing in a female through calling, or intercepting a female *en route* to the breeding pond, mating occurs during amplexus when the male mounts and grips the female throughout the entirety of egg-laying [122]. Fertilization is external with each female producing a long chain of 4000-8000 eggs in a gel membrane [122].

Following amplexus, tadpoles hatch within 7-10 days in most conditions [122]. Tadpoles are black in colour and tend to aggregate in shallow waters where temperatures are warmest and complete metamorphosis approximately 30 days after hatching although this is highly dependent on temperature [122]. Toads are hardy, and are able to withstand near freezing temperatures down to 2°C, however their tolerance may extend beyond this and have not been exhaustively tested [122]. To avoid freezing temperatures, toads overwinter by burrowing rearwards to below the frost line, with burrow depth depending on the severity of the cold [122]. This could potential allow them to inhabit any northern area without permafrost. The southern section of the American Toad range overlaps the ranges of several competing toad species including Woodhouse's Toad (*Anaxyrus woodhousii*), Fowler's Toad (*Anaxyrus fowleri*) and the Southern Toad (*Anaxyrus terrestris*), whereas in the northwest it encounters the Canadian Toad (*Anaxyrus hemiophrys*), which appears to act as a biotic limit for western expansion [126, 127]. However, a large part of the northern range of the American Toad is vacant of closely related species, and very sparse even in regards to other amphibian species. This provides the opportunity to examine abiotic drivers of range expansion with a significantly decreased confounding effect from competitive interactions. The American Toad therefore allows for the examination of abiotic and biotic limitations and drivers of species range.

Limb length has been used as a measure of dispersal capability in Cane Toads (*Bufo marinus*), with longer limbs allowing for individuals to travel greater distance and have a higher dispersal capability [128]. Consequently, this allows for selection for higher dispersal capability to be assessed through morphological variation [128, 129]. The

American Toad has also been observed to express ontogenetic dichromatism with males being yellower than females and dimorphism with females being larger in some populations [122]. However, the extent and generality of sexual dimorphism across the entire species is currently unknown.

When examining ectotherms as a group, there should be patterns in the range expansion of species with similar thermal tolerances following glacial retreat at the end of the Pleistocene. Generalities derived from species that have undergone phylogeographic studies should also help to elucidate areas where post-glacial secondary contact may have occurred in other species and generate hypotheses for species that have not been under scientific scrutiny. Furthermore, with the American Toad as a model, it should be possible to test whether there has been variation in the morphological traits between different populations and whether this variation follows latitudinal or environmental trends. Given how morphology seems to relate to multiple life history attributes of the American Toad, these findings could be indicative of local adaptation, and the influence of variation in selective pressures across the range. Additionally, using tadpoles from different populations, the physiological response to different thermal regimes should provide evidence as to the degree of adaptation within each population to their respective local climates. Such information may offer novel insights into the dynamics and drivers of range expansion and the extent to which generalizations based on a single population can be applied to a species across its entire range.

Chapter 2: Revision of COSEWIC's Terrestrial Amphibians and Reptiles Faunal Provinces Map

Linking Statement for Chapter 2:

This chapter is the justification to the update of the COSEWIC Terrestrial Amphibians and Reptiles Faunal Provinces Map. The existing map, COSEWIC's Terrestrial Amphibians and Reptiles Faunal Provinces map is a valuable tool for recognizing designable units within species including amphibian and reptiles. However, it was devised in 2003 and was principally based on congruencies in the geographic distribution of species of reptiles and amphibians. This update uses a robust statistical method for determining province boundaries as well as congruencies in the phylogeographic dispersal patterns of species following glacial retreat. This update establishes a firm basis for COSEWIC's use of the map for recognizing designatable units for assessment by providing a more complete biotic assessment of congruency.

The references in the following chapter have been renumbered and are included in the combined bibliography at the end of this thesis in numerical order following the sequence in which they appear throughout the entire thesis. This chapter has been formatted for submission to COSEWIC as a manuscript co-authored with Dr. David M. Green.

Introduction

To conserve and protect biological diversity, it is necessary to be able to assess and identify the conservation status of species [130]. Within this context, designable units (DU) have become a cornerstone of conservation and ecological planning[130]. Furthermore, a clear and justifiable map of species range and distribution patterns is a key component of defining and determining the geographic regions of a designable unit [130]. In light of contemporary climate change, these maps must also be predictive, being usable to create hypotheses about how species will respond to changes in climate.

The existing map, COSEWIC's Terrestrial Amphibians and Reptiles Faunal Provinces map (Fig. 1) has proven to be a valuable tool for recognizing designable units within these species of small, primarily terrestrial animals with limited dispersal capabilities. However, this map was devised in 2003 and was principally based on congruencies in the geographic distribution of species of reptiles and amphibians. The underlying principles were based on the biogeographic concepts of the postglacial dispersal of these animals following the Wisconsinan glacial maximum ca. 12,000 years ago, when almost all of Canada was glaciated. As amphibians and reptiles are not highly vagile, their postglacial range expansion northward follows along a limited number of corridors between and around a succession of physiogeographic barriers. Combined with climate, the limited number of corridors resulted in a pattern of distinct "biotic provinces" of species distributions. However, while geographic ranges may appear very similar between species, geographic genetic subdivisions within the species, such as recently discerned genetic subdivisions in chorus frogs [131], may remain elusive to detection using the current map. This is further exacerbated by recent work that confirms that there are significant and recoverable patterns of discontinuity between lineages of amphibians and reptiles in previously glaciated Canada [132, 133].

Herein, I present an update to COSEWIC's Terrestrial Amphibians and Reptiles Faunal Provinces map that would provide a basis for identifying zones of genetic diversity and biotic provinces of high congruency. This update establishes a firm basis for COSEWIC's use of the map for recognizing designatable units for assessment by providing a more complete biotic assessment of congruency through species distribution as well as climatic and biotic factors.

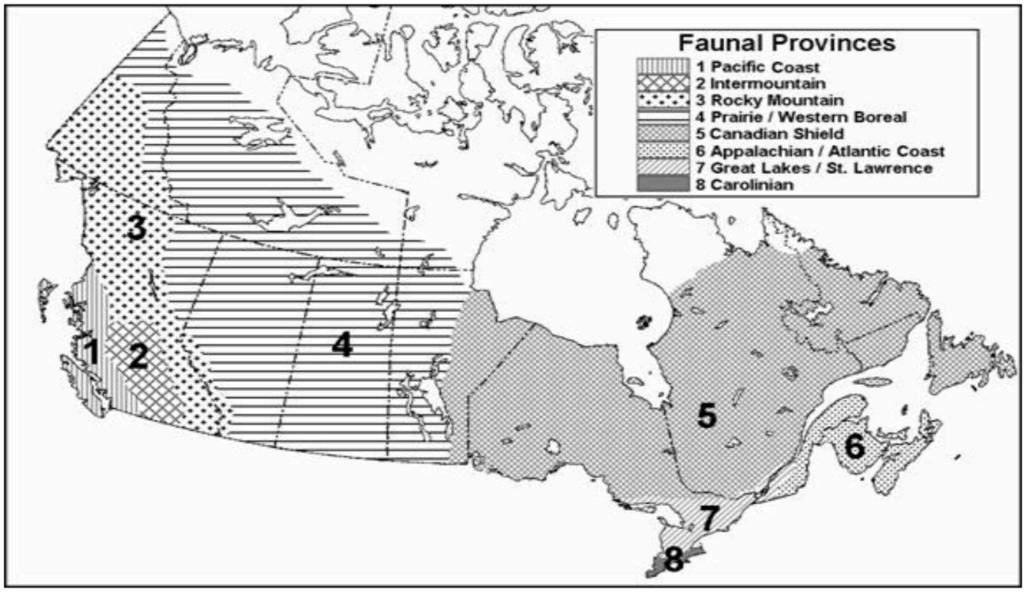


Figure 1: The previous COSEWIC Terrestrial Amphibians and Reptiles Faunal Provinces Map. As taken from Fig. 3, Appendix F5, O&P Manual page 278.

Methods

The work in creating this map was divided into three key steps: firstly, a systematic review was performed. The first systematic search used the following search parameters:

(phyloge* AND (Americ* OR Canad*) AND (Herto* OR Repti* OR Anur* OR Frog OR Toad OR lizard OR snake OR turtle OR terrapin OR tortoise)). Following this initial search, 1375 articles were found. These were sorted for relevance looking for those that pertained to amphibian or reptile species from Canada. Following this secondary selection process there remained 940 papers. These papers were examined and any possible phylogeographic maps were extracted. Of the 56 Canadian herp-species, including 29 amphibian species, and 27 reptile species, only 20 amphibian, and 16 reptile species have had phylogeographies performed [25, 126, 130-167]. When available phylogeographies had been used; maps for missing species were gathered from field guides, COSEWIC range reports, IUCN, AmphibiaWeb and species presence/absence reports. Following collection of species range and phylogeographic clade maps; climate maps, as well the eco-provinces map of Canada were collected.

Following data collection, maps were imported into ArcGIS and converted into .shp files at a resolution of at least 1:50,000. The species ranges were created using GIS technologies in ESRI's ArcMap 10.1 program. The range of each species was imported into ArcMap and georeferenced to the HydroSHEDS map of North America [168]. HydroSHEDS is a global spatial dataset of river/lake catchment units. It is widely accepted that the river/lake catchment is the most appropriate management unit for inland waters. Organizations such as the IUCN, WWF, and BioFresh use the HydroSHEDS system for their species assessments. After georeferencing, the exact range of each species was created using HydroSHEDS units and exported as an individual map. Using a common UTC coordinate system, all maps could be over-laid to improve and facilitate congruency analysis and determining the geographic delineation of borders between herpetofaunal provinces. Once range maps for each species had been created I used the occupancy data for each individual species to characterize the community assemblage of every hydroSHED unit across Canada.

I then used the hclust package in R to run a cluster analysis with no *a priori* assumption to group HydroSHED units based on community composition into the requisite number of provinces. I then performed a second cluster analysis using the divisions of the CEC Level 1 Ecoregions grouping as an *a priori* assumption for region division. I initially grouped hydroSHED units within each of the CEC Level 1 Ecoregions and analyzed each Ecoregion separately, dividing them into 3 or 5 regions depending on the complexity of the cluster analysis found within. I used these regions to create a second level map that combined the Level 1 divisions with the cluster analysis of the herpetofaunal communities. For both maps, I fine-tuned region boundaries using known phylogenetic and dispersal patterns. The maps produced without and with *a priori* division were highly similar, but the *a priori* division created a map that was more defendable and easier to apply in conservation efforts. The *a priori* assumption of region division was therefore used to produce the final map. These methods provide a statistically sound framework from which generalities of distribution patterns among and between taxa can be assessed and inferences about unknown species can be drawn.

Results

The final Amphibian and Reptile Faunal Provinces map that was produced using the *a priori* divisions of the CEC Level 1 Ecoregions map resulted in 12 provinces (Fig. 2).

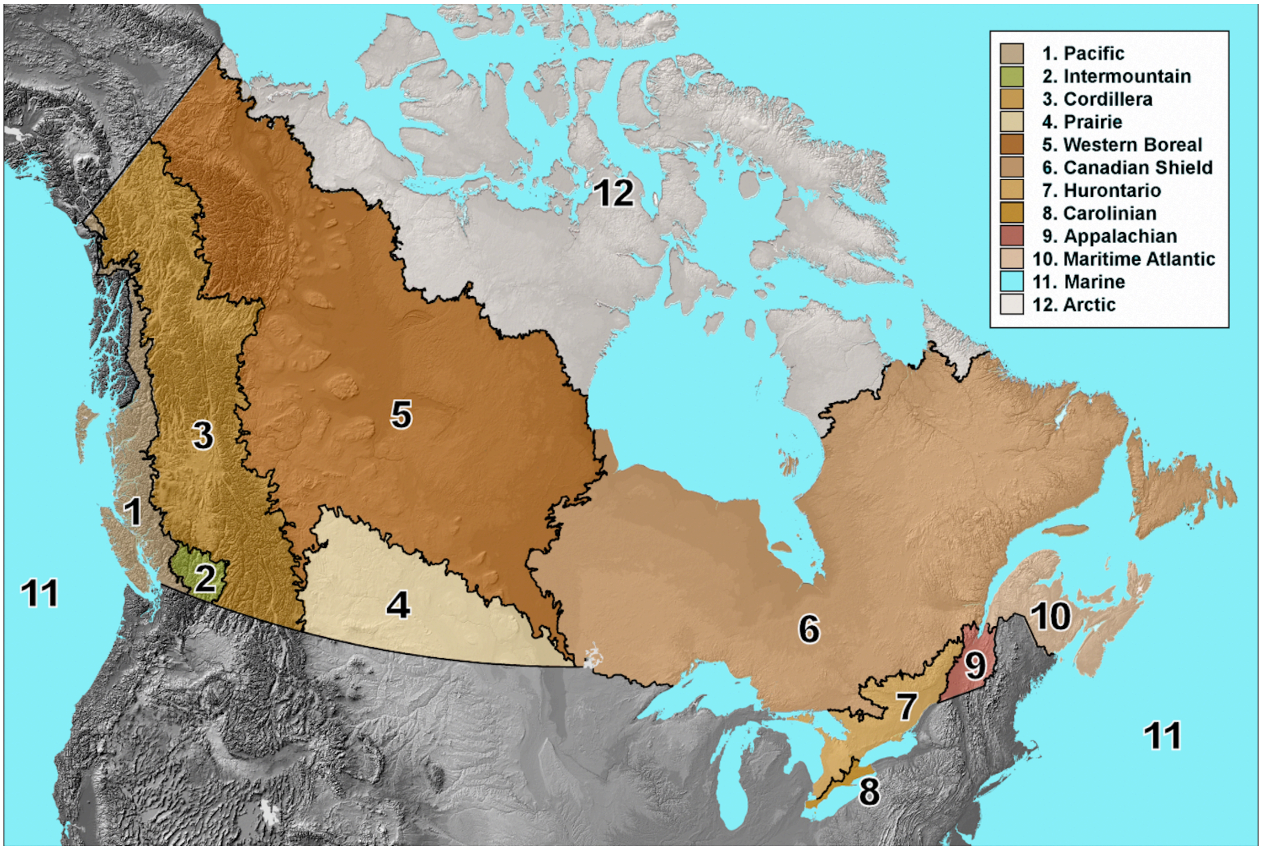


Figure 2: The Amphibian and Reptile Faunal Provinces Map of Canada showing the 12 faunal provinces as determined through analysis of the range and distribution of all Canadian endemic amphibian and reptile species, and phylogeographic patterns when these were available.

Phylogeographic Justification of Ecoprovince Distribution

Along an evolutionary and geological time scale current species distributions across eastern North America are a very modern occurrence, and these distributions are undergoing constant long-term change. In recent geological time, the Earth has undergone multiple ice-age events, resulting in the glaciation of large parts of the Earth's surface. The most recent of these occurred during the Pleistocene, with glaciers covering much of North America as recently at 10,000 years ago [169, 170]. Glaciers over several kilometers thick rendered the majority of northern North America completely inhospitable for amphibian species, which were confined to southern, unglaciated refuges [169]. It was only as the glaciers retreated that species were able to move northward into their modern distributions [169].

There has been an ever-increasing interest in phylogeography and accumulating phylogeographic data. However, there remains a lack of understanding of the general trends in amphibian phylogeography, particularly in northern North America. The most recent review of amphibian phylogeography was performed by Zeisset *et al*. in 2008 [16] who examined overall global phylogeographic trends but did not focus on North America. The most recent review of phylogeographic trends in eastern North America was that of Soltis *et al.* in 2006 [11]. However, their review focused on the unglaciated regions of eastern North America and looked at species other than just amphibians including plants and animals. Although Rissler *et al.* [171] reviewed geographic patterns in amphibian distributions across North America in 2010, their focus was on identifying hotspots of amphibian diversity and not on elucidating trends in past dispersal. For western North America, the most recent review was in 2010 by Shafer *et al.* However, Shafer *et al.* used both plant and animal species and did not concentrate on just herpetofauna [132]. Herein, I present the findings of phylogeographic studies from across North America as they pertain to the patterns found in the included map of Canadian Herpetofaunal Provinces.

Intraspecific variation, combined with geographic scale elucidates patterns in the history of a species and the species' dispersal and radiation events [172]. Although mitochondrial DNA assessment was the dominant method for performing phylogeographic work [11], allozyme analysis is also been used and nuclear DNA from next-gen sequencing is being used to a greater extent [173-176]. Furthermore, the predominant method of determining the statistical significance of phylogeographic inferences was through Nested Clade Phylogeographic Analysis (NCPA). However, studies have shown that even updated methods of NCPA result in very high rates of false positives of up to 75% [177-179]. Petit [180] identified many processes that affect the local haplotype frequency as causing problems in NCPA and it has become increasingly marginalized. Two alternatives are Analysis of molecular variance (AMOVA), and Bayesian statistics, such as Bayesian stochastic search variable selection [181-183]. As a result of the weaknesses in NCPA, and the uncertainty as to the validity of NCPA findings, I have therefore focussed on those studies which did not use NCPA in the determination of phylogeographic trends.

Eastern North America, Areas that were Unglaciated in the Pleistocene

The key to the initial dispersal of species following glacial retreat in the distribution of those species within southern refuge regions [169]. Across unglaciated eastern North America, genetically distinct clades within a species are separated by one of the following geographic structures: the Mississippi River, the Apalachicola River, and the Appalachian Mountains [11]. In examining nine caudate and four anuran species, many of the anurans had east-west distinctions between clades with species divided by the Mississippi [137, 149, 153, 184] however, there were sufficient exceptions that this could not be classified as a general trend (Fig. 3). Of the four species examined, *Lithobates pipiens, Lithobates catesbeianus,* and the *Pseudacris 'nigrita'* clade were described as having the Mississippi as the primary geographic barrier between clades and *Pseudacris crucifer* was described as being divided by both the Mississippi and the Appalachian Mountain range. Assuming an

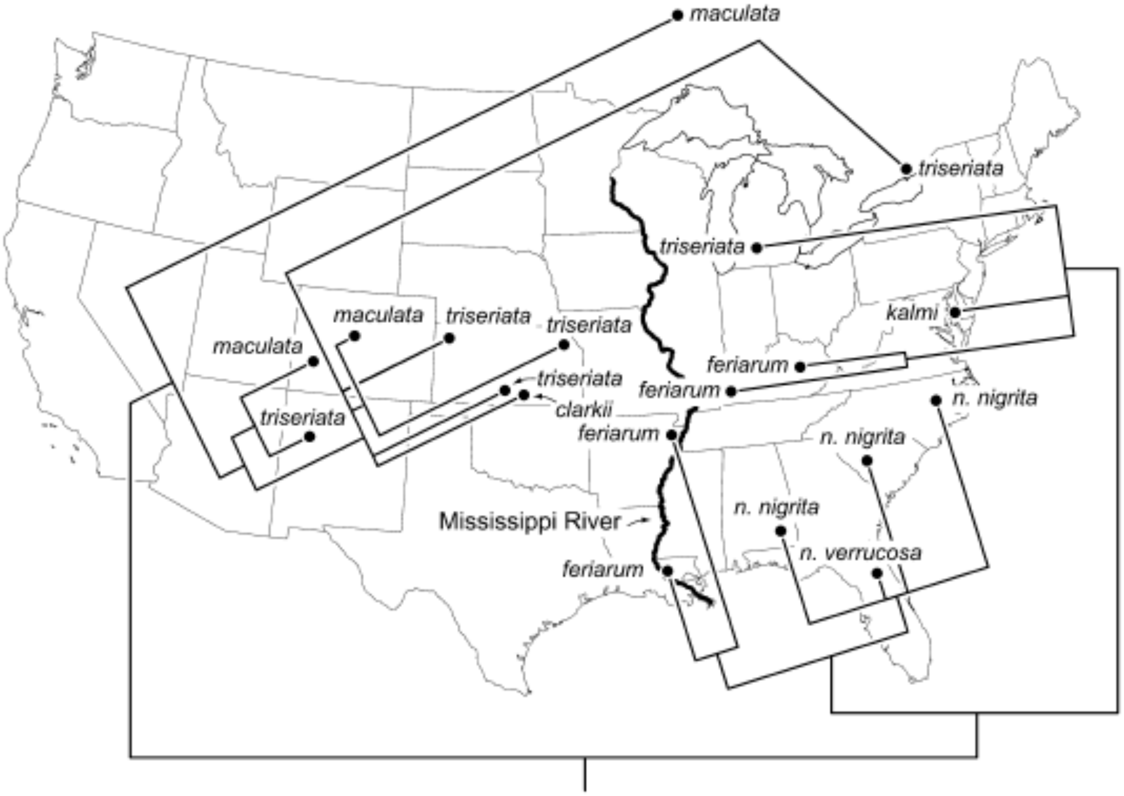


Figure 3: From Moriarty and Cannatella (2004) phylogeographic distribution of mtDNA clades within the *Pseudacris* chorus frogs of North America.

equal chance of having the Mississippi, the Appalachian Mountains, or both acting as geographic barriers, the apparent bias towards the Mississippi acting as a geographic barrier could have resulted from random chance (Chi-squared test: χ^2=3.375, d.f.= 3, p =0.185). Of the four species examined, only the *Pseudacris 'nigrita' clade* was divided by the Mississippi without interruption, and even then the western clade had been subdivided into 2 subclades [153] (Fig. 3). In the anuran *Lithobates catesbeianus,* although it is divided by the Mississippi River with two clades in the western- and three clades in the eastern-lineage, there was such overlap between the two lineages in the areas around the Mississippi River that it is likely that the western lineage arose from the eastern through range expansion and that the western haplotype arose from small numbers of individuals that had been isolated in refuge areas in the Gulf Coastal Plain (Fig. 4). Similar results were found in the distribution of Spring Peepers (*Pseudcris crucifer*), although they were divided

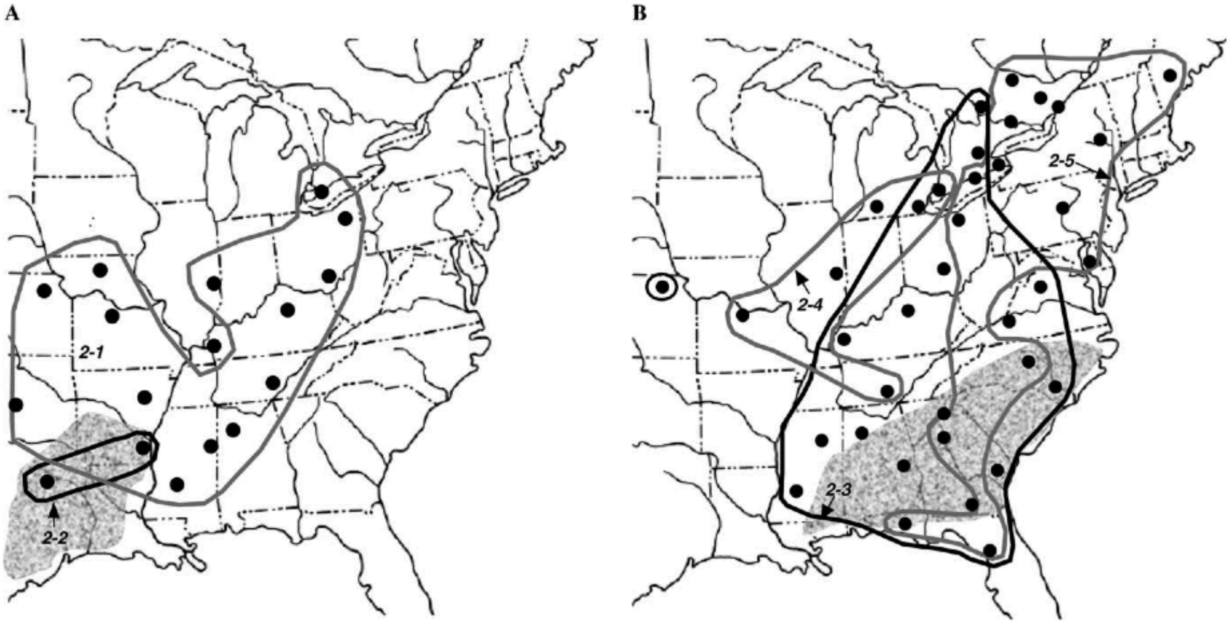


Figure 4: Taken from Austin *et al*. (2004). Geographic of *Lithobates catebeianus* clades as determined by nesting procedures from Templeton *et al.* (1987). Showing (A) the western group and (B) the eastern group. Grey areas represent major Pleistocene refuge areas.

by both the Mississippi River and the Appalachian Mountains [25, 137] (Fig. 5). For *P. crucifer* it was suggested that the current distribution arose from previous allopatric differentiation [137] and that the current populations arose from refuges in the Ozark Highlands and multiple southern Appalachian refugia [137]. Combined, these findings suggest that while the Mississippi and the Appalachians may reduce gene flow between populations, current haplotype divisions and distributions likely result from previous divisions into refugia [137].

