Range Expansion in the 21st Century – Ecology and Population Genetics of the Virginia Opossum

by

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Dedication

To my parents for raising me in nature, to my siblings for laughing with me, and to Evan for loving me through it all.

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Abstract

As climate change and anthropogenic disturbances threaten an array of communities, identifying factors that shape species' distributions is critical. Examining a species undergoing range expansion can reveal factors driving its distributional change. Determining traits that make a species resilient to ongoing changes will improve our ability to predict responses to fluctuating environmental pressures. With ancestors that evolved in the Neotropics, the Virginia opossum, *Didelphis virginiana*, baffled zoologists by its expansion into areas with harsh winters. **In my dissertation, I evaluated morphological, ecological, and genetic factors that may explain how the opossum expanded beyond its hypothesized climate niche**. For my first three chapters, I analyzed stable isotopes of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) from guard hairs from opossums in a wildlife center and from museum institutions. In my first chapter, I evaluated isotope values of captive and wild opossums for evidence of biannual or incomplete biannual molting, a mammalian adaptation to harsh winters. My results suggest opossums do not exhibit either winter molting strategy, and that guard hairs capture the opossum's lifetime diet. In my second chapter, I used isotope values to test the hypothesis that a Type A generalist's niche is positively correlated with habitat heterogeneity and to evaluate which climate variables best explained isotope values. I found a positive correlation between isotopic niche size and habitat-diversity indexes. Climate variables that represent aridity and C_4 plant abundance were positively associated with $\delta^{15}N$ and $\delta^{13}C$ values, respectively. The results reveal that opossums' isotopic compositions are shaped by the habitat in which they are found, as would be expected in a generalist species. In my third chapter, I used $\delta^{13}C$ values from opossums in the Midwest and

Northeast to test whether opossums rely on anthropogenic trash to survive extreme winters. There was no significant relationship between $\delta^{13}C$ and winter variables, but there was a significant increase in variance of Midwest opossums' $\delta^{13}C$ after the 1970 corn agricultural boom. The patterns observed for northern opossums suggest their foraging is influenced by agricultural land but not urban trash. In my final chapter, I used reduced-representation genome sequencing to evaluate how opossums spread into temperate North America. Bayesian clustering analysis identified four genetic clusters, with one cluster limited to the Yucatan Peninsula, one cluster found across Mesoamerica, and two clusters that largely co-occurred in temperate North America. I used approximate Bayesian computation to evaluate 19 phylogeographic scenarios. The best-performing scenario posited a single expansion into temperate North America, followed by divergence. Alternatively, the two temperate clusters may be a result of incomplete lineage sorting. Because opossums do not exhibit classic mammalian adaptations to winter, their expansion into areas considered to be too harsh for them has previously been explained using the conjecture that urban habitats facilitate their survival. My dissertation results suggest that the range expansion of a diet and habitat generalist can occur rapidly. Their ability to forage on a wide variety of food items in various habitats, including agricultural land, provides insight to the opossum's success in facing changing landscapes and indicates that their range expansion will continue. My results suggest that Type A generalists will be resilient to changing environments, even when they appear to lack adaptations specific to local habitats.

Introduction

For over a century, biologists have endeavored to understand the factors that shape species' geographic distributions. To examine these questions, scientists developed a variety of foundational concepts. Joseph Grinnell posited that a species distribution was shaped by the temperature and precipitation which yielded optimal habitat (1917). Charles Elton placed the species niche into a biotic context: for a given species, its niche is shaped by food availability and predators (1927). Hutchinson proposed a species' niche can be calculated as an n-dimensional hypervolume, where each dimension is a variable, both abiotic and biotic (1957).

With both evolutionary and ecological dynamics in flux, range expansions can serve as real-world laboratories to pursue species' distribution research. As species expand their range, they may encounter novel habitats and ecological interactions. Understanding the drivers of range expansion, therefore, is especially important for organisms that are pollinators, vectors for disease, or generalists that may out-compete specialists and affect the new ecosystems that they encounter. Because their ranges are actively changing, it is easier to identify the variables shaping their distribution, including climate change, behavioral changes, flexibility to novel environments, and microevolution (Garroway et al., 2011; Bridle et al., 2014). The utility and importance of range expansions are underlined by the more than 13-fold increase over the past two decades in Web of Science publications that contain the topic, "range expansion". Understanding how a species expands its distribution will help us better understand the impact of biological pressures including climate change and human modifications to the landscape.

Climate change is the most common mechanism cited for ongoing range expansions of animals in the past two decades (Parmesan et al., 2005; Rius et al., 2014). In recent years, multiple medium-sized North American mammals have expanded beyond their estimated climate thresholds (climate change not withstanding), suggesting that their expansion is not strictly due to climate change (Kanda and Fuller, 2004; Taulman and Robbins, 2014). In combination with milder winters, these mammals may be coping in northern habitats with the help of anthropogenic land-use change, through a behavioral response, rapid evolution, or a combination of these.