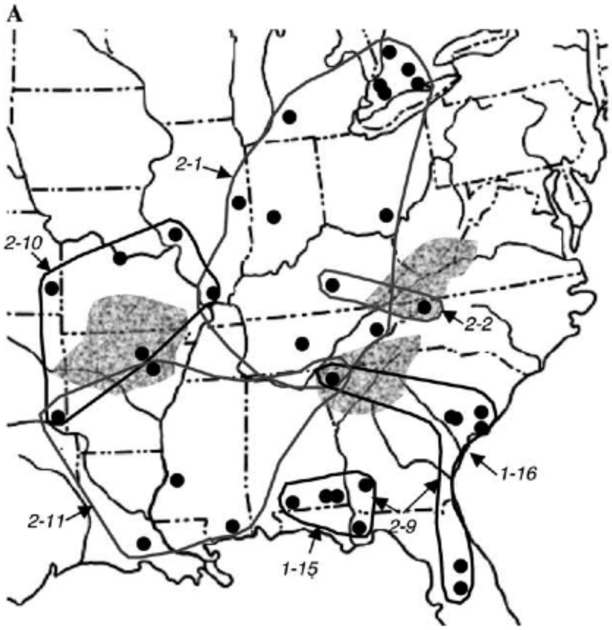


Figure 5: Taken from Austin *et al*. (2004). Geographic distribution of *Pseudacris crucifer* clades in the south eastern North America. Grey areas represent major Pleistocene refuge areas.

In contrast, salamanders show a distinctively different pattern of geographic division than the anurans. For salamanders, the primary barriers between east and west clades appear to be the Appalachian Mountains, and the Apalachicola River, not the Mississippi [185-191]. With eight species examined, five of which are divided by the Apalachicola River and the Appalachian Mountain, this pattern of distributions appears to differ significantly from random (Chi-squared test: χ^2=9.00, d.f.= 3, p =0.0293).

The phylogeographic patterns of the Tiger Salamander, *Ambystoma tigrinum,* demonstrate the possible barrier effect of the Appalachian mountains, with one main clade to the east and one west mountains [186] (Fig. 6). When considering the eastern clade of *A. tigrinum*, two refuges were present during the Pleistocene glaciation, one in the Blue Ridge Mountains and the second in the mid-Atlantic Coastal Plain [186]. Church *et al.* [186] suggest that post-glacial range expansion northward appears to have been entirely composed of emigrants from the coastal plain refuge, with the population in the mountains remaining isolated. Furthermore, the separation of the eastern and western clades, appears to have risen 0.75-2.1 million years ago, much before the Pleistocene glaciation [186]. Moreover, the main geographic barrier between these clades was likely the Apalachicola River basin, and not the Appalachian Mountains [186].

Unlike the Mississippi, which appears to be a transient barrier for anuran species, the Apalachicola appears to have had acted as a strong barrier to gene flow for salamander species. [174] suggest that the barrier effect of the Apalachicola River for the Flatwoods Salamander (*Ambystoma cingulatum*) resulted from repeated marine embayments during the Pliocene and Pleistocene interglacial periods.

Figure 6: Taken from Soltis *et al* (2006). Clade distribution of *Amybstoma tigrinum* demonstrating division due to the Appalachian Mountains. (Redrawn from Crown *et al.,* 2003)

Similarly, ancient changes in drainage patterns appear to have resulted in the allopatric fragmentation of the Northern Two-Lined Salamander (*Eurycea bislineata*), [190] (Fig. 7). While these haplotypes were pushed southern into refugia, as the glaciers retreated, the northern populations were able to expand their range northward. However, southern populations were restricted in their expansion by the presence of northern populations [190]. For the *Eurycea multiplicata* complex, (the Many-Ribbed, Oklahoma, Graybelly and Grotto salamanders) distributions are arranged along the Ozark Plateau-Ouachita mountains [188]. Bonet and Chippindale [188] found that for the *Eurycea multiplicata* complex, local habitat plays a stronger role in driving genetic differentiation between clades than geographic barriers. Crespi *et al.* [187] found that pygmy salamander (*Desmognaths wrighti*) haplotypes were divided into 4 genetically distinct clusters in the southern Appalachians. However, this arrangement of *D. wrighti* appears to be based on a much earlier separation and populations were unable to expand and reconnect during the Pleistocene [187]. For salamander species, general trends appear to be the result of a geographic barrier effect of the Appalachian Mountains and the Apalachicola River reducing gene flow between populations. Exceptions to this trend appear to arise in species that either respond rapidly to local conditions, or have undergone ancient separations that were not erased by modern geographic barriers.

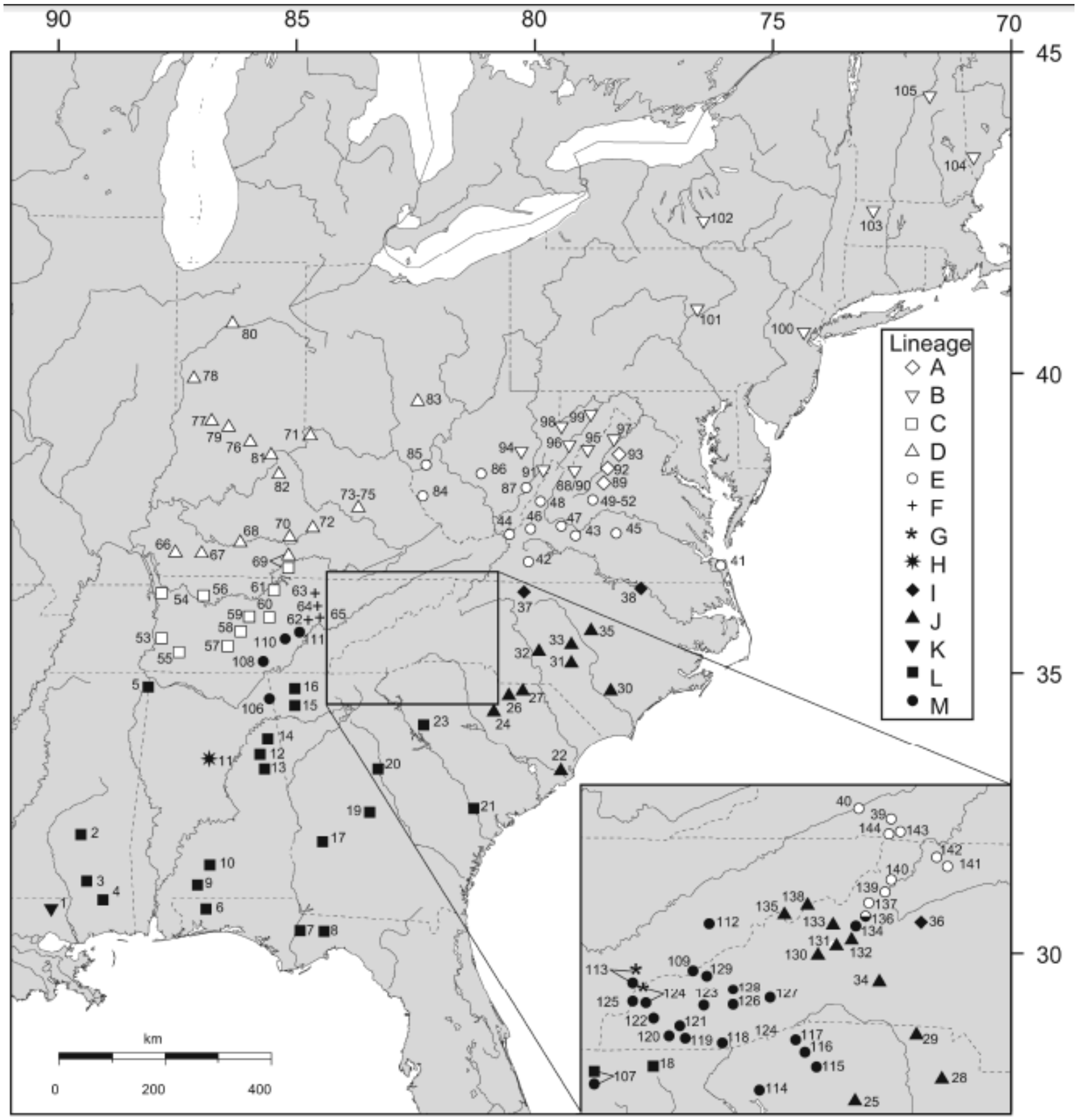


Figure 7: From Kozak *et al*. (2006). Geographic distribution of *Eurycea bislineata*-complex sampling locations. Open and shaded symbols represent the major clades and haplotype to which sampled individuals belong.

Eastern Canada, Areas Affected by Glaciation in the Pleistocene

During the Pleistocene glaciation the majority of Canada's surface area was glaciated, meaning that modern distributions of Canadian herpetofauna are all the result of post-glacial colonization events from southern refuges in modern Texas, Louisiana, Mississippi, Alabama, Georgia and Florida [170]. During these range expansions there were significant geographic barriers that largely determined the paths of dispersal and range expansion. In eastern North America, these barriers were the Mississippi River and the Appalachian Mountains, for east-west movement, and the Ohio River and the Great Lakes for north-south movement [11] (Fig. 8). However, studies of anuran species, including *Lithobates catesbeianus, Pseudacris crucifer,* and *Lithobates pipiens*, have demonstrated that the effect of geographic features predates the Pleistocene [25, 137, 184]. For these species, there are distinct east-west clade divisions that result from allopatric separation in the Pliocene nearly 2 million years ago [137, 184]. The east-west clade divisions did not arise post-glaciation due to the reduced gene flow across the Mississippi or the Appalachians. For these species, the Great Lakes presented a significant barrier to dispersal with populations forced to move around them. The Great Lake barrier was crossed through one or more of multiple dispersal pathways: in the east, through the Maritimes; by swinging westward around the lakes; from the Midwest, via the Lake Erie land bridge; or through the Niagara escarpment during the development of the Great Lakes. Once beyond the Great lakes however, there were no significant geographic barriers to east-west dispersal. Populations that had swung either through the Maritimes, or westward around the lakes were now able to turn southward and expand into southern Ontario and Québec where east and west clades made secondary post-glacial contact [137, 184]. As a result of this secondary contact the areas of highest genetic diversity for multiple eastern species is in areas of Ontario and Québec [137, 184]. This secondary contact, and high diversity is reflected in the number and density of herpetofaunal provinces in southern Ontario.

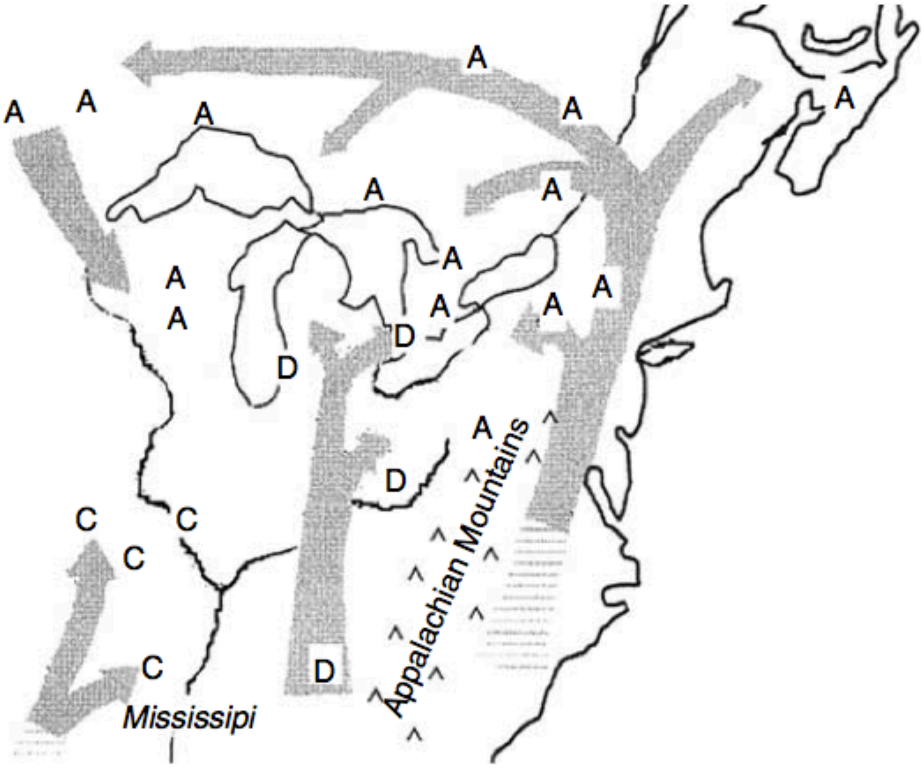


Figure 8: Taken from Zeigget and Beebee (2008), and based on Austin, *et al.* (2002). Proposed post-glacial colonization patterns for three clades of *Pseudacris crucifer*: (A, C, and D) Northern, Western and Eastern respectively. Note the distribution of clades with respect to the geological features of the Appalachian Mountains, the Mississippi, and the Great Lakes. This dispersal pattern is similar to that of *Lithobates catesbeianus.*

The eastern lineages in some species have a higher genetic diversity compared to the western lineages; however, the eastern lineages likely arose from migrants crossing the Mississippi from ancestral western populations [137, 184]. The current genetic variation of the western population seems to result from climatic variation, including glaciation events, that led to bottleneck events with rapid population decreases and loss of genetic variation [16]. The decrease in variation in western lineages could also be caused by rapid range expansion in the west from refuge areas following previous glaciation events [184]. Hoffman and Blouin [184] found that intraspecific genetic distance in *L. pipiens* was greater between eastern and western clades than the interspecific distance in some recognized species of ranid frogs. In a study of cricket frogs (Genus: *Acris*), Gamble *et al.* [192] found that the Ohio and Mississippi rivers acted as north-south and east-west barriers respectively, to the point where populations on the north-west side underwent a speciation event, with the genetically distinct *Acris blanchardi* arising from *Acris crepitans* following dispersal north-westward.

Although many species demonstrate east-west trends in genetic isolation, some species demonstrate north-south patterns. For the Fowler's Toad (*Anaxyrus fowleri*) three major clades: North, South and Central were found that could have arisen when northern populations were founded by individuals moving out of southern refuges as the glaciers retreated [126]. While the North and South clades of *A. fowleri* are highly distinct, the Central clade may be the result of hybridization or mtDNA introgression with *A. americanus*, and not indicative of dispersal or isolation; a challenge which will be discussed in greater detail below [126].

Western North America, Areas that were Unglaciated in the Pleistocene

Two major refuges existed in Northwestern North America during recent glacial events. Both Beringia, in modern Alaska, and the Pacific Northwest were refuge areas for

Canadian flora and fauna (Fig. 9). However herpetofauna was mostly restricted to the Pacific Northwest refuges south of the present Canadian-American border, although there is evidence that some species used the Haida Gwaii or Alexander Archipelago as well [147]. Species in this refuge were predominantly divided east-west by the Rocky mountains into two areas, the northern Rockies and the Cascade/Coast Range. Although each of these areas has undergone revisions to include north-south and east-west divisions into smaller identifiable refugia, this has only been in species other than herpetofauna. It would also appear that, similar to species in eastern North America, the geological divisions between clades greatly predate the Pleistocene glaciation [20, 193, 194]. While the predominant refugia are identified as being these two regions to the east and west of the Rocky Mountains, several herpetofaunal species have been identified as having specific refuge locations. Within the Cascade/Coast range, there is the Columbia River area which was a refuge for the Oregon Salamander, *Plethodon larselli;* and the Klamath-Siskiyou Mountains for the Coastal Tailed Frog, *Ascaphus truei* [195, 196]; and Rough Skinned Newt, *Taricha granulosa* [197].

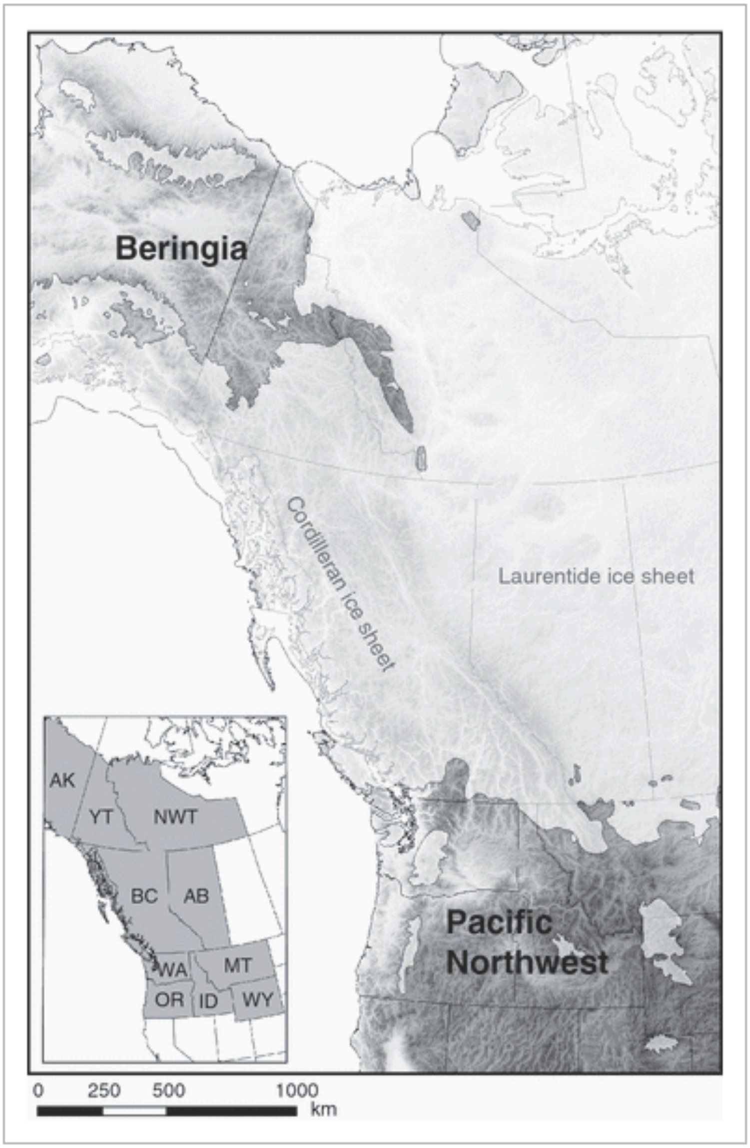


Figure 9: From Schafer *et al.* 2010. Shows the extent of glaciation across western North America and identifies the two main refuges, the Pacific Northwest and the Beringia

Western North America, Areas Glaciated in the Pleistocene

Although throughout the Prairies, species were able to simply expand northward relatively uninhibited by geological boundaries, species closer to the coast were less fortunate. Post-glacial expansion in western Canada followed a similar pattern to the original southern refugia, with species divided primarily by the Rocky Mountains (Fig. 10) [132]. However, following expansion there do appear to be significant north-south genetic differentiation in a number of species, for example *Dicamptodon aterimus/tenebrosi/copei* complex and *Plethodon vandykei* complex show an east-west split between the Cascades/Coast and the Rocky Mountains, with the Cascades/Coast then being further segregated into north south clades [20, 21, 198]. Similar divisions into north-south clades have been seen in *Ascapheus truei* which shows divisions of north coast, midcoast, and south coast which are each distinct, and in turn distinct from the interior population to the east of the Rocky Mountains [196]. The complex effects of the valley and highland areas of the Rocky Mountains and the Pacific coast are demonstrated in the map through the numerous and more complex patterns of herpetofaunal provinces found in this region.

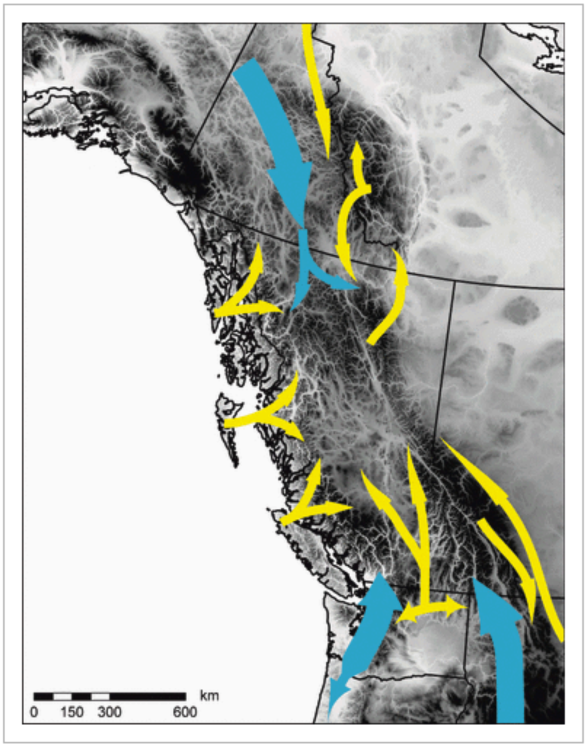


Figure 10: From Shafer *et al.* (2010) Shows the major recolonization routes of Western Canada post-glacial retreat. Major routes are marked in blue while minor routes are marked in yellow.

Conclusion

Herein, I used species ranges and phylogeographic patterns, for those species that have been studied, to create a robust and concise map of herpetofaunal provinces across Canada. However, there remains a lack of phylogeographic data for multiple Canadian species, especially in Northern Canada. While phylogeography has identified several patterns in post-glacial dispersal and distribution, the generality of these patterns across a wide range of amphibian species must be assessed. The post-glacial secondary contact between previously separated lineages, has resulted in complex patterns of species distribution across southern Canada, especially in Ontario and Québec. This map should provide a solid foundation through which to direct conservation efforts and identify areas of high conservation need, or of unique species assemblages.

Chapter 3: A simulation model of mitochondrial vs. nuclear discordance across the hybrid zone between two closely related species

Linking Statement for Chapter 3:

This chapter is an individual-based simulation model of hybridization and DNA introgression between two closely related and interbreeding species. The model was aimed at exploring the role that breeding behaviour and habitat selective pressures play on driving introgression of one species' nuclear or mitochondrial DNA into the range of the second species or on preserving the genetic differences between the two species. The hybridization between the American Toad and the Canadian Toad (*Anaxyrus hemiophrys*) was used as a case study to examine the role of behaviour in hybridization. The similar life history and differences in the breeding behaviour of these two species allows for the examination of how behaviour acts as a driver of hybridization and genetic introgression across the hybrid zone. This study should help to elucidate some of the drivers of hybridization and introgression and how these factors then influence species range boundaries.

The references in the following chapter have been renumbered and are included in the combined bibliography at the end of this thesis in numerical order following the sequence in which they appear throughout the entire thesis. This chapter has not yet been formatted for submission as a manuscript

Introduction

Interspecific hybridization, traditionally thought of as rare, has been increasingly shown to be a common occurrence between closely related taxa in multiple species groups. There have been recordings of hybrids in up to 25% of plant species, 6% of mammals [199, 200] and over 16% of avian species [201]. Amphibian species also show a remarkable proclivity for hybridization [202]. Hybridization events occur in 'hybrid zones' where the geographic ranges of species come into contact, or overlap and identifiable hybrids can be found [203]. Hybrid zones tend to be fairly limited in geographic scope. They are limited to narrow regions of species range overlap [204], or where significant decreases in hybrid fitness compared to parental types or hybrid sterility limits the areas where populations of hybrids are maintained [205, 206].

Although limited in size, hybrid zones can shift geographically, either by increased competition of one parental species over another, changing fitness landscapes, or through random drift [127, 200, 204]. These movements are often unidirectional [200, 204]. However, oscillations in the hybrid zone location can occur as exemplified in a recent observation of the hybrid zone between the American (*Anaxyrus americanus*) and Canadian Toad (*Anaxyrus hemiophrys*) [127]. This hybrid zone moved west by 38 km over 19 years and then east by 10km over the following 29 years [127].

In addition to creating visible hybrids that are combinations of both parental species in appearance, behaviour and/or fitness, hybridization can also have significant implications on the evolutionary trajectory of a species and can increase extinction risk in many cases [207]. Firstly, hybridization can result in introgression of nuclear DNA (DNA) and mitochondrial (mtDNA) deep into the range of either species, and individuals may appear to be pheno- or genotypically one species but have the mtDNA of another [208]. This discordance, when the DNA and the mtDNA of an individual do not match, can cause significant problems when attempting to identify the species of an individual or in the use of DNA barcoding techniques, when DNA barcoding may completely misidentify individuals [209, 210].

Even when there is very little range overlap between two species, this discordance can extend deep into the geographic range, drastically beyond the hybrid zone [208]. Introgression of genetic information is not limited to mtDNA and may extend to DNA, as demonstrated in the unidirectional influx of Polar Bear (*Ursus maritimus*) DNA into populations of Brown Bear (*Ursos arctos*) on the Admiralty, Baranof and Chichagof Islands near Alaska [211].

Although hybridization has been argued as a means of increasing diversity [207], there is a significant risk of species extinction; through outbreeding depression, where hybrids reduce overall fitness; through demographic swamping, where reproductive effort is lost in inferior hybrids and the species drops below the level required for replacement; or genetic assimilation, as hybrids displace the pure parental species [202, 212]. Conservation efforts may also be impeded by hybridization. In the European Brown Bear, introgression across a contact zone between two previously separated clades raised questions about the validity of a designated unit found therein [213]. *Arygyranthemum coronopifilum* was a rare plant species found on the Tenerife islands until roads caused a degradation of the barrier between *A. coronopifilum* and other *A. spp* at which point 3 of the 7 remaining populations were replaced with mixtures of hybrids and an invasive cogener with *A. coronopifilum* having been completely extirpated [214]. In North America, one of the rarest anuran species, the Florida Bog frog (*Lithobates okaloosae*), now shows evidence of hybridization with the much more widespread Green Frog (*Lithobates clamitans*) [215]. Recent invasions by the Woodhouse's Toad (*Anaxyrus woodhousii*) have resulted in hybridization with the native Arizona Toads (*Anaxyrus mircoscaphus*) over the last 30 years [216].

Multiple cases of genetic introgression have been found in the American Toad well beyond the hybrid zones with multiple other closely related conspecifics [127, 163, 208]. The proclivity for hybridization, and the overlap in breeding times between American Toads and closely related sympatrics [122] provide an interesting study system within which to examine the role that breeding behaviour plays in hybridization. Many anuran species, including both the Canadian and Fowler's Toads, use a stationary calling behaviour where sexual selection based on call structure results in sympatric speciation even if morphology and breeding timing are almost identical [217-219].

Although song structure can be a significant barrier to interbreeding, the song structure of hybrids can overlap the signal space of parental species, as observed in two sympatric Poison Frog species (*Oophaga histrionica* and *Oophaga lehmanni*) [220] and American Toads and Fowler's Toads [221]. American Toads, however, utilize multiple breeding strategies, with males either remaining stationary and calling, or actively pursuing females in a ‘scrambling’ behaviour and forming a ‘gauntlet’ of scrambling males that attempt to intercept any female that approaches or enters the breeding pond [125]. This scrambling behaviour has been shown to be highly unselective, and males have even been observed to attempt amplexus with beanbags tied to strings [125].

Herein, I used an individual-based simulation model to explore the role of breeding behaviour in driving both DNA and mtDNA introgression and DNA dilution between two distinct but interbreeding species.

Methods

I created an individual-based simulation model of two closely related but genetically distinct species, which were still capable of inter-breeding. The model was written in R (R 3.2.4 © The R Foundation for Statistical Computing) and source code is available upon request. The genome-wide DNA of an individual was a continuous variable, representing the average of all loci, where -1 is the homozygote genome of one parental species and 1 is the homozygote genome of the second parental species. These species were initially based off the American Toad (*Anaxyrus americanus*) and the Canadian Toad (*Anaxyrus hermiophrys*) as a biological framework around which to build the model. However, the model itself is not fixed to anuran species and bimodal life history was not included. I simulated a simple geographic landscape that mirrored where the ranges of the American and Canadian Toads collide [127]. The landscape was divided into seven different regions with varying habitat and was expressed as:

$$Habitat = -1 + \left(2 * \left(\frac{Location - 1}{Number\ of\ Locations - 1}\right)\right)$$

For example, the habitat of the fifth location in the simulation with seven total locations would be calculated as:

$$Habitat_5 = -1 + \left(2 * \left(\frac{5-1}{7-1}\right)\right) = 0.33$$

The habitat of the outer regions strongly selected for each of the parental species. The middle regions were survivable for both parental species, however hybrids in these regions had an advantage over, and were capable of out-competing parental species therein as is observed in the hybrid zone between American and Canadian Toads [127]. All regions were initially uninhabited except the outer locations that were seeded by one of the two parental species. Maximum lifespan for an individual in the simulation was approximately five iterations of the simulation. Maturation occurred after one full iteration, in the second iteration after having been born. Populations were density dependent, and fluctuated around a specific carrying capacity that was constant within and between regions.

Each iteration of the simulation had events occur in the following order: breeding, with offspring created immediately, mortality; all individuals were aged and lastly demographics were recorded for each region. Breeding events were instigated by randomly sorting all breeding aged females and pairing them with males, who were sorted by age. Under the null hypothesis, breeding pairs were entirely random within each region. When testing the impact of reproductive strategy, males of one species would only mate with females who were within a certain range of genetic similarity. The probability of breeding was expressed as:

$$if\ Male\ DNA \geq Female\ DNA - ((Male\ DNA * Choosiness\ )^2 +\ MinChoosiness)$$

or

$$if\ Male\ DNA \leq Female\ DNA + ((Male\ DNA * Choosiness\ )^2 +\ MinChoosiness)$$

However the second species continued to be promiscuous in its mate choice.

I chose to specifically limit the number of offspring to pre-emptively simulate first year mortality in offspring. Genetic inheritance was mixed, with DNA being a combination of parental types, and mtDNA being entirely inherited from the mother. Each pairing resulted in four offspring, with sex of the offspring being entirely random. For offspring, two remained residents in the location they were born, while two dispersed, one in each direction. This dispersal pattern is consistent with gene flow observed in wild populations of closely related anuran species [222, 223]. Dispersal was limited to the immediately neighbouring region and adults remained in the region in which they established, with no post-dispersal migration events to reflect the dynamics in wild populations [223-225]. Simulations were run for 500 iterations with 200 repetitions.

Mortality was density dependent with risk of death increasing with increasing population and with age.

$$P = Base\ Mortality + \left|\left(\frac{DNA - Habitat\ Quality}{Location} + \frac{Location\ Population}{4}\right)\right|$$

For example, if base mortality for a given population is set at 0.25 then an individual of species with DNA 1, in habitat 5, with a quality of 0.33 and in a population that is at 50% of the carrying capacity then the mortality probability of that individual would be 0.51.

As individuals were analogous to amphibian species, maximum expected lifespan was 5 years. Although there was no specifically stated maximum lifespan the probability of exceptionally long lifespans was incredibly low.

Results

Within the null model, there was the most discordance in the central location (Fig. 1), where the species were meeting. However discordance dropped drastically on either side of this central location, and was almost nonexistent at either extreme. However, when examining DNA, it was found that the central location rarely exhibited a pure hybrid DNA signature, rather it was often skewed on either side of the true hybrid genotype and was slightly closer to one of the ancestral species (Fig. 2). At the limits of the habitat, it was also found that there had been significant dilution of the DNA of each species, and likely if habitat selective forces were removed the two species would completely intermingle.

Inclusion of mate choice behaviour for one of the species resulted in a significantly different degree of discordance and a significantly different distribution of discordance across the habitat. While the null model with random mating showed maximum discordance in the centre of the landscape, the alternate model of breeding choice showed more discordance in both the centre location and deep into the habitat of the promiscuous species (Fig. 1). Interestingly, nuclear dilution patterns between the null and alternate behaviour model remained very similar, with DNA following the structure of the selection pressures across the landscape (Fig. 2).

Discordance and DNA were compared between each region across the landscape using t-tests and ANOVA. Across all values and regions taken together, there was no significant difference when controlling for Region (ANOVA: $F = 125.58$, $df = 1$, $P \ll 0.0001$), and when comparing between models (ANOVA: $F = 41.69$, $df = 1$, $P \ll 0.0001$). In multiple t-tests, α was adjusted by Bonferroni to 0.008 rather than 0.05. However, post-Bonferroni adjustment, there were significant differences in discordance between the null and choice models for Region 1 (t-test: $t = -8.853$, $df = 203.05$, $P \ll 0.0001$), Region 2 (t-test: $t = -8.384$, $df = 247.60$, $P \ll 0.0001$), Region 4 (t-test: $t = 6.496$, $df = 338.34$, $P \ll 0.0001$), Region 5 (t-test: $t = 8.855$, $df = 341.87$, $P \ll 0.0001$), Region 6 (t-test: $t = 6.477$, $df = 333.57$, $P \ll 0.0001$) and Region 7 (t-test: $t = 3.909$, $df = 360.96$, $P = 0.0001$) but not for Region 3 (t-test: $t = -1.251$, $df = 397.43$, $P = 0.212$).

Discussion

These results are highly suggestive of a role of behaviour in the introgression of mtDNA or DNA between two species. With random breeding behaviour, there was a normal distribution of discordance centred at the hybrid zone. Mate selection simulated behavioural patterns that have been observed for American and Canadian Toads. Canadian Toads depend upon call structure to attract females and mate with those females who are sufficiently genetically similar to be drawn by a Canadian call. American Toads however, in deploying two strategies, are able to attract American Toad females, or intercept any females approaching breeding ponds. This provides American Toad males the advantage of mating with females of both species. However, choosy breeding behaviour resulted in a decrease in discordance within the range of the selective species, and an increase in the discordance in the promiscuous species.

Although there were significant variations in the pattern of discordance between the two species, these patterns were not seen in the pattern of DNA across the landscape. The greatest variation in DNA and the highest concentration of hybrids were found in the middle of the landscape, as would be expected given the habitat type of this area. Similarly, at the edges of the landscape where habitat was most appropriate for either parental species, DNA was relatively preserved to that of the parental species.

These findings suggest that prezygotic barriers to breeding, such as breeding behaviour, play a crucial role in the introgression of mtDNA between species. Understanding how the presence of prezygotic barriers is essential as contact between previously allopatric species is expected to increase due to climate change and anthropogenically mediated introductions [226-228], I would expect in cases where no prezygotic barriers exist, a random breeding behaviour pattern would result, as seen between *A. coronopifilum* and other *A. spp* [214]. However, in species where prezygotic barriers exist in one or both species, then I could expect that the breeding pattern would more closely follow that of the American and Canadian Toad.

The lack of significant changes in DNA at the respective ends of the landscape suggest that selective pressure exerted by the habitat may be sufficient to maintain speciation. What is of concern is that although the DNA was approximately 25% diluted with the DNA of the competing species. In areas of extreme selective pressure, or where a small number of adaptations are essential for the survival of a species, a dilution of habitat specific mutations or decrease in selective pressure could rapidly result in the extinction of a species or loss of both species through genetic assimilation. In the case of the threatened native Westslope Cutthroat trout (*Oncorhynchus clarkii lewisi*), habitat selective pressures limited the spread of hybridization with invasive Rainbow trout (*Oncorhynchus mykiss*) until climate change reduced these pressures and hybrids spread rapidly through the Westslope Cutthroat range [228].

Habitat has also been cited as a possible control of ingress of Brown bear DNA into the Polar bear where arctic conditions result in such a significant selective pressure that the decrease in fitness from Brown bear DNA would be strongly deleterious [229]. However, this appears to work conjointly with behaviour, specifically both through male-male competition, wherein Polar bears gain advantage due to their larger size, and with a male-biased dispersal that would prevent mtDNA introgression between species [229]. Similarly, the American and Canadian Toad demonstrates a similar habitat-bounded hybrid zone, along the prairie-forest transition that stretches from Manitoba to Coastal Plains of the Gulf of Mexico, a transition area that is a contact zone for multiple species across North America [18, 171]. Along this transition, parental types are likely preserved as a result of habitat selective pressures on either side of the hybrid zone [127].

The significant long-distance ingress of mtDNA beyond the hybrid zone [208], congruent to that observed in the simulation model, would suggest that although environmental selective pressures may preserve the DNA composition of a species the behavioural differences between species can have significant effects on the direction and extent of genetic introgression between species.

Conclusion

The American and Canadian Toad with their hybrid zone along the forest-prairie habitat transition represent an opportunity to examine the drivers of hybridization, hybrid zone dynamics, and genetic exchange between conspecifics. Observational studies of sexual selection and the role of dispersal and behaviour should help to further improve our understanding of the role that behaviour plays in driving hybrid zone dynamics. An improved understanding of these drivers should increase the precision of predictions regarding species interactions, movement of hybrid zones and gene flow across contact zones. Understanding the dynamics of hybrid zones are especially important given the potential damage that hybridization can inflict on the long-term persistence of a species and the predicted increase in species contact through modern climate and anthropogenic introductions.

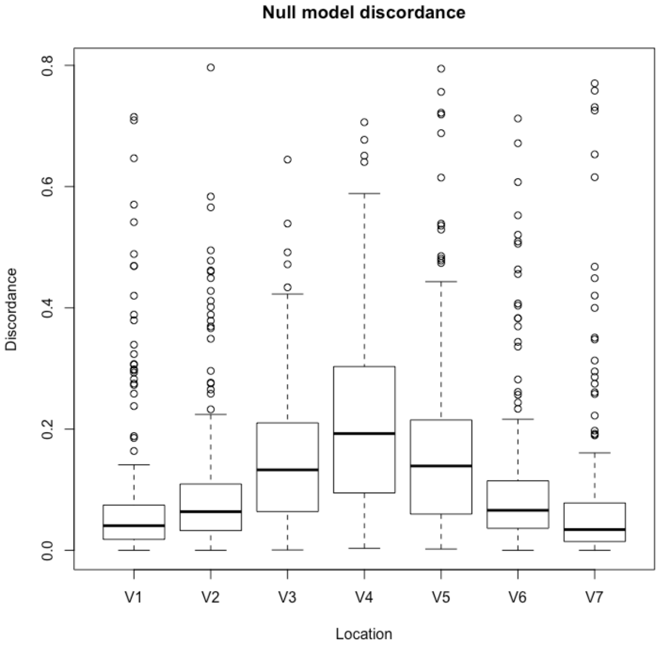
Figures and Legends

Figure 1 — Absolute nuclear and mitochondrial discordance within populations when breeding is a result of A) random mating and B) a selective mating process for one species and random mating for the second species.

Figure 2 — Nuclear DNA distribution across landscapes for A) random mating and B) a selective mating process for one species and random mating for the second species.

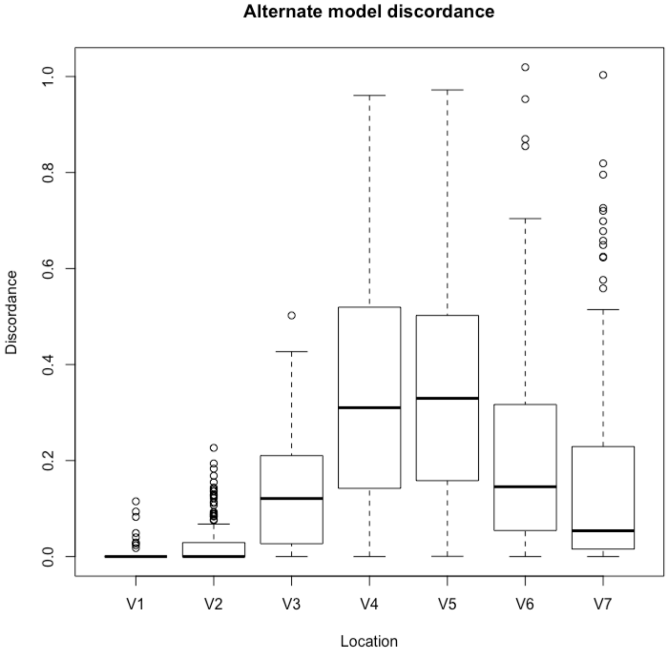
Figure 1:

A)



Null model discordance
Discordance
0.0
0.2
0.4
0.6
0.8
V1
V2
V3
V4
V5
V6
V7
Location

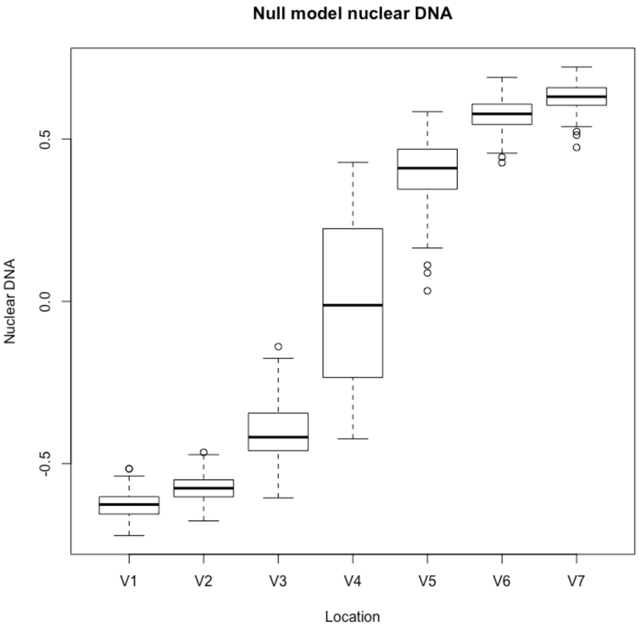
B)



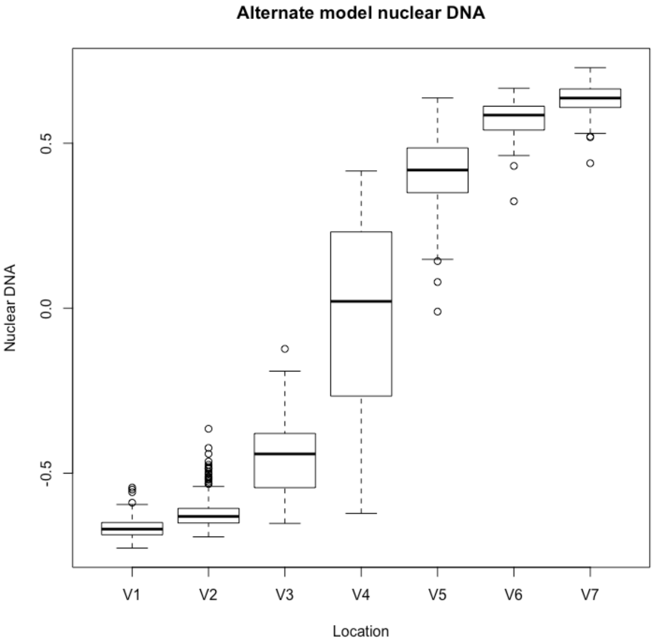
Alternate model discordance
Discordance
0.0
0.2
0.4
0.6
0.8
1.0
V1
V2
V3
V4
V5
V6
V7
Location

Figure 2

A)


Null model nuclear DNA
Nuclear DNA
0.5
0.0
-0.5
V1
V2
V3
V4
V5
V6
V7
Location

B)


Alternate model nuclear DNA
Nuclear DNA
0.5
0.0
-0.5
V1
V2
V3
V4
V5
V6
V7
Location

Chapter 4: Latitudinal variation in morphology in American Toads, *Anaxyrus americanus*

Linking Statement for Chapter 4:

This chapter covers my analysis of morphological variation with latitude among ethanol preserved specimens and live caught specimens of the American Toad. These specimens were chosen as they were fully developed mature adult specimens. Furthermore, these specimens were physically intact, with no dissections that could affect the measurement of morphological traits. Specimens were also selected to ensure that the populations sampled represented the most diverse and widespread coverage of the geographic range of the American Toad as possible.

The drastic changes in climate and environmental conditions that occur along the latitudinal range of a species may have a significant effect on the morphology of the species. Morphological variation, driven by altered behaviour in response to varying light conditions or changes in seasonality or selection for dispersal-associated traits, can reflect the mosaic of selective pressures encountered by populations of the same species. This is the first such study of morphological variation in the American Toad. Despite previously having a northern subspecies (*A. americanus copei*) this is the first such study of morphological variation across the entire range of the American Toad.

The references in the following chapter have been renumbered and are included in the combined bibliography at the end of this thesis in numerical order following the sequence in which they appear throughout the entire thesis. This chapter has been formatted for submission to Herpetologica as a co-authored manuscript with Dr. David M. Green. All measured morphological traits, and a listing of all specimens that were examined is found in **Appendix III**.

Abstract: Species with extremely large geographic ranges, especially those with large latitudinal ranges, may be exposed to a high degree of variation in environmental conditions. While average daily temperature may be the most obvious environmental factor to vary with latitude; populations may also respond to light levels, daylight hours, diurnal temperature variation, and active season length, which may also vary greatly. I examined 428 preserved and live-caught American Toads (*Anaxyrus* (=*Bufo*) *americanus* Bufonidae) from Mississippi, USA, to northern Quebec, Canada, which represents the extent of their latitudinal range. I evaluated morphological traits on these specimens that are known to be associated with specific behaviours, and I tested whether those traits varied latitudinally, varied within previously identified subspecies ranges, or varied sexually. There were correlations between cranial and limb morphology and latitude across the range when grouping all individuals together, but I found that these correlations were not consistent when grouping by sex. While SVL was not affected by latitude, forelimb length increased for both sexes with increasing latitude. For female toads, hindlimb length increased significantly with latitude, however there was no significant relationship in male toads. As the correlations between latitude and morphological traits were not consistent between the sexes, my findings suggest a behavioural and sexual basis or an interaction effect between sex and environment for this selection rather than a general environmental or dispersal influence.

Key words: American Toad; Amphibian; *Anaxyrus americanus*; Behaviour; Environmental interactions; Latitudinal gradient; Morphological variation; Morphology

THE EXTENT of environmental variation experienced by species with large latitudinal ranges is often considered to be just a matter of a temperature gradient. However, between low latitudes and high latitudes, animals must contend with the complex interactions of multiple abiotic factors, including marked differences in the directness of solar radiation, exposure to UV radiation, day length, seasonal duration, and diurnal temperature variation, as well as average annual temperature [230, 231]. At latitudes approaching or within the Arctic or Antarctic Circle, for instance, the summer season is incredibly short compared to lower latitudes, but is also highly productive because of the very long days [232-234]. The drastically different environmental conditions that characterize habitats at greatly differing latitudes should impose strong selective pressures [235-237].

These pressures have tangible effects upon the morphology, behaviour, and physiology of multiple taxa, including insects [238-240], amphibians [241-243] and mammals [244]. One of the most common suggested generalized trends in morphology with latitude is Bergmann's rule, which stipulates an increase in body size with increased latitude, yet has seen considerable controversy in generality and applicability (Reviewed in: [245]). Furthermore Allen's Rule, which would suggest that relative limb length should decrease with increased latitude, was also not found during a detailed examination of limb morphology in the Common Frog (*Rana temporaria*) [246]. While these trends do appear to be significant in avian and mammalian species [247], there remains a significant degree of ambiguity as to how these trends apply to herpetofauna [248, 249].

Numerous amphibian species occur over very large latitudinal ranges in the Northern Hemisphere and may show genetic or phenotypic variability in response to varying environmental conditions across latitudinal gradients. Amphibians reaching far northern latitudes could be subjected to the extremes of these variations, and it is among those species that have the greatest latitudinal range that the variation between northern and southern populations should be the most evident. European Common Frogs (*Rana temporaria* Ranidae)*,* for example, show evidence of phenotypic variability in response to varying environmental conditions from north to south [241]. Likewise, the breeding biology of amphibians should be affected by the environmental variation that occurs with changes in latitude. It has been observed in assorted species, including American Toads (*Anaxyrus (=Bufo) americanus* Bufonidae), Northern Leopard Frogs (*Lithobates (=Rana) pipiens* Ranidae) and Wood Frogs (*L. sylvaticus* Ranidae) that populations breed later at higher latitudes than at lower latitudes [243, 250, 251].

Although many North American anuran species are found across a wide range of latitudes [242], few approach the range of latitude covered by American Toads. From 30°N near Baton Rouge, Louisiana; to nearly 56°N at Kuujjuarapik, Quebec; above 55°N in Winish Ontario; and possibly even further north [242]. In total, the range of American Toads extends north to south over 2970 km and more than 25° of latitude. Across this range there is tremendous variation in day length, temperature regime, and the length of the toads' active season.

When southern populations of American Toads at the latitude of Vicksburg, Mississippi (32.3 ° N) begin calling in February, there are fewer than 12 hours of daylight, but when far northern populations at the latitude of Grande-Rivière, Quebec (53.6 ° N), begin their breeding season after the spring thaw in July, there are over 16 hours of daylight [252]. During July at Grande-Rivière, daytime air temperatures average 17.3 ° C but night-time temperatures average only 4.2 ° C [252], and can easily drop below freezing on clear nights. Furthermore, summer nights at high latitude are never truly dark; the sun remains so near the horizon that, at its darkest, the night sky is still in twilight [253]. Such conditions make it unlikely that any anuran in the far north can breed strictly at night, or even in darkness, as they usually do towards the south. Northern amphibians such as Wood Frogs [254-256] and American Toads [257-259] will call both day and night. American Toads from localities 45 ° N – 50 ° N in Quebec not only breed during the day but also at air and water temperatures approaching 0 ° C and 4 ° C respectively [257-259].

Akthough many amphibians only engage in either singing behaviour or scrambling behaviour, where males actively pursue females, American Toads engage in both [260-262]. Although persistent singers can have the highest reproductive success rate, singing has a high energetic cost and predation risk and can become unsupportable at lower temperatures [261, 263]. Conversely scramblers have decreased success but also decreased energy demand and predation risk [261, 263]. Furthermore, calling vs. scrambling behaviours appear to favour differing morphologies [264, 265]. Thus, the variation in breeding season conditions experienced by populations across the range of the American Toad may be manifested in morphological changes driven by changes in breeding strategy.

If there is morphological adaptation along the latitudinal range of the American Toad then there are multiple possible, and not mutually exclusive, hypotheses. Firstly is whether it is latitude, or phylogeny that determines morphological variation. Although inter-species variations are dependent upon phylogeny, are subspecies subdivisions a greater predictor of intraspecific trends in morphology over latitudinal gradients or are these subdivisions the result of general trends within the species. If this trend is in relation to latitude, then there should be a parallel latitudinal trend in morphological variation observed in both sexes. However, if trends are driven by changes in behaviour due to environmental selective pressures then trends in morphology should be sex-dependent or resultant from interactive effects between sex and environment.

Materials and Methods

Specimens

I used a combined total of 428 ethanol preserved American Toads from 22 localities from the collections of the American Museum of Natural History (New York, NY, USA), Canadian Museum of Nature (Ottawa, ON, Canada), Smithsonian Institute National Museum of Natural History (Washington, D.C, USA), and the Redpath Museum (Montreal, QC, Canada), and live specimens captured from northern and southern Quebec (Appendix I). American Toads are currently divided into two subspecies: the Dwarf American Toad (*A. americanus charlesmithi*), and the Eastern American Toad (*A. americanus americanus*), which makes up the majority of the species' range that have been examined in this study [126].

Dwarf American Toads are found in the south-central United States, namely: Illinois, Indiana, Kentucky, Tennessee, Arkansas, Missouri, Kansas, Oklahoma, and Texas. A previously recognized subspecies was the "Hudson's Bay" American Toad (*A. americanus copei*) which had a designated range in Northern Québec and Ontario starting at approximately 50°N latitude prior to being [266]. However, the "Hudson's Bay" American Toad is no longer recognized, and was merged with the more southerly Eastern American Toad, due to insufficient difference in isozymes between them [267].

Before performing any further analysis, I first tested whether there were differences between the morphology of toads collected from the recognized ranges or either the Eastern (*n* = 266), Dwarf (*n* = 70), or "Hudson's Bay" American Toad (*n* = 92). I used only sexually mature individuals, identified by the presence of fully developed cranial crests [242]. My samples came from as far south as Vicksburg, MS, USA, at 32.35 ° N latitude, and as far north as Radisson, QC, Canada at 53.92 ° N latitude, thus spanning 21.58 ° of latitude (2581 km) (Fig. 1).

Data Collection

Using dial callipers, I measured 28 different morphological traits, including limb lengths, snout to vent length (SVL), head shape and cranial features involving the eye, cranial crests, parotoid glands, and tympani. Shrinkage during preservation has been shown to be insignificant for these characters in toads, and so I did not need to make allowance for possible shrinkage effects [268].

I used digital photographs for the analysis of cranial landmarks. All images had a standard background that contained multiple measurement scale references. I used tpsDig2 [269] to record 24 landmarks on the head of each toad, marking the eyes, cranial crests, and parotoid glands for each individual.

To compare corporeal morphologies among specimens, I used hand-measured length measurements of limbs and corporeal traits.

Analysis

Initially I assessed differences in toad morphology based upon the previously recognized subspecies ranges. I used ANOVA testing both when dividing by subspecies for all toads together and then for each of the two sexes, and I followed up on significant results through Tukey Honest Significant Difference *post-hoc* testing.

I assessed the relationship of snout-vent length (SVL) between male and female toads using t-tests. Following this, I performed a partial correlation of total hindlimb length, total forelimb length, and cranial width with SVL in order to remove any influence of SVL prior to analysis of limb lengths and cranial width in relationship to latitude. Following partial correlations, I performed regressions of total hindlimb length, total forelimb length and cranial width each in relation to latitude. I assessed assumptions of tests throughout. When comparing SVL and dimorphism in SVL across latitude, I performed ANOVA in R v3.0.2 (The R Foundation for Statistical Computing, Vienna, Austria) for all toads together, and then for males and females separately using latitude as a continuous predictor variable.

I performed a geometric morphometric analysis [270] using Principal Components Analyses (PCA) of geometric shape data using the "geomorph" package in R v3.0.2 [271]. Rotation was controlled for using the axis of the cranial length, and geomorph controlled for allometry between specimens through cranial length and SVL for cranial traits. Following PCA, I performed linear regressions between the primary components and latitude, while controlling for sex, SVL, and longitude. All values are presented as mean ± standard error and then the range with the minimum – maximum recorded values.

Results

Snout-vent Length

I found little difference in snout-vent length (SVL) among toads in relation to either sex or latitude, however there were distinct differences in regards to the subspecies range divisions.

When all toads were grouped together, I found that female toads were slightly, but not significantly larger than male toads (Table 1) (t-test: $t = 1.02$; $P = 0.310$; $n = 404$) (Fig. 2).

I found that there were significant differences in the SVL for all toads when divided by subspecies range (ANOVA: $F = 11.06$, $df = 2$, $P < 0.001$). Toads from both the Dwarf range and the "Hudson's Bay" Range were significantly smaller than those from the Eastern range (Tukey HSD: $P = 0.004$ and $P < 0.001$ respectively), but there was no significant difference between those of "Hudson's Bay" and Dwarf range (Tukey HSD: $P = 0.914$). This relationship held true for male toads analyzed alone (ANOVA: $F = 36.37$, $df = 2$, $P < 0.001$), with those from the Dwarf and "Hudson's Bay" range being smaller than those from the Eastern range (Tukey HSD: $P < 0.001$, Tukey HSD: $P < 0.001$ respectively), and no significant difference between those from the Dwarf and "Hudson's Bay" range (Tukey HSD: $P = 0.421$). However, in contrast, when examined alone, there was no significant difference between the size of females when grouped by origin (ANOVA: $F = 0.122$, $df = 2$, $P = 0.885$).

I found that there was no significant correlation between latitude and SVL for all toads (ANOVA: adjusted $R^2 = 0.002$, $F = 1.680$, $df = 426$, $P = 0.200$) nor was there a significant correlation when individuals were divided by sex for males (ANOVA: adjusted $R^2 = 0.007$, $F = 3.060$, $df = 297$, $P = 0.080$) or females (ANOVA: adjusted $R^2 = -0.002$, $F = -0.005$, $df = 127$, $P = 0.570$) (Fig. 3).

Limb Length

I found that there were significant differences in limb morphology for toads when divided by subspecies range, however these differences were sex specific and varied between unadjusted and values adjusted for SVL. Although I did not find any significant difference in unadjusted fore- or hindlimb length between the sexes (Table 1), I found slight, significant trends in unadjusted forelimb length amongst toads in relation to latitude when sexes were grouped together, and amongst male but not female toads. I did not find similar trends in unadjusted hindlimb length in relation to latitude amongst all toads or either sex. Once adjusted for SVL, I found trends in both forelimb and hindlimb length in relation to latitude for grouped toads (Fig. 4). Although both sexes individually showed trends in adjusted forelimb length in relation to latitude, only females showed a trend in adjusted hindlimb length in relation to latitude (Fig. 4).

I found a significant difference in the unadjusted hindlimb length when all toads were grouped by subspecies range (ANOVA: $F = 26$, $df = 2$, $P < 0.001$). With those from the Dwarf range having smaller hindlimbs than either those from “Hudson’s Bay” (Tukey HSD: $P = 0.020$) or Eastern (Tukey HSD: $P < 0.001$), and those from the “Hudson’s Bay” smaller than from Eastern (Tukey HSD: $P < 0.001$). However, this relationship disappeared once when limb lengths were controlled for SVL (ANOVA: $F = 0$, $df = 2$, $P = 1$).

When divided by sex, males showed a significant difference in hindlimb length based on subspecies range (ANOVA: $F = 62.58$, $df = 2$, $P < 0.0001$) with those from the Dwarf range having shorter hindlimbs compared to either those from Eastern (Tukey HSD: $P < 0.0001$) and “Hudson’s Bay” (Tukey HSD: $P < 0.0001$) and those from “Hudson’s Bay” having smaller hindlimbs than those from the Eastern range (Tukey HSD: $P < 0.011$). Contrastingly, females showed no significant differences based on the subspecies range where they were collected (ANOVA: $F = 0.511$, $df = 2$, $P = 0.601$).

Significant differences were also found in the forelimb (ANOVA: $F = 15.55$, $df = 2$, $P < 0.0001$). Although those from the Dwarf range had shorter forelimbs than either Eastern (Tukey HSD: $P < 0.0001$) or “Hudson’s Bay” (Tukey HSD: $P = 0.001$), there was no difference between those from the “Hudson’s Bay” range and those from the Eastern range (Tukey HSD: $P = 0.273$). When divided by sex, males showed significant differences in forelimb size (ANOVA: $F = 36.07$, $df = 2$, $P < 0.0001$), with those from the dwarf range having the shortest forelimbs compared to those of Eastern (Tukey HSD: $P < 0.0001$) and “Hudson’s Bay”

(Tukey HSD: *P* = 0.0002), and "Hudson's Bay" having shorter forelimbs than Eastern (Tukey HSD: *P* = 0.0014). Females did not show any significant differences due to the subspecies range divisions (ANOVA: *F* = 0.533, *df* = 2, *P* = 0.588).

Adjusted for SVL, there was no significant difference when dividing hindlimb lengths by subspecies range (ANOVA: *F* = 0.00, *df* = 2, *P* = 1.00) and this held true amongst males (ANOVA: *F* = 0.00, *df* = 2, *P* = 1.00), but not for females where there was a significant difference (ANOVA: *F* = 3.176, *df* = 2, *P* = 0.0451). Amongst females those from the Dwarf range had shorter adjusted rearlimb lengths than those from the Eastern (Tukey HSD: *P* = 0.0384) and were marginally non-significantly smaller than those from "Hudson's Bay" range (Tukey HSD: *P* = 0.0506). There was no significant difference between those from the Eastern compared to "Hudson's Bay" range (Tukey HSD: *P* = 0.9871).

Once adjusted for SVL, there was a significant difference in the forelimb length of all toads based on subspecies range (ANOVA: *F* = 23.31, *df* = 2, *P* < 0.0001). Those from the Dwarf range had shorter limbs compared to either those from Eastern (Tukey HSD: *P* < 0.0001) or "Hudson's Bay" (Tukey HSD: *P* < 0.0001) and those from "Hudson's Bay" had longer forelimbs than those from the Eastern range (Tukey HSD: *P* = 0.0044). This relationship held true when I examined just males (ANOVA: *F* = 35.58, *df* = 2, *P* < 0.0001). With those from "Hudson's Bay" again having the longest forelimbs compared to either Eastern (Tukey HSD: *P* = 0.0003) or Dwarf range (Tukey HSD: *P* < 0.0001), and those from the Eastern having longer forelimbs than those from the Dwarf range (Tukey HSD: *P* < 0.0001). For females, there was a significant difference in forelimb length based on subspecies range (ANOVA: $F = 3.471$, $df = 2$, $P = 0.0341$). Although there was no significant differences between the adjusted forelimb length of those from the Eastern and either the Dwarf range (Tukey HSD: $P = 0.098$) or the "Hudson's Bay" range (Tukey HSD: $P = 0.3707$) those from the Dwarf range were significantly smaller than those from the "Hudson's Bay" (Tukey HSD: $P = 0.0269$).

When initially examining unadjusted limb length regressed onto latitude, I found that forelimb length for all toads increased slightly with latitude ($ß = 0.286$, $F = 6.632$, $P = 0.010$, $R^2 = 0.013$; $df = 426$) and increased slightly for males ($ß = 0.296$, $F = 4.440$, $P = 0.036$, $R^2 = 0.011$; $df = 297$), however there was not a significant relationship for females ($ß = 3.140$, $F = 2.790$, $P = 0.097$, $R^2 = 0.014$; $df = 127$). Unadjusted hindlimb measurements for all toads were not significantly associated with changes in latitude ($ß = 0.023$, $F = 0.010$, $P = 0.919$, $R^2 = –0.002$; $df = 426$), and neither was hindlimb for male ($ß = –0.232$, $F = 0.596$, $P = 0.440$, $R^2 = –0.001$; $df = 297$) or female toads ($ß = 3.380$, $F = 0.824$, $P = 0.367$, $R^2 = –0.001$; $df = 127$). Once adjusted for SVL, forelimb length was found to increase marginally with increased latitude ($ß = 0.404$, $F = 42.40$, $P < 0.001$, $R^2 = 0.088$; $df = 426$) for all toads, as well as for male ($ß = 0.491$, $F = 36.420$, $P < 0.001$, $R^2 = 0.106$; $df = 297$) and female toads ($ß = 4.040$, $F = 16.380$, $P < 0.001$, $R^2 = 0.107$; $df = 127$). Hindlimb length of toads was also found to increase with latitude ($ß = 0.267$, $F = 4.440$, $P = 0.0358$, $R^2 = 0.008$; $df = 426$). When separated by sex this was only found in the case of females ($ß = 5.190$, $F = 7.020$, $P = 0.009$, $R^2 = 0.045$; $df = 127$) and not for males ($ß = 0.197$, $F = 1.324$, $P = 0.251$, $R^2 = 0.001$; $df = 297$).

Cranial Length and Width

I found significant differences in cranial morphology between subspecies ranges. However I did not find significant differences in the unadjusted cranial width or length between the sexes (Table 1). I found differences in cranial width and length in relation to latitude both when unadjusted and adjusted for SVL amongst all toads grouped together and for male toads, but not amongst female toads. However, I did find trends in morphology in relation to latitude amongst all, and amongst individual sexes, when I used a geometric morphometric analysis of cranial features.

There was a significant difference in the unadjusted head width when toads were divided by species range (ANOVA: $F = 19.36$, $df = 2$, $P < 0.0001$), with no difference between Dwarf and Eastern (Tukey HSD: $P = 0.7583$) but with those from the "Hudson's Bay" range having a narrow head than either the Eastern (Tukey HSD: $P < 0.0001$) or Dwarf (Tukey HSD: $P = 0.0001$). Once controlled for SVL, although there was still a significant difference in cranial width across all species ranges (ANOVA: $F = 22.29$, $df = 2$, $P < 0.0001$), Dwarf toads had the widest heads compared to both Eastern (Tukey HSD: $P = 0.0024$) and "Hudson's Bay" (Tukey HSD: $P < 0.0001$), and Eastern had wider heads than the "Hudson's Bay" range (Tukey HSD: $P < 0.0001$).

Male toads followed a similar pattern, with a significant difference in cranial width depending on subspecies range (ANOVA: $F = 47.41$, $df = 2$, $P < 0.0001$). There was no difference between those from the Dwarf and Eastern ranges (Tukey HSD: $p = 0.3131$), but toads from the “Hudson’s Bay” range had narrower heads than either the Dwarf (Tukey HSD: $P < 0.0001$) or Eastern (Tukey HSD: $P < 0.0001$) subspecies.

Once adjusted for SVL, there were significant differences as a result of subspecies range (ANOVA: $F = 34.32$, $df = 2$, $P < 0.0001$), with Dwarf having the widest heads, followed by Eastern and then “Hudson’s Bay” (Tukey HSD: $P < 0.0001$ in all cases). For females, there was no difference in cranial width based on subspecies range both when not controlled for SVL (ANOVA: $F = 1.088$, $df = 2$, $P = 0.34$) and when controlled for SVL (ANOVA: $F = 2.195$, $df = 2$, $P = 0.116$).

Regressions of unadjusted cranial width resulted in a trend of decreasing cranial width with increasing latitude for all toads when grouped together ($ß = –0.275$; $F = 25.90$; $P < 0.001$; $R^2 = 0.060$; $df = 426$) (Fig. 4). When divided by sex, males had narrower heads with increased latitude ($ß = –0.495$; $F = 70.700$; $P < 0.001$; $R^2 = 0.190$; $df = 297$). However, females did not demonstrate a relationship between cranial width and latitude ($ß = –1.150$; $F = 1.420$; $P = 0.237$; $R^2 = 0.003$; $df = 127$) (Fig. 4). Cranial width for all toads, when adjusted for SVL, was found to decrease with latitude ($ß = –0.217$; $F = 49.970$; $P < 0.001$; $R^2 = 0.103$; $df = 426$). This was also the case for males ($ß = –0.412$; $F = 119.600$; $P < 0.001$; $R^2 = 0.285$; df = 297) but not for females ($ß = –0.678$; $F = 1.770$; $P = 0.186$; $R^2 = 0.006$; $df = 127$).

Unadjusted cranial lengths were found to decrease with latitude for all toads ($ß = –0.166$; $F = 24.000$; $p < 0.001$; $R^2 = 0.051$; $df = 426$) as well as for males ($ß = –0.263$; $F = 37.400$; $P < 0.001$; $R^2 = 0.101$; $df = 127$) but not for females ($ß = –0.817$; $F = 2.060$; $P = 0.154$;

R^2 = 0.008; *df* = 127). Once adjusted for SVL, length was found to decrease with latitude when all toads were grouped together (*ß* = –0.131; *F* = 38.150; *P* < 0.001; R^2 = 0.080; *df* = 426) and for just males (*ß* = –0.202 *F* = 54.560; *P* < 0.001; R^2 = 0.157; *df* = 297) but not for females (*ß* = –0.561; *F* = 2.475; *P* = 0.118; R^2 = 0.011; *df* = 127).

Through geometric morphometric analysis, cranial morphology and latitude were correlated (ANOVA: $F_{1,259}$ = 46.600; *P* < 0.001; R^2 = 0.221; *df* = 160) when all toads were considered together and SVL was controlled for (Fig. 5). Toads at higher latitudes tended to have relatively narrower heads than those at lower latitudes. This was also true when only males were considered (ANOVA: $F_{1,102}$ = 27.000; *P* < 0.001; R^2 = 0.200; *df* = 103) and only females(ANOVA: $F_{1,540}$ = 18.20; *P* < 0.001; R^2 = 0.235; *df* = 55) (Fig. 6).

Discussion

I found that there were significant relationships between latitude and morphology among American Toads. Additionally, I found that those elements of the observed variation that were differentiated using the reported ranges of current and previously identified subspecies, were often sex specific, or were congruent to the more general latitudinal trends. Although relationships between limb length and latitude were found when all toads were grouped together, when these same analyses were repeated and toads were differentiated by sex, there were distinct differences in these relationships between the sexes.

Dispersal selection, commonly referred to as the 'Olympic village' effect, may be suspect in the morphological patterns observed, but this does not seem to be the case. Among the toads examined, I did not see a consistent pattern of selection for longer limbs. Both males and females had longer forelimbs with increased latitude; however, only female toads demonstrated a relationship between hindlimb length and latitude. This would appear to exclude a selection for dispersal similar to that observed on the invasion front of Cane Toads (*Rhinella marina* Bufonidae) in Australia [272-274]. Given that the American Toad has also been established in these previously glaciated areas for 5,000 to 10,000 years [126], a dispersal effect similar to that of the Cane Toad, with its much more recent dispersal and colonization, also seems unlikely.

Possible random changes that may arise through genetic drift across geographic distance or allele surfing likewise should not result in a sexual bias for these changes. Furthermore, I would not expect that the relationship between latitude and morphological traits would be the result of changes in thermal regime alone, given the disparity observed between males and females.

Our data is also suggestive of a peak in SVL in the middle of the range which tapers down towards the periphery, a finding in contrast with Bergmann's rule, which predicts a trend of increased SVL with latitude. However, Bergmann's rule appears to be far from universal as several other species also have reverse trends (Ashton, 2002; Bancila, et al. 2009). Generally, larger body size in males has been found to increase success during male-male physical competition [275-278] however selection for a smaller body size has been found in fighting toads [276-278] and this could result in the counter to Bergmann's rule in male American Toads. The observed decrease in body size could also more simply be the result of resource limitation overwhelming selective pressures for a larger body size, as has been seen in some mammals [279]. My findings are also contradictory to Allen's rule which would suggest that body size should increase while relative limb length decreases. However, findings in the Common Frog *(Rana temporaria*) demonstrated that Allen's rule may not be applicable to amphibians [246].

Of the possible drivers for the relationship between morphological variation and latitude may be due to changes in length of the active season. It has been suggested that for ectothermic species, which are necessarily active only during the summer season, may be particularly affected by both the long periods of winter dormancy and limited darkness during summer at higher latitudes [280, 281]. The shortened period of activity encountered by ectotherms at high latitudes has been previously demonstrated to have important impacts including a transition from oviparity to viviparity in various reptiles with increased latitude [282-285] especially in response to decreased length of breeding season [282].

In the American toad, the decreased length of the breeding season at higher latitudes could result in increasingly explosive breeding with increased chorus density [286].In American Toads, this could have a significant effect as American Toads have a bimodal breeding behaviour with individual males alternating between a static singing behaviour and a dynamic scrambling behaviour where they actively pursue females with the proportion of animals engaged in one or the other behaviour is often density dependent

[260-262]. These scrambling males form a gauntlet to intercept females as they approach the singing males and this may increase male-male competition [125]. Although it has been shown that persistent singers have the highest reproductive success rate, it is energetically expensive and has a high predation risk whereas, scramblers have decreased success but also decreased energy demand and predation risk [261, 263]. Furthermore, at lower temperatures, the energetic cost of singing becomes unsupportable [261]. Calling vs. scrambling behaviours appear to favour differing morphologies [264, 265] with studies of Common Toads (*Bufo bufo* Bufonidae) [263] and American Toads [265, 287, 288] finding that larger body size was selected for among calling toads, while longer limbs would be advantageous in scrambling males [128, 264, 289, 290].

If my hypothesis that grappling behaviour can drive morphological changes in the American Toad is correct, then I therefore predict that there would be a greater proportion of male toads engaged in scrambling behaviour at higher latitudes, or that those engaged in scrambling behaviour would have a higher reproductive success. This scrambling behaviour could favour males with longer legs and narrower heads and an increased importance of visual cues, and therefore male toads in the north should have relatively longer legs and narrower heads (transitivity), compared to southern populations [291, 292].

My findings demonstrate a correlation between latitude and morphology within a widespread North American ectotherm. However, the fundamental drivers of the relationship and the selective pressures acting on the species remain elusive. My observations of changes in morphology have been found in areas that have been occupied through post-glacial re-colonization of northern regions in the last 10,000 years ago. While temperature may play a strong role in determining species range, my findings suggest that other environmental factors may have an effect, and that the influence of breeding behaviour on morphological variation may be playing a greater role than previously suspected.

Acknowledgements. — I would like to thank A. Howell, D. Kizirien, J. Jacobs and M. Steigerwald for their assistance. All legal and ethical guidelines were followed in the collection and care of both living and preserved specimens. All handling of animals was performed under McGill Animal Use Protocol No. 4569 as well as collection and handling permit No. 2014–05–23–018–00–S–F from the Government of Québec.

TABLE 1.—Morphometric measurements for all toads across the entire latitudinal range. Values are presented for all toads grouped together and then when toads are divided according to sex. SVL and unadjusted limb measurements are presented in mm and in the following format: mean ± standard error (minimum value – maximum value). Adjusted measurements are presented in the following format: minimum value – maximum value ± standard error.

	All toads	Female toads	Male toads
SVL (mm)	57.7 ± 0.42 (11.6 – 95.3)	58.6 ± 1.2 (11.6 – 95.3)	57.3 ± 0.65 (29.6 – 73.3)
Unadjusted forelimb (mm)	47.4 ± 0.4 (20.7 – 72.2)	47.6 ± 0.9 (20.7 – 72.2)	47.4 ± 0.3 (23.9 – 67.2)
Unadjusted hindlimb (mm)	89.5 ± 0.3 (40.0 – 135.6)	89.4 ± 1.8 (40.3 – 135.6)	89.5 ± 0.7 (40.0 – 115.5)
Adjusted forelimb	−10.9 – 48.1 ± 0.2	−10.1 – 47.6 ± 0.5	−9.0 – 20.3 ± 0.2
Adjusted hindlimb	−20.8 – 90.9 ± 0.4	−27.3 – 88.2 ± 1.0	−20.8 – 17.1 ± 0.4
Unadjusted cranial width (mm)	23.1 ± 0.2 (10.2 – 39.3)	23.5 ± 0.5 (11.4 – 39.3)	22.9 ± 0.5 (11.4 – 39.3)
Unadjusted cranial length (mm)	16.0 ± 0.1 (7.6 – 24.8)	16.1 ± 0.3 (7.6 – 24.8)	16.0 ± 0.1 (8.1 – 23.9)
Adjusted cranial width	−8.8 – 22.4 ± 0.1	−5.7 – 22.6 ± 0.3	−8.5 – 4.4 ± 0.1
Adjusted cranial length	– 10.2 – 13.0 ± 0.1	−10.0 – 12.5 ± 0.2	−3.1 – 7.2 ± 0.1

Figure Legends

FIG. 1.—Sampling localities of specimens overlaid onto a map of the geographic range of the American Toad.

FIG. 2.—Box plots of body sizes (SVL) male and female American Toads irrespective of location of capture.

FIG. 3.—Linear regression analyses of body size (SVL) vs. latitude for individual female (A) and male (B) American Toads. For female toads, adjusted $R^2 = -0.002$; for male toads adjusted $R^2 = 0.007$.

FIG. 4.—Hindlimb length (A, B) forelimb length (C, D) and head width (E, F) vs. latitude among individual female (A, C, E) and male (B, D, F) American Toads. All data are plotted as the residuals of a regression against SVL in order to compensate for variation in individual body size.

FIG. 5.—Variation in head morphology among individual female and male American Toads. Showing the solid consensus points with surrounding variation between individuals for all 24 landmarks recorded per individual.

FIG. 6.—Relative head shape from geometric morphometric analysis in relation to latitude for (A) female and (B) male American Toads. For female toads, $R^2 = 0.007$; for male toads $R^2 = 0.076$.

Figure 1.

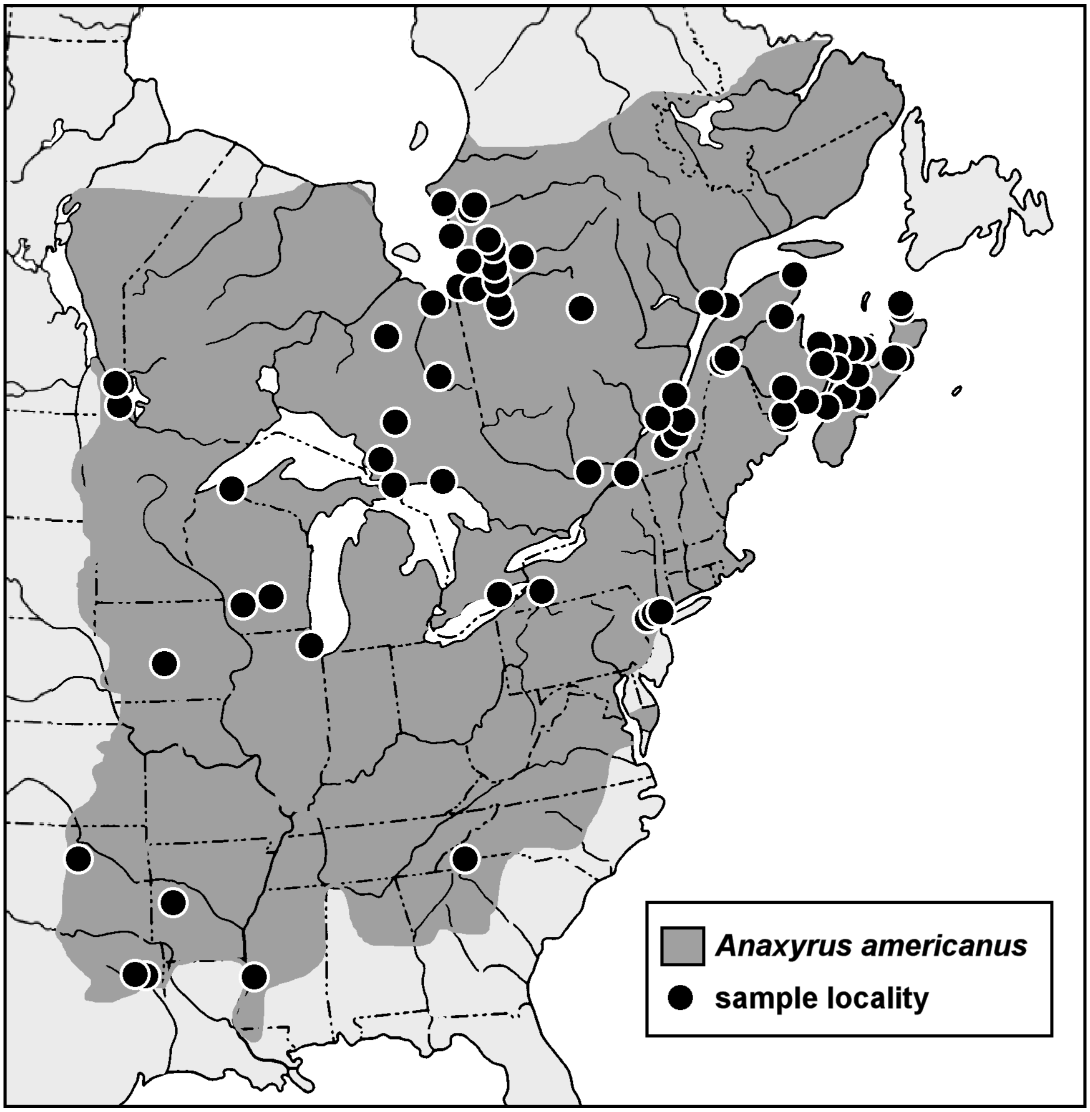


Figure 2.

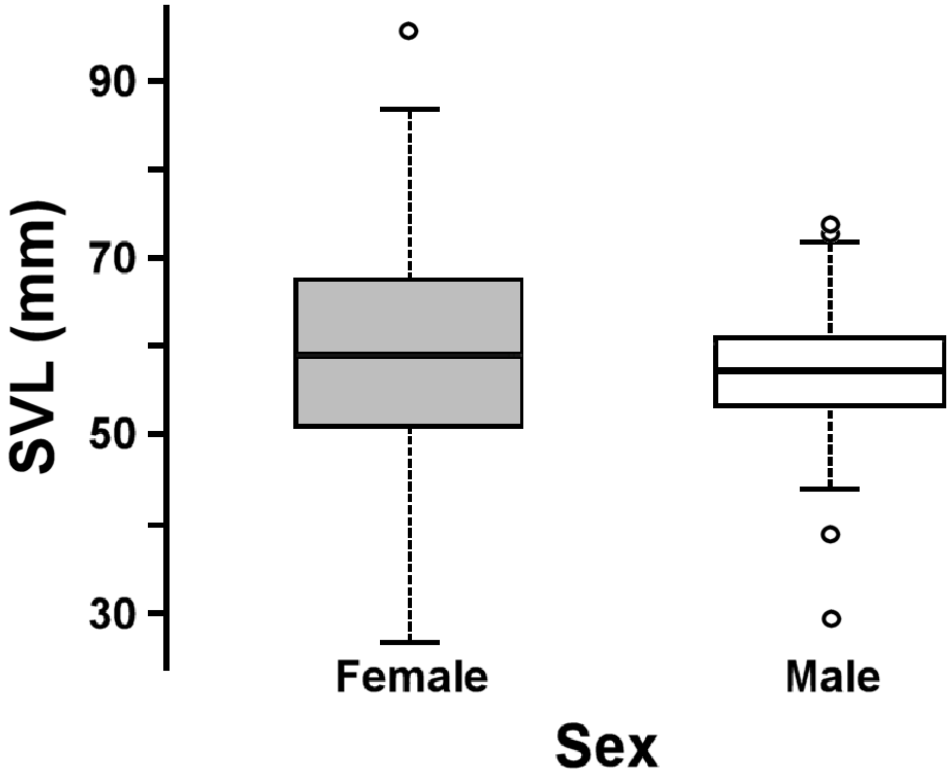


Figure 3.

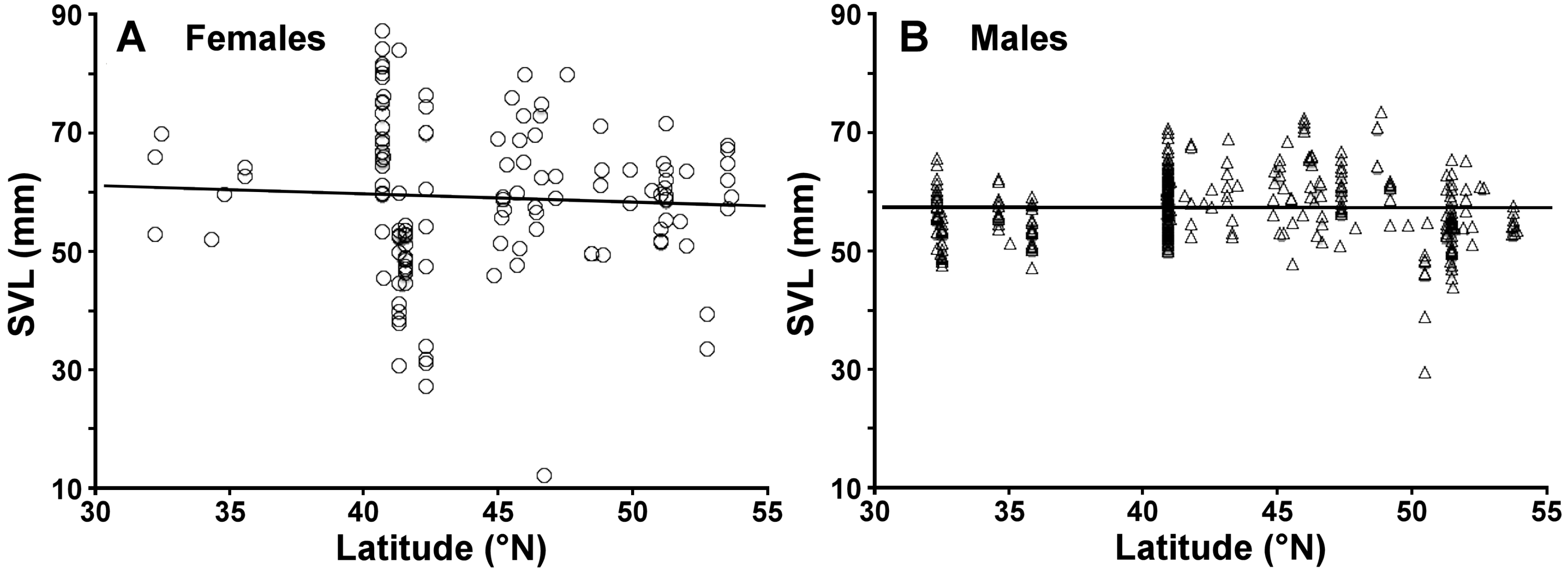

A
Females
B
Males
SVL (mm)
Latitude (°N)
90
70
50
30
10
30
35
40
45
50
55

Figure 4.

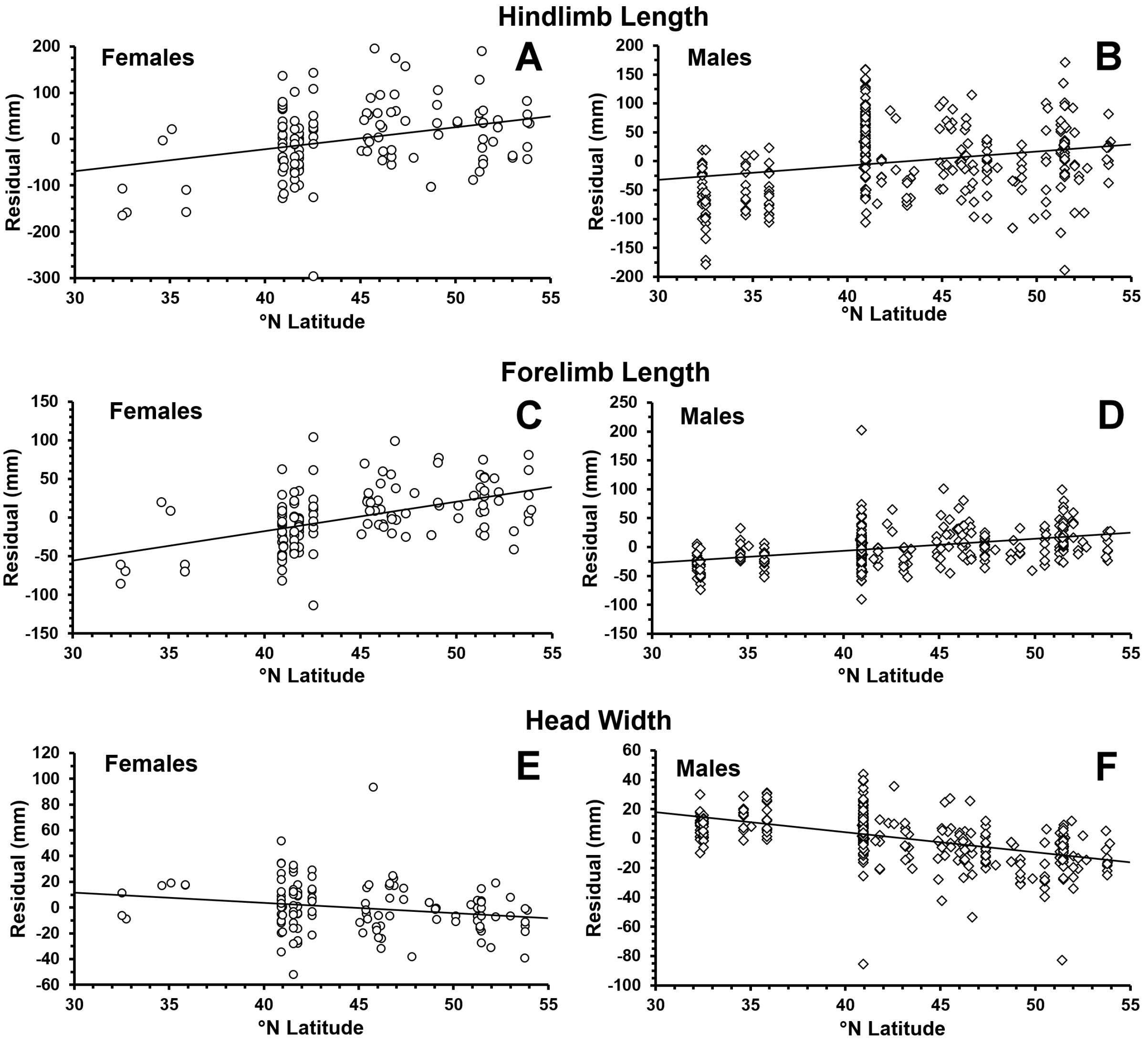

Hindlimb Length
Females
A
Males
B
Residual (mm)
°N Latitude
200
100
0
-100
-200
-300
150
50
-50
-150
30
35
40
45
50
55
Forelimb Length
Females
C
Males
D
250
Head Width
Females
E
Males
F
120
80
60
40
20
-20
-40
-60
-80

Figure 5.

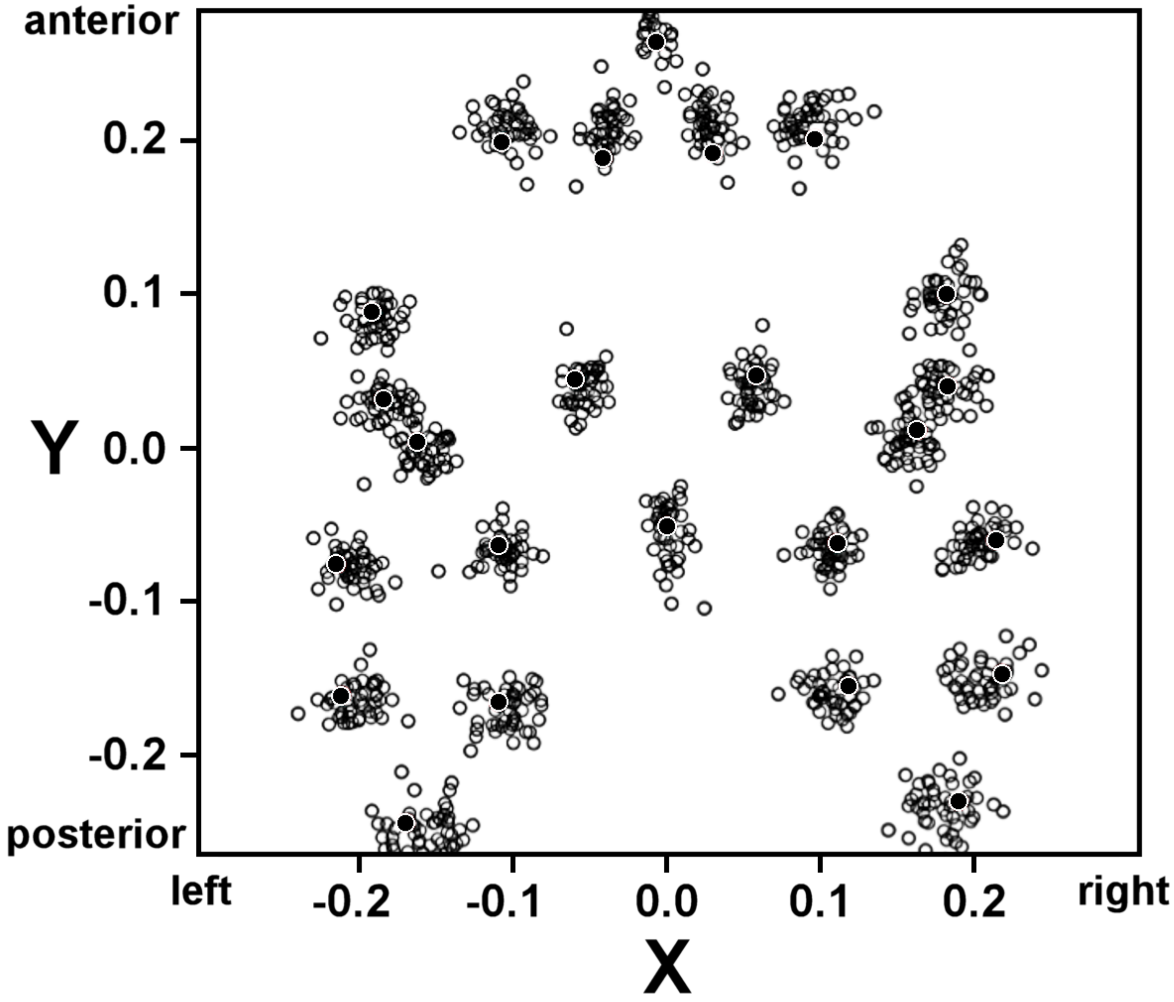
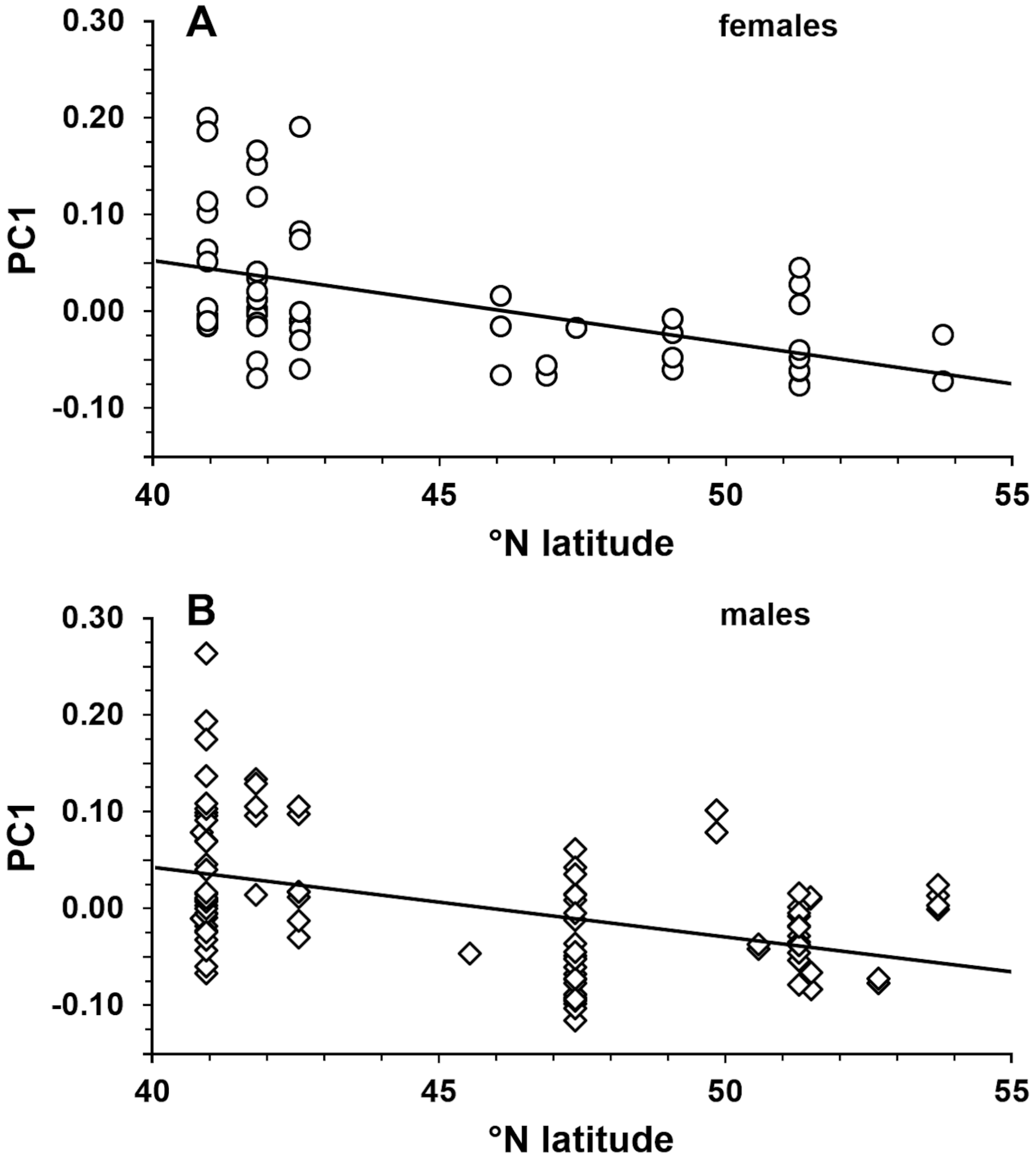


Figure 6.


A
females
PC1
0.30
0.20
0.10
0.00
-0.10
40
45
50
55
°N latitude
B
males
PC1
0.30
0.20
0.10
0.00
-0.10
40
45
50
55
°N latitude

Chapter 5: Evidence of Adaptation to Local Climate According to Latitude Among Populations of the American Toad, *Anaxyrus americanus*

Linking Statement for Chapter 5:

Herein, I present my findings of growth rates of American Toad tadpoles from the extremes and middle of the range and how these populations may be adapting to the local climate regimes where they are found.

Growth and development times of tadpole development can be important factors in both juvenile survival and adult fitness. As a result, exposure to temperatures outside of the optimum range can have a dramatic effect on a species, and adaptation to continued exposure to temperature extremes would be highly beneficial. For the American Toad, with a range extending over 2,600km there are significant differences in the growth conditions of tadpoles of southern populations versus those of northern populations. However the extent to which different populations have adapted to their local climatic conditions is largely unknown. This presents a significant problem with regards to predicting species' response to climate change. Recent theoretical models suggest that local adaptations may play a crucial role in species' response. Herein I have used a common garden experiment with American Toad tadpoles to empirically explore the degree of response to local climate as reflected in the variation in growth rate within and between populations of American Toad tadpoles when raised at different temperature and light conditions. This study represents novel work exploring the degree of local adaptation in the temperature dependent growth rate of larvae in a species with a large geographic and latitudinal range. My findings herein have important implications in the modelling of species ranges, dynamics and response to climate change.

The references in the following chapter have been renumbered and are included in the combined bibliography at the end of this thesis in numerical order following the sequence in which they appear throughout the entire thesis. This chapter has been formatted for submission to American Midland Naturalist as a manuscript co-authored with Dr. David M. Green.

Abstract .—Species with very large ranges have classically been considered more resilient to climate change than species with small ranges. This idea is based on an assumption that a change in climate in one region of a large range will likely result in a climate that is similar to one found elsewhere in the range. Therefore the species will have sufficient phenotypic plasticity to cope. However, if populations are specifically adapted to their local climates, how each individual population may respond to climate change may be significantly different. If each population's temperature tolerances are narrow, then the species as a whole may be at risk in the face of climate change. Accordingly, I tested the extent of adaptation to local climate regimes in tadpoles of the American Toad, an amphibian whose range covers a very large latitudinal extent, from subtropical to subarctic climates. I subjected tadpoles sampled from widely separated populations to a common garden experiment that assessed survival and development times under thermal and light conditions mimicking those of northern, southern and middle latitudes. I found that tadpoles from 54° N grew significantly faster and survived significantly better in cold temperature/long day conditions compared to tadpoles from either 45° or 34° N, but that all tadpoles had similar survival rates and development times when raised in warm temperature/short day conditions. These findings are consistent with the presence of adaptations to local climate, including increased plasticity, in northern populations that may shield them from the effects of rising climatic temperatures.

Introduction

Non-migratory species of animals that occur over very large geographic ranges almost invariably have populations that inhabit regions characterized by differing climatic conditions. Therefore, if such species are treated as though all populations throughout the range have similar temperature tolerances, this climatic heterogeneity and its impact on the evolutionary and adaptive history of these species will greatly underestimated [56, 58, 293]. For example, when contemporary species range models, like ecological niche modelling (ENM), treat species as homogeneous (e.g. [294-297], the possible impacts of phenotypic plasticity and natural selection on species responses to changing or variable environments can be underestimated [28].

The local climate that any one population is exposed to may represent only a subset of the total range of climates encountered by that species [56, 293]. Because climate has its effects at the resolution of populations and not the species, an altered climate may be novel for a specific population even if other populations of the same species already experience those climatic conditions [58]. Therefore, the extent to which local populations have adapted to their respective climates determines the extent of heterogeneity in the species' response to climatic change as a whole [28, 298]. This can be especially important in species with very large ranges, which have often been considered more resilient to climate change *ipso facto* [58, 293], but may actually exhibit considerable local adaptation in response to climate change that is not, as yet, well understood [251, 299-309].

A potentially good example of a widespread species exhibiting adaptation to local climatic regimes is the American Toad, *Anaxyrus americanus*, which is a widespread and abundant amphibian found throughout most of eastern North America [242]. Its range, which spans a greater extent of latitude than almost any other amphibian, extends from Baton Rouge, Louisiana (30°N latitude) to Kuujjuarapik, Québec (56°N latitude), a distance of over 2,790 km [242]. From north to south across this huge range, there exists tremendous variation in summer day length, average annual temperature and average daily and monthly temperatures. At their southern limit, American Toads breed in February or March, when daylight is less than 12 h per day and average daytime temperatures can approach 20°C [260]. At their northern limit, American Toads reproduce in June, when daylight can exceed 16 to 18 h per day and average daytime temperatures can still be as low as 0°C to 4°C [310].

Among the developmental processes most likely to be affected by latitude in the American Toad are the timing, rate and duration of tadpole development. Compared to the southernmost populations, the northernmost populations of American Toad experience a summer that is much shorter but with many more hours of daylight. If populations of American Toads at such differing latitudes are locally adapted with regards to tadpole development, then tadpoles from different latitudes, if reared under common conditions controlling for temperature and day length, should be expected to grow and develop best under those conditions characteristic of their place of origin.

Methods and Materials

American Toad egg clutches or recently hatched tadpoles were collected at the onset of breeding and transported to the Redpath Museum in Montréal, QC, from three populations (Fig. 1): Athens, GA (33°57'N, 83°22'12"W; eggs collected 15 March, 2014), Ste-Anne-de-Bellevue, QC (45°25'48"N, 73°55'48"W; eggs collected 14 April, 2014) and Radisson, QC (53°45'36"N, 77°34'12"W; tadpoles, Gosner stage 24 [311], collected 10 June, 2014). Eggs from Athens were put in plastic bags with pond water upon collection and shipped overnight on ice in a Styrofoam cooler. Eggs from Ste-Anne-de-Bellevue were placed in pond water in 20L buckets transported by vehicle. Tadpoles from Radisson were placed in a 40L cooler and transported by vehicle immediately upon collection. Sibship of eggs and tadpoles could not be determined but multiple egg masses were sampled in the breeding ponds. After arrival at the Redpath Museum's animal facility, eggs were held at 16°C in Pyrex pans until hatching.

After hatching, tadpoles were placed individually in 1L polyethylene deli containers to preclude intraspecific competition, which could otherwise complicate the outcome of a common garden experiment [312]. All tadpoles were reared in reconstituted water which was brought to a medium level of hardness from distilled water with the addition of Kent Marine Liquid R/O Right (Kent Marine, Walnut Creek, CA). Water changes consisted of removing and replacing 1/3 of the volume of the container and were performed every three days. Tadpoles were fed *ad libitum* with frozen chopped spinach that was cooked as per the instructions on the package. Any uneaten spinach was removed and replaced on a daily basis.

All tadpoles were photographed upon placement into a container. Once a tadpole had reached Gosner stage 42 [311], i.e. the emergence of all four limbs, the time from placement in the treatment until limb emergence was recorded. Immediately upon reaching stage 42, a tadpole was removed from its container, weighed, photographed and placed into a nursery. Nurseries were circular flat-bottomed Pyrex containers, within which 5 mm of water and paper towels provided a refuge for metamorphs and prevented the emergent tadpole from either drowning or desiccating. Nurseries were kept in the water baths of their respective temperature treatments and the temperature of the water and the air in the nursery was maintained at the temperature of the tadpole treatment. Upon complete reabsorption of the tail (Gosner stage 46), newly metamorphosed toadlets were removed from their nurseries, re-photographed and re-weighed. The time, in days to go from stage 42 to 46 was recorded. Toadlets were euthanized in a solution of MS-222 before preservation in 95 % ethanol.

Tadpoles were raised under three different temperature treatments combined with two different light treatments. Tadpole deli containers were placed in one of three water baths with constant water circulation to ensure that all individuals in a treatment were raised at the same temperature. All experiments were conducted in a room air-conditioned to a constant 16° C, which resulted in the coldest treatment being at this ambient temperature. For the 20°C and 23°C treatments, immersion aquarium heaters placed in the water baths to constantly maintain the desired rearing temperature. Current air and water temperatures, as well as the minimum and maximum temperatures from the previous 24 hours, were recorded twice daily. To simulate different day/night regimes, tadpoles were raised under either a 16:8 h light-dark cycle, intended to emulate the sub-arctic conditions at Radisson, QC, or a 12:12 h light-dark cycle, emulating the sub-tropical conditions of Athens, GA. One half of each water bath was under the 16:8 h cycle and the other half was under the 12:12 h cycle

The absolute time from introduction into the experiment to either Gosner stage 42 or Gosner stage 46 varied between tadpoles from different populations, precluding direct comparisons between populations. Therefore, to improve comparisons between locations, relative development times were used. The time required to reach Gosner stage 42 or stage 46 of each tadpole was normalized to the average time, to either stage 42 or stage 46 respectively, for tadpoles from that location in the 23°C treatment. The 23°C treatment was used as this was the treatment with the least amount of time from introduction to stage 42 and 46, and therefore all relative measurements were multiples of this fastest development time.

The relationships between both mass and time of development versus temperature and light regime were initially assessed with single variable ANOVA for tadpoles from each of the locations of origin. Linear mixed effects regressions (LMER) were then used to test for covariant effects, with temperature and light regime as fixed effects and mass and the time to reach either Gosner stage 42 or 46, or from Gosner stage 42 to 46, as random effects. Significance was determined through maximum likelihood tests for each mixed model against its null, which did not contain the fixed effect being examined. All statistical analyses were performed in R (v3.2.0 R Institute for Statistical Computing Vienna, Austria) using the lme4 package for LMER analysis. Analyses were performed for the effect of origin between tadpoles from only Athens and Ste-Anne-de-Bellevue as well as between all three locations of origin. This was done as the tadpoles from Radisson were introduced into the experiment at a much later development stage than either the tadpoles from Athens or Ste-Anne-de-Bellevue.

Results

A total of 720 tadpoles were initially introduced into the treatments. Of these, 252 tadpoles survived through to complete metamorphosis. Raised at 23°C, tadpoles from Athens had a 52.5% survival rate, tadpoles from Ste-Anne-de-Bellevue had the highest survival at 61.3% and tadpoles from Radisson had the lowest survival at 32.5% (Table 1). In the 20°C treatment, tadpoles from Athens had 41.3% survival rates, while tadpoles from Ste-Anne-de-Bellevue had a 50% survival rate and tadpoles from Radisson also had a 41.3% survival (Table 1). In the 16°C treatment, tadpoles from Athens and Ste-Anne-de-Bellevue had survival rates of 13.8% and 1.3%, respectively, whereas tadpoles from Radisson had the highest survival at 21.3% (Table 1).

Tadpoles from Radisson developed more rapidly but weighed less than tadpoles from either Athens or Ste-Anne-de-Bellevue at comparable stages of development (Table 2). Across all treatments the time taken to reach Gosner stage 42 from hatching and introduction into the experiment, differed significantly among tadpoles from Athens vs. Ste-

Anne-de-Bellevue (LMER: df = 2, χ^2 = 96.418, $P < 0.001$) and the three different sites (LMER: df = 2, χ^2 = 144.73, $P < 0.001$); tadpoles from Athens took 43.46 (± 23.37) days, those from Ste-Anne-de-Bellevue took 55.79 (± 7.68) days, and those from Radisson took 7.92 days (± 3.33). To go from Gosner stage 42 to Gosner stage 46 (complete absorption of the tail), tadpoles from Athens took 4.87 ± 2.91 days, those from Ste-Anne-de-Bellevue took 4.61 ± 2.24 days, and those from Radisson took 3.06 ± 2.46 days (Table 2), showing significant differences between tadpoles from Athens vs. Ste-Anne-de-Bellevue (LMER: df = 2, χ^2 = 4.186, $P = 0.041$) and among the three sites (LMER: df = 2, χ^2 = 2.392, $P < 0.0001$). There was an overall reduction in overall time from introduction, upon hatching at stage 20 for Athens and Ste-Anne-de-Bellevue tadpoles and stage 26 for Radisson tadpoles, to stage 46 (Table 2) of tadpoles from Athens and Radisson by 30.8 % (48.3 ± 24.78 days) and 68.6 % (11.0 ± 5.44 days), respectively, when compared to tadpoles from Ste-Anne-de-Bellevue (60.4 ± 7.28 days), with significant differences between tadpoles from Athens vs. Ste-Anne-de-Bellevue (LMER: df = 2, χ^2 = 87.478, $P < 0.001$) and among the three sites (LMER: df = 2, χ^2 = 119.34, $P < 0.001$).

Tadpoles from Athens, Ste-Anne-de-Bellevue and Radisson weighed, on average across treatments, 0.26 ± 0.07 g, 0.20 ± 0.05 g, and 0.16 ± 0.05 g, respectively; when they reached stage 42 whereas, at stage 46, they weighed, on average, 0.16 ± 0.04 g, 0.15 ± 0.04 g, and 0.09 ± 0.03 g, respectively. There was a significant difference between localities in terms of average tadpole mass at Gosner stage 42; Ste-Anne-de-Bellevue and Radisson tadpoles had 11.5 % and 12.6 % less mass than Athens tadpoles (Table 2), these differences were significant between tadpoles from Athens vs. Ste-Anne-de-Bellevue (LMER: df = 2, χ^2 =

4.140, P = 0.042) and among the three sites (LMER: df = 2, χ^2 = 9.875, P = 0.007). However, there was no significant difference between localities in terms of mass at stage 46 when comparing between tadpoles from Athens vs. Ste-Anne-de-Bellevue (LMER: df = 2, χ^2 = 1.193, P = 0.167) and those from all three sites (LMER: df = 2, χ^2 = 2.228, P = 0.328).

Overall, tadpoles reared at 20°C took 66.3 % longer to reach stage 42 than those raised at 23°C whereas those raised at 16°C took 171.0 % longer to reach stage 42 than tadpoles raised at 23°C, when controlling for location of origin and light effects. These timings were significantly different for tadpoles from Athens vs. Ste-Anne-de-Bellevue (LMER: df = 2, χ^2 = 234.51, P < 0.001) and for when tadpoles from all three locations were included (LMER: df = 2, χ^2 = 187.90, P < 0.001). Tail absorption between stage 42, and until stage 46 when absorption was complete, was retarded by 5.0 % and 191.0 % for tadpoles reared at 20°C and 16°C respectively compared to those in 23°C. These timings were significantly different among the three temperatures for tadpoles from Athens vs. Ste-Anne-de-Bellevue (LMER: df = 2, χ^2 = 72.468, P < 0.001) and for when tadpoles from all three locations were included (LMER: df = 2, χ^2 = 131.64, P < 0.001). Overall tadpole development from introduction into the experimental treatment until stage 46 was 55 % longer in 20°C rearing temperatures and 173.8 % longer in 16°C rearing temperatures compared to 23°C rearing temperatures. These changes were significantly different between the three temperatures for tadpoles from Athens vs. Ste-Anne-de-Bellevue (LMER: df = 2, χ^2 = 207.71, P < 0.001) and for when tadpoles from all three locations were included (LMER: df = 2, χ^2 = 191.54, P < 0.001). Tadpoles reared at 20°C and 16°C had 9.3 % and 17.8 % greater mass respectively than those reared at 23°C at stage 42, the time of emergence. These differences in mass were significantly different among all three temperatures for tadpoles from Athens vs. Ste-Anne-de-Bellevue (LMER: df = 2, $\chi^2 = 26.273$, $P < 0.001$) and for when tadpoles from all three locations were included (LMER: df = 2, $\chi^2 = 8.784$, $P = 0.012$). Following absorption of the tail newly metamorphed toadlets from 16°C treatments weighed 17.2 % more than those from 23°C treatments and those in 20°C treatments weighed 18.5 % more than those from 23°C. These changes in mass following complete reabsorption were also significantly different for all three temperatures for tadpoles from Athens vs. Ste-Anne-de-Bellevue (LMER: df = 2, $\chi^2 = 17.372$, $P < 0.001$) and for when tadpoles from all three locations were included (LMER: df = 2, $\chi^2 = 21.14$, $P < 0.001$).

Tadpoles raised at different temperatures had significant differences in the time required from introduction to reach Gosner stage 42, the emergence of all four limbs when examining only tadpoles from Athens and Ste-Anne-de-Bellevue (ANOVA: $F_{2,171} = 173.7$, $P < 0.001$) and those from all three locations (ANOVA: $F_{2,245} = 74.79$, $P < 0.001$), and from Gosner stage 42 until Gosner stage 46, the complete reabsorption of the tail for tadpoles from Athens and Ste-Anne-de-Bellevue (ANOVA: $F_{2,149} = 33.6$, $P < 0.001$) and when those from Radisson were also included (ANOVA: $F_{1,219} = 94.91$, $P < 0.001$). Total time from introduction into the experiment until Gosner stage 46, was therefore also significantly different depending upon rearing temperature for tadpoles from Athens and Ste-Anne-de-Bellevue (ANOVA: $F_{2,151} = 152.2$, $P < 0.001$) and those from all three locations (ANOVA: $F_{2,222} = 88.67$, $P < 0.001$). Tadpoles reared in different temperatures also had significant differences in mass at Gosner stage 42 for tadpoles from Athens and Ste-Anne-de-Bellevue (ANOVA: $F_{2,171} = 15.5$, $P < 0.001$) and those from all three locations (ANOVA: $F_{2,245} = 4.23$, P

= 0.02) and at stage 46 for tadpoles from Athens and Ste-Anne-de-Bellevue (ANOVA: $F_{2,151}$ = 8.319, P = 0.001) and those from all three locations (ANOVA: $F_{2,222}$ = 10.43, P < 0.001).

When controlling for locality of origin and temperature, tadpoles raised under the light regime of Athens took 2.65 days longer to reach Gosner stage 42 (LMER: df = 1, χ^2 = 2.324, P = 0.127), and had a 0.02 day reduction in time to absorb their tails (LMER: df = 1, χ^2 = 0.566, P = 0.452) and had an overall increase by 2.69 days in development time (LMER: df = 1, χ^2 = 0.637, P = 0.425) when compared to those raised under the longer subarctic days of Radisson, these differences were not significant when tadpoles from all locations were examined and remained the case when tadpoles from Radisson were excluded (LMER: df = 1, χ^2 = 0.409, P = 0.522, df = 1, χ^2 = 0.062, P = 0.803, df = 1, χ^2 = 0.411, P = 0.522 respectively). The mass of toadlets at stage 46, complete metamorphosis was significantly different depending on light treatment. Those raised under the Athens light regime weighed 8.2 % less than those raised under the Radisson light regime and this difference was significant when all tadpoles were considered (LMER: df = 2, χ^2 = 5.552, P = 0.018) and when tadpoles from Radisson were excluded from the analysis (LMER: df = 2, χ^2 = 4.817, P = 0.028).

The time from introduction into the experiment to Gosner stage 42 was significantly different depending upon the light regime under which the tadpole was raised when all tadpoles were included (ANOVA: $F_{1,246}$ = 4.91, P = 0.03) and when tadpoles from Radisson were excluded from the analysis (ANOVA: $F_{1,172}$ = 6.38, P = 0.01) but the time from stage 42 to stage 46 was not different for all tadpoles (ANOVA: $F_{1,219}$ = 0.33, P = 0.57) nor for only tadpoles from Athens and Ste-Anne-de-Bellevue (ANOVA: $F_{1,150}$ = 1.61, P = 0.21). Interestingly, total time from introduction into the experiment until stage 46 was not different between tadpoles raised under different light regimes when all tadpoles were examined (ANOVA: $F_{1,223}$ = 2.90, P = 0.09), however it was different when tadpoles from Radisson were excluded (ANOVA: $F_{1,152}$ = 5.13, P = 0.02). Tadpoles from the three different populations also did not have significantly different mass at stage 42 dependent upon their rearing light regime for all tadpoles (ANOVA: $F_{1,246}$ = 0.004, P = 0.95) and when tadpoles from Radisson were excluded (ANOVA: $F_{1,172}$ = 0.29, P = 0.62). However, when controlling for other variables, the mass at complete reabsorption of the tail was different for metamorphs raised under different light conditions when tadpoles from Radisson were included (ANOVA: $F_{1,222}$ = 4.24, P = 0.04) but not when tadpoles from Radisson were excluded (ANOVA: $F_{1,152}$ = 2.47, P = 0.12).

Discussion

My findings are consistent with a hypothesis of local adaptation within a very widespread species in that I found significant indications that tadpole growth in American Toads from higher latitudes appears to have improved performance under conditions of relatively longer days and colder temperatures. The decreased development time of the Radisson tadpoles was, however, in part related to their introduction into the experimental treatment as hatched tadpoles. The time between Gosner stage 20, when tadpoles from Ste-Anne-de-Bellevue and Athens were introduced in the experiment, and Gosner stage 26 could not be accounted for Radisson tadpoles as they were introduced into the experiment

Gosner stage 26. However my findings were consistent when comparing between all three locations of origin and also when the Radisson tadpoles were excluded and comparisons were made only between tadpoles from Athens and Ste-Anne-de-Bellevue.

The apparent propensity for increased growth rate in cold temperatures among more northern populations does not seem to be at the expense of rapid growth in warm temperatures, since high latitude tadpoles, like others tested, maintained their most rapid growth in the warmest treatment. The nature of the selective pressures on far northern populations may thus favour greater phenotypic plasticity compared to more southerly populations, as demonstrated in Pool Frogs, *Pelophylax lessonae* in northern Sweden [312] where previous studies had shown very low variability in genetic markers between populations [313, 314]. Northern populations of both Pool Frogs and American Toads experience fewer episodes of warm weather and, as a result, the ability to maintain a high growth and developmental rate at cool temperatures should be highly adaptive. The retention of growth performance of tadpoles of these northern populations in consistently warm conditions may be a retained trait in consequence of their recent penetration of previously glaciated territory. Similar results of increased growth with increased temperature have been observed in many other animals, including the Brackish Water Mysid Shrimp, *Neomysis integer* (Fockedey *et al*. 2005), Atlantic Salmon, *Salmo salar* (Handeland *et al*. 2008) and Eastern Indigo Snake, *Drymarchon couperi* [315-319].

Observations of adaptability to environemental change has also been observed in other amphibian species, including salt tolerance in Wood Frogs, *Lithobates sylvaticus* [320] and Spotted Salamanders, *Ambystoma maculatum*, [321] and acid tolerance in Moor Frogs, *Rana arvalis,* [322, 323]. However, environmental change is not neccesarily beneficial for organisms. Eelgrass, *Zostera marina*, for example, suffers increased mortality and decreased overall growth at increased temperatures, possibly because it is already living at temperatures approaching the upper limit of its physiological tolerance [324]. Similarly, the European Common Frog, *Rana temporaria*, demonstrates adaptation to small, chronic increases in nitrate, but suffers high mortality when exposed to acute, high levels of nitrate [325]. It seems then, that although species do seem to express adaptations to small or gradual environmental change and stresses, significant mortality can occur due to exposure beyond the limits of this adaptability.

Range edge populations may have reduced genetic variation either through founder effects or bottlenecks during range expansion or simply through small population size (Willi *et al.*, 2006, Rehm *et al.*, 2015). Within the American Toad there appears to be a considerable amount of genetic variation among populations on both sides of the Wisconsinan glacial terminal moraine [326-328]. A possible explanation for this is that populations could have novel elements of genetic diversity as a result of local selective pressures [327, 328], which may represent novel adaptations that contribute to accelerated growth in cold conditions of high latitude populations [329]. However, the American Toad has not been studied in this regard.

My empirical work indicates that there may be local adaptation in populations in cold climates that may not be detrimental in a warming climate if ancestral temperature tolerances are retained. However I was unable to rigorously discriminate between adaptive and plastic response, to do so would have required multigenerational testcrosses [330], which were unfeasible in this specific context. These results mirror the finding of increased phenotypic plasticity in northern populations of Pool Frogs [312], and help to demonstrate the effect that a highly heterogeneous environment can have on the temperature tolerances among populations of a species whether these effects drive phenotypic plasticity or adaptive response.

If populations' thermal niches are too narrow and highly optimized to their local conditions, then the species as a whole may be at risk, especially if cold adapted populations block potential range shifts by more locally adapted populations [58]. Furthermore, decreased diversity and increased local adaptation may also increase extirpation risk as high diversity may be of increasing importance to allow for a greater *in situ* response of species under stress from climate change [331]. While phenotypic plasticity may be responsible for many of the observed adaptations to climate change it may insufficient to compensate for all the impacts of climate change [332-334]. Although the precise genetic basis underlying adaptation to climatic differences in ectotherms remains largely unclear [34], it could be the result of either novel adaptation or variation in gene expression [335]. However, plasticity may also be responsible for increases in the upper tolerance limits of a species, although its role in lower limits is likely much more limited [336]. Furthermore, the possibility remains that local adaptation in range-edge populations of widespread species may be important reservoirs of genetic diversity [337]. Clarifying the generality of adaptive vs. plastic responses by species to environmental changes, i.e. their eco-evolutionary dynamics [338], will enhance our abilities to predict such responses.

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Table 1.—Survival of tadpoles from hatching to development stage 46 for tadpoles when ordered by location of origin, and rearing temperature.

Locality of Origin	Latitude	Rearing Temperature	Absolute and Percent Survival of Tadpoles from Hatching to Stage 46	
			Absolute Survival (out of 120)	Percent Survival (%)
Radisson, QC	53°48' N	16°C	9	21.3
		20°C	17	41.3
		23°C	39	32.5
Ste-Anne-de-Bellevue, QC	45°24' N	16°C	1	1.3
		20°C	20	50.0
		23°C	74	61.3
Athens, GA	33°57' N	16°C	6	13.8
		20°C	17	41.3
		23°C	63	52.5

Table 2.—Mean growth times, in days, from hatching to development stage 42 or 46 for tadpoles when ordered by location of origin, rearing temperature and light regime.

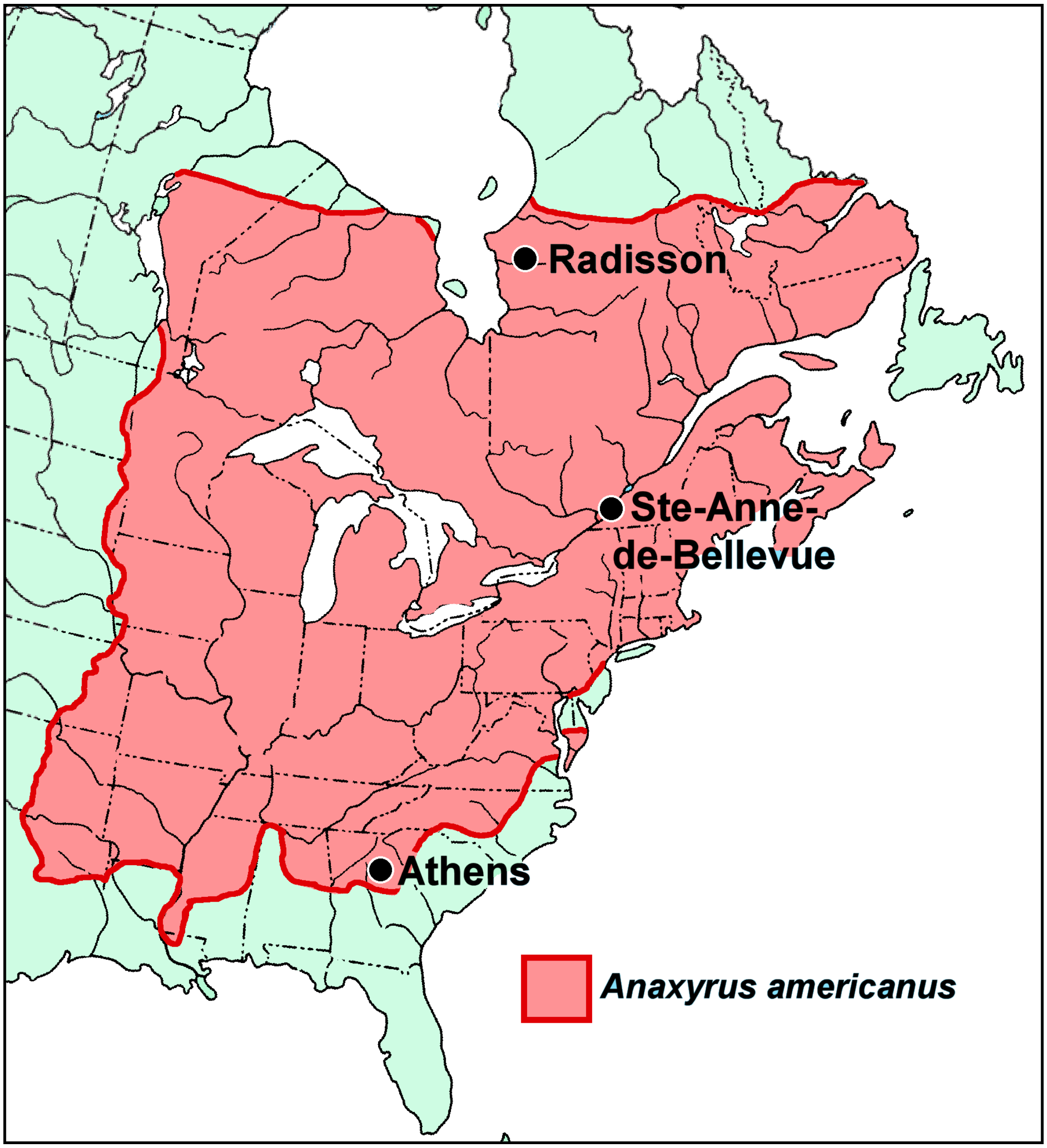
Locality of Origin	Latitude	Rearing Temperature	Light Regime	Time, in Days, from Hatching to Development Stage (Mean ± S.E.)	
				Stage 42 (4-limbs)	Stage 46 (tail reabsorption)
Radisson, QC	53°48' N	16°C	16h day	8.57±1.81	13.17±2.86
			12h day	13.60±5.96	20.50±8.80
		20°C	16h day	7.44±1.51	10.00±2.00
			12h day	6.82±1.14	8.37±1.42
		23°C	16h day	6.83±1.60	10.33±0.82
			12h day	6.60±0.94	8.20±1.15
Ste-Anne-de-Bellvue, QC	45°24' N	16°C	16h day	-	-
			12h day	103	109
		20°C	16h day	59.94±3.49	62.67±0.98
			12h day	60.05±3.66	64.00±3.88
		23°C	16h day	51.69±4.03	56.42±3.27
			12h day	51.04±4.62	56.88±3.50
Athens, GA	33°57' N	16°C	16h day	80	88
			12h day	87.3±3.68	97.75±6.02

20°C	16h day	58.33±2.93	62.75±3.36
	12h day	56.19±4.29	60.55±5.14
23°C	16h day	19.77±1.64	23.92±2.93
	12h day	23.23±3.64	27.47±4.50

Figure Legend

FIG. 1.— Native range of the American Toad, *Anaxyrus americanus*, in eastern North American including locations of studied populations.

FIG. 1.



Chapter 6: Discussion

Linking Statement for Chapter 6:

Herein, I have summarized the key findings from each of my chapters as well as placed them into the context of existing literature and proposed directions for future research based upon those questions that remain unanswered.

Overall, I have identified important barriers to range expansion and the distributions of herpetofaunal species in Canada. These findings should help to identify areas of post-secondary contact, and also assist in conservation practices of herpetofaunal species. Furthermore I assessed the role that behaviour can play in the establishment of hybrid zones, and genetic introgression between species. Although focused on the hybridization between the American Toad and the Canadian Toad, the model I present here can quickly be generalized and adapted to almost all hybridization interactions. Contact between species may increase in the future due to climate change and artificial introductions, and understanding the role of behaviour in hybridization and competition is essential to predict species' reactions.

In a detailed examination of the American Toad, I found that there were significant trends in morphological variation with latitude. These trends were sex-specific, and often counter-intuitive compared to generally accepted ecological rules and past subspecies differentiations. Additionally, I examined tadpole growth and development in a common garden and found that populations from higher latitudes appear to be showing signs of adaptation to colder growing conditions.

This study represents an essential update to a powerful conservation tool in Canada, A novel work exploring the drivers of hybridization and genetic introgression between species. Additionally, this is the most detailed morphological assessment of the American Toad ever performed, and the first study to examine local adaptation in this species. My findings herein have important implications in the modelling of species ranges, dynamics and understanding how a species' evolutionary history may be influenced by habitat heterogeneity across a large geographic range.

The references in the following chapter have been renumbered and are included in the combined bibliography at the end of this thesis in numerical order following the sequence in which they appear throughout the entire thesis.

Discussion:

Using a multitude of approaches, herein I sought to determine some of the mechanisms that allow species to have very large ranges, and assess some of the drivers behind range dynamics and the limiting factors of range size. With data from previous studies, examination of museum specimens dating back to the turn of the 20th century, live caught specimens, common garden experiments and simulation modelling I have demonstrated general trends in Canadian herpetofaunal distributions with the COSEWIC Terrestrial Amphibians and Reptiles Faunal Provinces Map, as well as identified trends in morphology and physiological performance in the American Toad with relation to latitude, and explored drivers behind mtDNA introgression between species across a hybrid zone. I examined patterns of distribution of the ranges of closely related species and how these correspond to ecotonal patterns and geographic barriers in determining general trends across multiple species and genera. I explored how behaviour can play a significant role in the exchange and introgression of genetic material where the ranges of two closely related species collide. I also examined how an extensive range, and the resultant heterogeneous climate experienced by different populations, has impacted the morphological and physiological variation between and within populations of a single widespread species. I have found evidence of large-scale trends in morphology with latitude as well as adaptation to local-climate conditions. While the exact drivers of these changes remain elusive I present several hypotheses that may explain these observed trends.

Understanding the applicability of generalized trends to a single species can be a valuable tool in predicting patterns that should exist in species that have yet to be studied.

Furthermore, an improved understanding of how past geography and climate have impacted individual populations within a wide ranging species is essential to understanding how species develop, maintain, and are limited to their specific range and identifying possibly cryptic biodiversity within species [339, 340]. Through examining the distributions of species, it is possible to identify assemblages of species that are occupying similar habitats and identify where ectonal changes result in barriers to range expansion of individual species' and assemblages of species. By examining and delineating these congruencies in species assemblages I was able to identify distinct ecoprovinces of herpetofauna across Canada. However, when examining general trends in species range distributions patterns, I found that for both eastern and western North America, there were similar geographic barriers between for different species, however, in many cases clades within species themselves had arisen at a much earlier point. Furthermore, contemporary boundaries appear to either be reinforcing or maintaining much older divisions. In performing this review of phylogeographies across Canada, I determined that there were significant weaknesses in the coverage, and completeness for Canadian herpetofauna, weaknesses that I discuss in detail below.

Species may also be limited through their interactions with competitive species. In areas where closely related and competing species' ranges meet, hybrid zones may develop. Although hybridization may be common, hybrid zones tend to be fairly limited in geographic scope as it can be limited to regions of species range overlap [204], or where extensive overlaps exist but significant decreases in hybrid fitness limits the areas where populations of hybrids are maintained [205, 206]. Furthermore, hybrid zones can shift geographically, by increased competition of one parental species over another, changing fitness landscapes, or through simple random drift [127, 200, 204]. These movements are often unidirectional [200, 204], but there have been observations of oscillations in the hybrid zone location [127].

These hybrid zones may have important implications in the genetic composition of species as well as mtDNA introgression between species, and discordance between the mtDNA and DNA of individuals in a population is possible. In the case of movement of a hybrid zone or encroachment of one species into the range of another, mtDNA may remain in the same location, resulting in mtDNA flow from the local species into the invading species [341]. However introgression between species may also have a selective advantage, and this may also result in discordance between DNA and mtDNA [341].

There may also be additional behavioural elements that can act as drivers of mtDNA introgression between species. Herein I examined the role that differences in breeding behaviour can play in the introgression of mtDNA from one species to another. I found that differences in breeding strategy had a significant effect on the direction and extent of introgression but that habitat quality maintained DNA differences between the species. However, with modern climate change, gradients in habitat selective pressure may be changing as well. This was seen in the case of the threatened native Westslope Cutthroat Trout (*Oncorhynchus clarkii lewisi*), where climate change reduced the habitat selective pressures that had previously limited the spread of hybridization with invasive Rainbow

Trout (*Oncorhynchus mykiss*) and hybrids spread rapidly through the Westslope Cutthroat range [228].

In addition to geographic barriers, local climate also plays a significant role on the evolutionary development of a species. I found herein that there is evidence suggestive of local adaptation to temperature within the American Toad. I found that growth performance of American Toads were highly suited to their local thermal conditions. Although I was unable to discriminate whether this performance was based on heritable genetic changes or plasticity as required to rigorously demonstrate local adaptation, my findings were highly suggestive that local adaptation could be occurring. I found that American Toad tadpoles from northern populations had significantly improved growth performance in colder conditions compared to tadpoles from southern populations. Furthermore, the growth rate of tadpoles from northern populations in colder temperatures did not appear to have been be at the expense of growth in warm temperatures. Northern tadpoles, like other tadpoles tested, retained their most rapid growth in the warmest of the treatments. However, temperature may not encompass all the differences in environmental condition between populations as northern populations also experience significantly shorter growth seasons, as well as longer days in addition to colder water and air temperature compared to southern populations.

My findings suggest that for American Toads, individuals at higher latitudes are under a selective pressure for improved performance in cold conditions. The differences between low and high latitude populations are especially surprising given that American

Toad populations at high latitudes have only been established recently, as these regions were covered with either glaciers, or the sea as recently as 5,000 years ago. The maintenance of rapid growth rate of tadpoles in hot conditions may result in these high latitude populations being less vulnerable to climate change, and some warming may actually be beneficial to their growth rate. Similar findings have been found in the Brackish Water Mysid (*Neomysis integer*), Atlantic Salmon (*Salmo salar*), Domestic Chickens (*Gallus gallus domesticus*) and Eastern Indigo Snake (*Drymarchon couperi*), when they were tested at temperatures above those currently experienced, but below their physiological limit as well [315-319].

For species effectively at or approaching their thermal tolerance, such as Eelgrass (*Zostera marina*), any increase in temperature can result in increased mortality and decreased overall growth [316-319, 324]. These findings would suggest that there may not be an overall general trend in the effect of increased temperature on a species and that care must be taken to assess species at risk of temperature increases. In the case of the American Toad, the accelerated growth of high latitude populations in cold conditions may also represent a novel adaptation absent from southern populations and could be a form of cryptic biodiversity [329]. If the cold temperatures that are responsible for the selective pressures that drive this adaptation are lost then there could be a loss of cryptic biodiversity and this could have repercussions on the future development of the species [329]. Furthermore, any loss of genetic biodiversity can be especially important as a large diverse gene pool increases the resilience of a species to climate change [309, 331].

However, to ascertain the extent of cold climate adaptations in northern populations, further studies will be required, including a phylogeography of the American Toad with a much broader sampling area and greatly increased number of Northern samples to assess variation in the genetic composition of northern versus southern populations.

In addition to variation in the growth rate of populations in response to temperature, I also found that there were significant relationships between latitude and morphology between populations of American Toads, particularly with regard to limb length and head shape, but these latitudinal trends are not congruent for both sexes. In examining American Toads from localities spanning nearly 22° of latitude, head width decreased and forelimb length increased with increasing latitude regardless of sex whereas a positive correlation between hindlimb length and latitude was only found among female toads.

I found evidence that the classically reported sexual dimorphism in SVL for American Toads may not be the case among all populations. I found that there was not a consistent difference between the average SVL for male and female toads when examining toads from across the entire range. This goes in contrast with the literature regarding the American Toad, including the most recent book by Dodd: Frogs of the United States and Canada [122]. I did however find that those toads expressing a sexual dimorphism of SVL were in regions where most of the classical research was done. This could therefore explain the apparent discrepancy between the reported sexual dimorphism and the lack of a dimorphism when all populations were examined.

Furthermore, the correlations I observed between latitude and morphology among American Toads appear to be manifested largely in areas that have been re-occupied following post-glacial colonization of northern regions less than 10,000 years ago. This would suggest that these changes are relatively recent and have occurred in a short period of time. If this is the case then the observed variations could be tied to the colonization events, and range expansion that occurred following glacial retreat. However, the exact drivers of these trends remain elusive.

Although temperature regime may play a strong role in determining species range, other environmental factors may also have an effect, and the influence of behaviour on morphological variation must be considered. A similar environmental effect on morphological variation has been observed in Blotched Snakes (*Elaphe sauromates*), which showed increased dimorphism with latitude [342]; in Green Anoles (*Anolis carolinensis*), which had strong correlations between cranial morphology and environmental conditions [343] and in Bobcats (*Lynx rufus*), which exhibited both a latitudinal and longitudinal gradient in morphology [344]. My observations, and those from across different taxa, demonstrate that there are significant relationships between morphological variation and environment by proxy of latitude however, the exact drivers of this variation are not entirely clear.

My findings are consistent with the hypothesis that changes in breeding behaviour are associated with changing environmental conditions with increasing latitude. Migrant and resident populations of Blackcaps (*Sylvia atricapilla*) in Spain had similar morphology, but were morphologically distinct from migratory populations that bred in central Europe, suggesting an effect of breeding habitat on morphological variation [345]. The butterfly *Pararge aegeria*, which has three distinct breeding periods, has significant variations in morphology that correspond to the breeding time cohort that the butterfly belonged to, again suggestive of a breeding habitat effect on morphology [346]. Northern American Toad populations are exposed to very short cold nights, with breeding postponed by cold temperatures until early summer. Southern populations conversely, experience long warm nights very early in the year, with breeding possible as early as February in some locations. Given the drastic differences in climatic conditions across its range, environment would be expected to drive changes in breeding behaviour in the American Toad. However there are currently no studies that have examined variation in breeding behaviour between populations. A detailed examination of behaviour of multiple populations across the entire geographic range, or at least comparing the two extremes of the range would help to illuminate the role of behaviour in driving morphological variation.

Strengths and Weaknesses:

When examining species distribution patterns across species, I remarked that there remains a consistent lack of phylogeographies that extend north of the Canadian-US Border. While there has been a considerable effort in recent years to perform phylogeographies on Canadian herpetofaunal species, there remains a considerable paucity of data on northern species. Even among species with extensive northern ranges, or where the majority of the range is in high latitudes, phylogeographic studies rarely include specimens from northern populations. Furthermore, in some cases of widespread species, northern populations are often proportionally under-represented with few specimens or specimens that have not been attributed to the correct geographic location. However, despite a general lack of studies from Canadian populations, those studies that have focused specifically on Canadian populations are extensive and robust in their approach.

In modelling the hybrid zone between two species, selective pressures and adaptive responses were kept as simplified as possible. Although, a more comprehensive approach would see the inclusion of adaptive responses to habitat as well as variation in the selective pressures exerted on the species, both of these as well as fitness advantages of mtDNA types were not included in this model. The habitat was kept static as well, ensuring that parental types could be preserved. Within the context of this model, the emphasis was on the behavioural factors that can affect hybridization and genetic discordance and not on the role of habitat stochasticity, or fitness variation due to different mtDNA. As a result the model was still robust in exploring behaviour and hybridization across a range of conditions.

When examining the physiological response of tadpoles to temperature and light quantity during development, it would be advantageous to examine their performance at temperatures that exceed those currently experienced by southern populations and below those at the northern limit of the range. By pushing tested temperature limits beyond what is currently experienced by tadpoles in a natural setting, it would have been possible to gain an improved estimate of the potential climatic range and thermal tolerances of the

American Toad. Despite not exceeding current limits, my findings still present evidence of adaptations to increasingly cold conditions with increased latitude. As this is the first such study in the American Toad, the complete curve of the relationship of physiological performance vs. temperature remains unknown. Using expanded temperature limits and a greater resolution of temperatures, it would be possible to create a more precise response curve for each of the populations examined. Additionally, an increased sampling range, with higher geographic resolution would result in a more precise geographic delineation of where adaptations arise. Through these curves, the influence and extent of local adaptation within and between populations could be further assessed and the exact adaptations identified.

Although tadpoles were collected at different times of the year, this was unavoidable as the timing of the breeding season of different locations varies drastically with latitude. While southern populations may breed in February, northern populations do not begin to breed until May, or possibly June as northern water bodies remain largely ice-locked and inhospitable. As a result, although eggs and tadpoles were collected at different chronological times of the calendar year they were collected at very similar times of their respective breeding season. With the exception of the tadpoles from northern populations, the eggs from Ste-Anne-de-Bellevue and Athens, Georgia were collected within hours of being laid, and hatched in the lab. The tadpoles from Radisson were collected within a week of hatching. As a result, the timing of their growth rate is very comparable. Although the offset in introduction between the different tadpoles is an important weakness, I addressed this using multiple analyses: the first by examining tadpoles from Ste-Anne-de-Bellevue and

Athens separately from tadpoles from Radisson and then secondly by examining tadpoles from all three locations together. Furthermore, the use of the accepted and well defined Gosner growth stages [311] as landmarks of development means that comparisons are also very robust.

When examining morphological variation, I took great care was taken to ensure that I had a large number of specimens from each location. There have been observations of a population effect on individual size in Fowler's Toads, a closely related species, with larger populations being composed of generally smaller individuals, and the opposite the case for small populations [347]. By using individuals of a population that were all collected in the same year, I can ensure that they were coming from one population, and variations were not the effect of interannual changes in individual size with fluctuations in population. In order to prevent any possible confounding effect from variation in SVL between populations, all measurements of morphology and all morphological variation was first controlled for SVL before any examination. In this way, interpopulational and even intrapopulational variation in SVL did not affect the trends observed in regards to morphological variation with latitude. Since there have been observed fluctuations in body size with population size, it would be of interest to examine populations of toad across multiple years to assess whether these fluctuations are constant with latitude, or whether the effect of population size on the size of individuals varies with latitude. By controlling for SVL, I also controlled for any allometric effect, and for SVL dimorphism between the sexes. Although previous studies have examined morphology across a very small latitudinal range, mine is the first such study to examine American Toad populations from across the entire range of the American Toad, and my findings did go against the classical observations of dimorphism in the American Toad [122]. This is a surprising result, and suggests that updates may be required for the species description in order to incorporate the trends I observed here. While American Toad females do appear to have a higher maximum size than males, the average size is not significantly different. In sexing American Toads, many of the common indicators of sex in males, including a darkened throat and the use of a release chirp upon capture, were no longer present as I was using preserved specimens. However, every specimen examined was sexed using the presence of throat slits that are only found in males. Only those toads that could be properly sexed were included. A size-cut off was also implemented and those that were too small, as well as juveniles which lacked fully formed cranial crests, and those where it was not possible to assess sex were not used. In this way I only included specimens that could be definitively confirmed as being mature males or females.

Conclusion:

Overall, my thesis provides the first empirical evidence of local variation within a species with a very large latitudinal range and how a single species expresses significant variation in its morphology and physiological response to latitude as a proxy for environmental variation. Variations in environmental conditions across latitude appear to have resulted in local adaptation at the population level. Covering nearly half of North America, the American Toad has great value as a model organism with which to examine these evolutionary and ecological impacts of range size. Of further interest in these toads is that over half of their current range is a very recent development following post-glacial range expansion at the end of the Pleistocene glaciation. As with other species that expanded their range and colonized previously glaciated areas, the American Toad may still be experiencing range expansion, and may not yet have arrived at its northern limit. This provides the exciting opportunity for further work examining selection and adaptive processes at the range edge, and throughout range expansion. My work here has provided a solid foundation with which to continue to explore range dynamics and how variation in the environmental factors, and stochastic or ecological dynamics within and across a species' range can be drivers of evolutionary change.

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Appendices

Appendix A – Catalogue ID number, sex, location of capture and the subspecies range wherein the individuals were captured for all individuals used in morphological anlyses.

Catalogue ID	Sex	Latitude	Longitude	Latitude Group	Subspecies Area
64984	Male	32.345	-90.873	1	Charlesmithi
64969	Male	32.345	-90.873	1	Charlesmithi
64970	Male	32.345	-90.873	1	Charlesmithi
64979	Male	32.345	-90.873	1	Charlesmithi
64980	Male	32.345	-90.873	1	Charlesmithi
64981	Male	32.345	-90.873	1	Charlesmithi
64971	Male	32.345	-90.873	1	Charlesmithi
64972	Male	32.345	-90.873	1	Charlesmithi
64974	Male	32.345	-90.873	1	Charlesmithi
64983	Male	32.345	-90.873	1	Charlesmithi
64973	Male	32.345	-90.873	1	Charlesmithi
64982	Male	32.345	-90.873	1	Charlesmithi
64977	Male	32.345	-90.873	1	Charlesmithi
64978	Male	32.345	-90.873	1	Charlesmithi
64968	Male	32.345	-90.873	1	Charlesmithi
64967	Male	32.345	-90.873	1	Charlesmithi
64975	Male	32.345	-90.873	1	Charlesmithi
64976	Male	32.345	-90.873	1	Charlesmithi
83815	Male	32.505	-94.642	1	Charlesmithi
83814	Male	32.505	-94.642	1	Charlesmithi
83811	Female	32.526	-94.963	1	Charlesmithi
83805	Female	32.526	-94.963	1	Charlesmithi
83800	Male	32.526	-94.963	1	Charlesmithi
83801	Male	32.526	-94.963	1	Charlesmithi
83808	Male	32.526	-94.963	1	Charlesmithi
83806	Male	32.526	-94.963	1	Charlesmithi
83803	Male	32.526	-94.963	1	Charlesmithi
83796	Male	32.526	-94.963	1	Charlesmithi
83809	Male	32.526	-94.963	1	Charlesmithi
83804	Male	32.526	-94.963	1	Charlesmithi
83807	Male	32.526	-94.963	1	Charlesmithi
83810	Male	32.526	-94.963	1	Charlesmithi
83802	Male	32.526	-94.963	1	Charlesmithi
83799	Male	32.526	-94.963	1	Charlesmithi
83798	Male	32.526	-94.963	1	Charlesmithi

83795	Male	32.526	-94.963	1	Charlesmithi
83797	Male	32.526	-94.963	1	Charlesmithi
46185	Female	32.755	-91.759	1	Charlesmithi
470205	Female	34.633	-93.581	1	Charlesmithi
470201	Male	34.633	-93.581	1	Charlesmithi
470204	Male	34.633	-93.581	1	Charlesmithi
470208	Male	34.633	-93.581	1	Charlesmithi
470206	Male	34.633	-93.581	1	Charlesmithi
470209	Male	34.633	-93.581	1	Charlesmithi
470203	Male	34.633	-93.581	1	Charlesmithi
470200	Male	34.633	-93.581	1	Charlesmithi
470207	Male	34.633	-93.581	1	Charlesmithi
470202	Male	34.633	-93.581	1	Charlesmithi
469967	Male	34.633	-93.581	1	Charlesmithi
470199	Male	34.633	-93.581	1	Charlesmithi
87291	Male	35.054	-83.189	2	Charlesmithi
315489	Female	35.113	-83.205	2	Charlesmithi
94265	Female	35.877	-96.876	2	Charlesmithi
94275	Female	35.877	-96.876	2	Charlesmithi
94274	Male	35.877	-96.876	2	Charlesmithi
94273	Male	35.877	-96.876	2	Charlesmithi
94270	Male	35.877	-96.876	2	Charlesmithi
94281	Male	35.877	-96.876	2	Charlesmithi
94280	Male	35.877	-96.876	2	Charlesmithi
94278	Male	35.877	-96.876	2	Charlesmithi
94279	Male	35.877	-96.876	2	Charlesmithi
94271	Male	35.877	-96.876	2	Charlesmithi
94276	Male	35.877	-96.876	2	Charlesmithi
94264	Male	35.877	-96.876	2	Charlesmithi
94277	Male	35.877	-96.876	2	Charlesmithi
94269	Male	35.877	-96.876	2	Charlesmithi
94267	Male	35.877	-96.876	2	Charlesmithi
94268	Male	35.877	-96.876	2	Charlesmithi
94272	Male	35.877	-96.876	2	Charlesmithi
94266	Male	35.877	-96.876	2	Charlesmithi
114792	Male	40.867	-74.577	3	Americanus
116837	Male	40.867	-74.577	3	Americanus
101557	Female	40.948	-74.083	3	Americanus
11988	Female	40.948	-74.083	3	Americanus
101554	Female	40.948	-74.083	3	Americanus
12840	Female	40.948	-74.083	3	Americanus
12853	Female	40.948	-74.083	3	Americanus
51528	Female	40.948	-74.083	3	Americanus

11996	Female	40.948	-74.083	3	Americanus
52382	Female	40.948	-74.083	3	Americanus
12835	Female	40.948	-74.083	3	Americanus
12836	Female	40.948	-74.083	3	Americanus
12831	Female	40.948	-74.083	3	Americanus
12848	Female	40.948	-74.083	3	Americanus
11994	Female	40.948	-74.083	3	Americanus
11986	Female	40.948	-74.083	3	Americanus
11995	Female	40.948	-74.083	3	Americanus
12846	Female	40.948	-74.083	3	Americanus
12832	Female	40.948	-74.083	3	Americanus
12841	Female	40.948	-74.083	3	Americanus
11998	Female	40.948	-74.083	3	Americanus
11991	Female	40.948	-74.083	3	Americanus
41331	Female	40.948	-74.083	3	Americanus
144929	Male	40.948	-74.083	3	Americanus
101547	Male	40.948	-74.083	3	Americanus
1571	Male	40.948	-74.083	3	Americanus
41361	Male	40.948	-74.083	3	Americanus
12847	Male	40.948	-74.083	3	Americanus
41354	Male	40.948	-74.083	3	Americanus
144904	Male	40.948	-74.083	3	Americanus
144914	Male	40.948	-74.083	3	Americanus
144932	Male	40.948	-74.083	3	Americanus
41338	Male	40.948	-74.083	3	Americanus
144911	Male	40.948	-74.083	3	Americanus
144925	Male	40.948	-74.083	3	Americanus
101546	Male	40.948	-74.083	3	Americanus
11987	Male	40.948	-74.083	3	Americanus
144931	Male	40.948	-74.083	3	Americanus
41341	Male	40.948	-74.083	3	Americanus
101549	Male	40.948	-74.083	3	Americanus
41340	Male	40.948	-74.083	3	Americanus
101551	Male	40.948	-74.083	3	Americanus
101553	Male	40.948	-74.083	3	Americanus
41343	Male	40.948	-74.083	3	Americanus
144905	Male	40.948	-74.083	3	Americanus
41353	Male	40.948	-74.083	3	Americanus
12842	Male	40.948	-74.083	3	Americanus
41356	Male	40.948	-74.083	3	Americanus
144935	Male	40.948	-74.083	3	Americanus
11997	Male	40.948	-74.083	3	Americanus
11985	Male	40.948	-74.083	3	Americanus

12851	Male	40.948	-74.083	3	Americanus
12839	Male	40.948	-74.083	3	Americanus
144903	Male	40.948	-74.083	3	Americanus
144934	Male	40.948	-74.083	3	Americanus
144928	Male	40.948	-74.083	3	Americanus
144930	Male	40.948	-74.083	3	Americanus
101558	Male	40.948	-74.083	3	Americanus
11992	Male	40.948	-74.083	3	Americanus
12837	Male	40.948	-74.083	3	Americanus
12844	Male	40.948	-74.083	3	Americanus
144939	Male	40.948	-74.083	3	Americanus
41358	Male	40.948	-74.083	3	Americanus
12845	Male	40.948	-74.083	3	Americanus
12830	Male	40.948	-74.083	3	Americanus
101555	Male	40.948	-74.083	3	Americanus
18749	Male	40.948	-74.083	3	Americanus
11990	Male	40.948	-74.083	3	Americanus
144910	Male	40.948	-74.083	3	Americanus
144917	Male	40.948	-74.083	3	Americanus
144918	Male	40.948	-74.083	3	Americanus
35502	Male	40.948	-74.083	3	Americanus
41360	Male	40.948	-74.083	3	Americanus
144908	Male	40.948	-74.083	3	Americanus
101552	Male	40.948	-74.083	3	Americanus
144941	Male	40.948	-74.083	3	Americanus
144924	Male	40.948	-74.083	3	Americanus
12849	Male	40.948	-74.083	3	Americanus
101556	Male	40.948	-74.083	3	Americanus
144938	Male	40.948	-74.083	3	Americanus
144945	Male	40.948	-74.083	3	Americanus
41339	Male	40.948	-74.083	3	Americanus
144913	Male	40.948	-74.083	3	Americanus
144907	Male	40.948	-74.083	3	Americanus
144943	Male	40.948	-74.083	3	Americanus
144922	Male	40.948	-74.083	3	Americanus
144927	Male	40.948	-74.083	3	Americanus
101548	Male	40.948	-74.083	3	Americanus
12852	Male	40.948	-74.083	3	Americanus
144940	Male	40.948	-74.083	3	Americanus
144944	Male	40.948	-74.083	3	Americanus
12854	Male	40.948	-74.083	3	Americanus
144937	Male	40.948	-74.083	3	Americanus
11993	Male	40.948	-74.083	3	Americanus

144920	Male	40.948	-74.083	3	Americanus
144946	Male	40.948	-74.083	3	Americanus
41355	Male	40.948	-74.083	3	Americanus
144915	Male	40.948	-74.083	3	Americanus
35500	Male	40.948	-74.083	3	Americanus
12850	Male	40.948	-74.083	3	Americanus
101550	Male	40.948	-74.083	3	Americanus
12843	Male	40.948	-74.083	3	Americanus
12834	Male	40.948	-74.083	3	Americanus
12838	Male	40.948	-74.083	3	Americanus
144933	Male	40.948	-74.083	3	Americanus
11989	Male	40.948	-74.083	3	Americanus
41357	Male	40.948	-74.083	3	Americanus
12833	Male	40.948	-74.083	3	Americanus
70271	Female	41.011	-74.305	3	Americanus
67028	Female	41.011	-74.305	3	Americanus
71621	Female	41.011	-74.305	3	Americanus
72574	Male	41.011	-74.305	3	Americanus
67029	Male	41.011	-74.305	3	Americanus
72573	Male	41.011	-74.305	3	Americanus
537025	Female	41.576	-93.615	3	Americanus
537023	Female	41.576	-93.615	3	Americanus
537024	Female	41.576	-93.615	3	Americanus
537022	Female	41.576	-93.615	3	Americanus
537021	Female	41.576	-93.615	3	Americanus
537020	Female	41.576	-93.615	3	Americanus
537014	Female	41.576	-93.615	3	Americanus
537019	Female	41.576	-93.615	3	Americanus
537016	Female	41.576	-93.615	3	Americanus
537017	Female	41.576	-93.615	3	Americanus
537018	Female	41.576	-93.615	3	Americanus
14758	Female	41.576	-93.615	3	Americanus
13926	Female	41.576	-93.615	3	Americanus
537015	Male	41.576	-93.615	3	Americanus
1181	Female	41.812	-87.894	3	Americanus
1214	Female	41.812	-87.894	3	Americanus
1184	Female	41.812	-87.894	3	Americanus
1213	Female	41.812	-87.894	3	Americanus
5584	Female	41.812	-87.894	3	Americanus
1216	Female	41.812	-87.894	3	Americanus
1215	Female	41.812	-87.894	3	Americanus
1185	Female	41.812	-87.894	3	Americanus
1183	Female	41.812	-87.894	3	Americanus

1186	Female	41.812	-87.894	3	Americanus
5587	Female	41.812	-87.894	3	Americanus
5586	Female	41.812	-87.894	3	Americanus
1180	Female	41.812	-87.894	3	Americanus
5585	Female	41.812	-87.894	3	Americanus
1182	Male	41.812	-87.894	3	Americanus
5582	Male	41.812	-87.894	3	Americanus
5583	Male	41.812	-87.894	3	Americanus
5589	Male	41.812	-87.894	3	Americanus
5588	Male	41.812	-87.894	3	Americanus
41432	Male	42.270	-78.685	3	Americanus
rm2352	Female	42.559	-80.290	3	Americanus
rm2349	Female	42.559	-80.290	3	Americanus
rm2350	Female	42.559	-80.290	3	Americanus
rm2343	Female	42.559	-80.290	3	Americanus
rm4242	Female	42.559	-80.290	3	Americanus
rm2344	Female	42.559	-80.290	3	Americanus
rm2345	Female	42.559	-80.290	3	Americanus
rm2347	Female	42.559	-80.290	3	Americanus
rm2398	Female	42.559	-80.290	3	Americanus
rm2399	Female	42.559	-80.290	3	Americanus
rm2400	Female	42.559	-80.290	3	Americanus
rm2396	Male	42.559	-80.290	3	Americanus
rm2346	Male	42.559	-80.290	3	Americanus
207390	Male	43.138	-90.510	3	Americanus
207391	Male	43.138	-90.510	3	Americanus
207387	Male	43.152	-90.496	3	Americanus
207389	Male	43.152	-90.496	3	Americanus
207388	Male	43.152	-90.496	3	Americanus
207392	Male	43.159	-90.486	3	Americanus
207384	Male	43.337	-89.375	3	Americanus
207386	Male	43.337	-89.375	3	Americanus
207383	Male	43.337	-89.375	3	Americanus
136	Male	43.533	-65.617	3	Americanus
1636-1	Male	44.867	-63.717	3	Americanus
1636-2	Male	44.867	-63.717	3	Americanus
3218	Male	44.896	-65.240	3	Americanus
3774	Male	44.896	-65.240	3	Americanus
16234	Male	45.067	-67.050	4	Americanus
4963	Male	45.067	-67.050	4	Americanus
3524	Female	45.083	-64.367	4	Americanus
34822	Male	45.084	-67.063	4	Americanus
rm2676	Male	45.087	-74.175	4	Americanus

31343	Female	45.233	-67.068	4	Americanus
25762	Male	45.233	-67.068	4	Americanus
31345	Male	45.233	-67.068	4	Americanus
20633	Female	45.367	-65.967	4	Americanus
rm2417	Female	45.391	-61.499	4	Americanus
rm2416	Male	45.391	-61.499	4	Americanus
742	Female	45.417	-75.683	4	Americanus
1938	Female	45.450	-61.700	4	Americanus
rm2411	Female	45.499	-61.814	4	Americanus
rm2412	Male	45.499	-61.814	4	Americanus
6590	Male	45.533	-63.550	4	Americanus
36526-1	Female	45.564	-72.140	4	Americanus
36526-2	Male	45.564	-72.140	4	Americanus
36526-3	Male	45.564	-72.140	4	Americanus
rm2410	Female	45.779	-71.606	4	Americanus
2433	Male	45.925	-64.221	4	Americanus
6806	Female	45.942	-66.641	4	Americanus
6807	Female	45.983	-66.583	4	Americanus
32853 (4)	Male	46.017	-81.767	4	Americanus
32853 (3)	Male	46.017	-81.767	4	Americanus
32853 (2)	Male	46.017	-81.767	4	Americanus
32853 (1)	Male	46.017	-81.767	4	Americanus
36356-2	Female	46.065	-71.269	4	Americanus
36356-1	Female	46.065	-71.269	4	Americanus
2494	Female	46.185	-62.534	4	Americanus
6563	Female	46.190	-64.600	4	Americanus
3646 (1)	Male	46.197	-62.892	4	Americanus
3646 (2)	Male	46.197	-62.892	4	Americanus
3633	Male	46.217	-62.999	4	Americanus
32839-1	Female	46.233	-83.833	4	Americanus
32839-2	Male	46.233	-83.833	4	Americanus
3646(2)	Male	46.283	-63.250	4	Americanus
3646(1)	Male	46.283	-63.250	4	Americanus
36495	Male	46.374	-72.168	4	Americanus
3695	Male	46.483	-63.367	4	Americanus
1579	Male	46.567	-63.933	4	Americanus
207373	Male	46.609	-90.682	4	Americanus
2639-6	Female	46.631	-60.439	4	Americanus
2639-5	Male	46.631	-60.439	4	Americanus
696	Female	46.650	-60.650	4	Americanus
3821 (1)	Female	46.667	-60.400	4	Americanus
3821 (2)	Female	46.667	-60.400	4	Americanus
3821(1)	Male	46.679	-60.492	4	Americanus

3821(2)	Male	46.679	-60.492	4	Americanus
3781	Male	46.683	-64.350	4	Americanus
36529	Female	46.817	-71.217	4	Americanus
36228	Female	46.874	-71.259	4	Americanus
1846	Female	46.883	-60.517	4	Americanus
18514	Female	46.983	-84.167	4	Americanus
2013	Male	47.350	-68.683	4	Americanus
17501-12	Female	47.387	-68.378	4	Americanus
17501-9	Female	47.387	-68.378	4	Americanus
17501-11	Male	47.387	-68.378	4	Americanus
17501-16	Male	47.387	-68.378	4	Americanus
17501-2	Male	47.387	-68.378	4	Americanus
17501-7	Male	47.387	-68.378	4	Americanus
17501-13	Male	47.387	-68.378	4	Americanus
17501-3	Male	47.387	-68.378	4	Americanus
17501-15	Male	47.387	-68.378	4	Americanus
17501-6	Male	47.387	-68.378	4	Americanus
17501-4	Male	47.387	-68.378	4	Americanus
17501-5	Male	47.387	-68.378	4	Americanus
17501-8	Male	47.387	-68.378	4	Americanus
17501-10	Male	47.387	-68.378	4	Americanus
17501-1	Male	47.387	-68.378	4	Americanus
17501-14	Male	47.387	-68.378	4	Americanus
33681	Female	47.833	-65.500	4	Americanus
16902	Male	47.917	-83.317	4	Americanus
33676-3	Female	48.734	-67.644	4	Americanus
33676-3	Female	48.734	-67.644	4	Americanus
33676-1	Male	48.734	-67.644	4	Americanus
33676-2	Male	48.734	-67.644	4	Americanus
33676-1	Male	48.734	-67.644	4	Americanus
33676-2	Male	48.734	-67.644	4	Americanus
33677	Male	48.865	-64.211	4	Americanus
34332-3	Female	49.067	-81.017	4	Americanus
34332-2	Female	49.067	-81.017	4	Americanus
36354	Female	49.107	-68.190	4	Americanus
36357	Female	49.131	-68.365	4	Americanus
11133-3	Male	49.206	-95.306	4	Americanus
11133-5	Male	49.206	-95.306	4	Americanus
11133-2	Male	49.206	-95.306	4	Americanus
11133	Male	49.206	-95.306	4	Americanus
11133-1	Male	49.206	-95.306	4	Americanus
11133-4	Male	49.206	-95.306	4	Americanus
32658	Male	49.850	-95.533	4	Americanus

rm2414	Female	50.137	-74.016	5	Copei
rm2413	Female	50.137	-74.016	5	Copei
1740	Male	50.500	-83.000	5	Copei
1744	Male	50.500	-83.000	5	Copei
1743	Male	50.500	-83.000	5	Copei
1741	Male	50.500	-83.000	5	Copei
1742	Male	50.500	-83.000	5	Copei
1738	Male	50.500	-83.000	5	Copei
1739	Male	50.500	-83.000	5	Copei
35501	Male	50.583	-77.565	5	Copei
35614	Female	50.946	-77.647	5	Copei
34337-1	Female	51.283	-80.650	5	Copei
34337-3	Male	51.283	-80.650	5	Copei
34337-4	Male	51.283	-80.650	5	Copei
34337-2	Male	51.283	-80.650	5	Copei
34337-5	Male	51.283	-80.650	5	Copei
34339-9	Female	51.283	-80.650	5	Copei
34339-17	Female	51.283	-80.650	5	Copei
34339-10	Female	51.283	-80.650	5	Copei
34339-15	Male	51.283	-80.650	5	Copei
34339-10	Male	51.283	-80.650	5	Copei
34339-3	Male	51.283	-80.650	5	Copei
34339-14	Male	51.283	-80.650	5	Copei
35531 (1)	Female	51.415	-77.438	5	Copei
35531 (2)	Male	51.415	-77.438	5	Copei
35527-1	Female	51.429	-78.655	5	Copei
35527-3	Male	51.429	-78.655	5	Copei
35527-2	Male	51.429	-78.655	5	Copei
35513	Female	51.478	-78.679	5	Copei
35505-4	Female	51.484	-78.722	5	Copei
35505-1	Female	51.484	-78.722	5	Copei
35518-1	Female	51.484	-78.722	5	Copei
35516-4	Female	51.484	-78.722	5	Copei
35516-5	Female	51.484	-78.722	5	Copei
35516-1	Female	51.484	-78.722	5	Copei
35505-7	Male	51.484	-78.722	5	Copei
35505-10	Male	51.484	-78.722	5	Copei
35505-5	Male	51.484	-78.722	5	Copei
35505-12	Male	51.484	-78.722	5	Copei
35505-6	Male	51.484	-78.722	5	Copei
35505-11	Male	51.484	-78.722	5	Copei
35505-8	Male	51.484	-78.722	5	Copei
35505-2	Male	51.484	-78.722	5	Copei

35505-9	Male	51.484	-78.722	5	Copei
35505-3	Male	51.484	-78.722	5	Copei
35518-3	Male	51.484	-78.722	5	Copei
35518-2	Male	51.484	-78.722	5	Copei
35518-4	Male	51.484	-78.722	5	Copei
35516-13	Male	51.484	-78.722	5	Copei
35516-3	Male	51.484	-78.722	5	Copei
35516-6	Male	51.484	-78.722	5	Copei
35516-10	Male	51.484	-78.722	5	Copei
35516-2	Male	51.484	-78.722	5	Copei
35516-8	Male	51.484	-78.722	5	Copei
35516-9	Male	51.484	-78.722	5	Copei
35516-7	Male	51.484	-78.722	5	Copei
35516-12	Male	51.484	-78.722	5	Copei
35516-11	Male	51.484	-78.722	5	Copei
35514	Male	51.485	-78.743	5	Copei
35525	Male	51.504	-79.223	5	Copei
35617	Male	51.508	-79.273	5	Copei
35536	Male	51.911	-77.403	5	Copei
35540	Female	52.000	-76.000	5	Copei
35554(2)	Male	52.000	-76.000	5	Copei
35554(1)	Male	52.000	-76.000	5	Copei
35506	Male	52.000	-76.000	5	Copei
35537	Male	52.000	-76.000	5	Copei
35545	Female	52.242	-78.501	5	Copei
35539	Female	52.244	-78.506	5	Copei
35542-2	Male	52.244	-78.501	5	Copei
35542-1	Male	52.244	-78.501	5	Copei
35557	Male	52.508	-77.321	5	Copei
35612	Male	52.681	-77.371	5	Copei
35966	Female	53.020	-78.992	5	Copei
35967	Female	53.020	-78.992	5	Copei
35601-1	Male	53.709	-77.841	5	Copei
35601-2	Male	53.709	-77.841	5	Copei
35558	Female	53.787	-77.620	5	Copei
35568	Female	53.791	-77.625	5	Copei
35564-1	Female	53.793	-77.606	5	Copei
35564-2	Female	53.793	-77.606	5	Copei
35564-3	Male	53.793	-77.606	5	Copei
35564-7	Male	53.793	-77.606	5	Copei
35564-4	Male	53.793	-77.606	5	Copei
35564-6	Male	53.793	-77.606	5	Copei
35564-5	Male	53.793	-77.606	5	Copei

35558	Female	53.794	-77.618	5	Copei
35565	Male	53.798	-77.622	5	Copei
35587	Male	53.918	-78.852	5	Copei
35585	Female	53.923	-78.854	5	Copei
2496	Male	46.279	-63.298	4	Copei
35525	Male	51.571	-79.203	5	Copei