By analyzing a species with an evolutionary history in the Neotropics that has adapted to temperate regions, my dissertation uses the Virginia opossum (*Didelphis virginiana*) to illuminate the ways in which mammals might cope with changing climate in the years to come. In the following chapters, I evaluate how a mammal species may be surviving in regions previously deemed too harsh for them. I also evaluate range-wide ecological and genetic patterns to better understand how the species reacts to its local environment.

Study System

The Virginia opossum (henceforth opossum) is a generalist mesopredator found from Central America to the Midwest and most of New England (Gardner and Sunquist, 2003). It is the largest extant species in the marsupial family, Didelphidae, and molecular analysis indicates that *D. virginiana* is the most divergent species within *Didelphis* (Voss and Jansa, 2009). Over the last half century, the opossum has expanded its range into the northern Midwest and New England, providing an opportunity to study parallel range expansions within a mammalian species (Godin, 1977; Baker, 1983; Walsh *et al.,* 2017).

Opossums occur in a variety of habitats, and while deciduous forests and wetlands were historically considered their preferred habitat (Gardner and Sunquist, 2003), a study conducted in Massachusetts observed more opossums in urban and suburban environments (Kanda et al., 2006). Research in the Midwest found that gene flow was not impeded by agriculture (Beatty et al., 2012), and there was higher genetic diversity in counties with more farms (Walsh and Tucker, 2018). Opossums readily cross roads and have been observed crossing bridges and swimming across small bodies of water (Gardner and Sunquist, 2003). As a habitat generalist with nomadic behavior, there is likely little preventing the opossum's continued dispersal northward, provided they can survive northern winters.

Opossums weighing at least 2.5 kg are able to maintain their body temperature when temperatures reach 0°C, but smaller opossums are unable to regulate their body temperature below freezing temperatures (reviewed by Gardner and Sunquist, 2003). One study conducted in Michigan found that captive opossums lost approximately 40% of their body weight in the winter. Out of 120 days in winter, a large adult required 30 days of full foraging to survive winter (Brocke, 1970). A thermoregulation modeling study conducted in Massachusetts, representing the edge of the opossums' northeastern range, predicted that the winter survival of opossums was approximately 31% (Kanda and Fuller, 2004). This is far below the survival rate necessary to maintain a northern population (calculated at 81%), indicating that climate change alone cannot explain the northern populations of opossums. Kanda and Fuller (2004) speculated that winter survival was higher than their models predicted because of anthropogenic food sources.

Summary of Chapters

Chapter 1: Stable Isotope Values Suggest Opossums (Didelphis virginiana) Do Not Seasonally Molt.

In this chapter, I evaluated the evidence of seasonal molting, a winter adaptation, in Virginia opossums (Beltran et al., 2018). Identifying the molt pattern in the Virginia opossum will provide a deeper understanding of their natural history and inform the seasonal nature of stable isotopic compositions in ecological analyses. Guard hairs from two captive opossums with known diet shifts as juveniles were serially cut and analyzed. The stable isotope values of both opossums changed through time, mirroring their recorded diet shifts in captivity. Opossums trapped in the northern edge of their range during summer were compared to opossums trapped in the fall and winter, and no difference in isotope $\delta^{13}C$ or $\delta^{15}N$ values was found. The results from both captive and wild-caught opossums do not support seasonal molting but suggest guard hair captures the opossum's lifetime diet.

Chapter 2: Isotopic Niche Breadth Varies Across the Range of a Generalist Mesopredator.

In this chapter, I examined range-wide ecological patterns and compared how the opossum interacts with its environment across its range. I used $\delta^{13}C$ and $\delta^{15}N$ values to compare opossums from six regions, based on the opossum's expansion in North America. Mesoamerica and the Gulf Coast are ancestral regions, the Midwest and Northeast are recent northward expansion regions, and California's Central Valley and Coast are two regions occupied due to human-facilitated introductions (Gardner and Sunquist, 2003). I calculated the isotopic niche breadth (SEA_B) for each region and evaluated for correlation with habitat heterogeneity. The opossum isotope values were also analyzed collectively with WorldClim bioclimate data using correlation and conditional forest analyses. I found a strong positive correlation between SEA_B and two habitat-diversity indexes, suggesting Type A generalist niches are correlated with landscape heterogeneity.

Chapter 3: The Opossum's Diet Isn't Trash! Opossum Carbon Isotope Values Through Time Do Not Match Uptick of Corn in Our Foods.

This chapter directly examined a hypothesized anthropogenic factor driving the opossum's range expansion: anthropogenic trash. Because opossums in the Midwestern "Corn Belt" may encounter corn (a C_4 plant with higher $\delta^{13}C$ values than C_3 plants) in either urban refuse or cornfields, I used the Northeast as a control for corn agriculture. I evaluated correlations between latitude or winter harshness and $\delta^{13}C$ values and found no significant relationship. Because the use of corn additives increased over the past century, I also looked for stepwise changes in $\delta^{13}C$ values through time. Variance in $\delta^{13}C$ values significantly increased in the Midwest after the industrialization of corn agriculture began in 1970 (Philpott, 2008).

Chapter 4: Historical Context of the Virginia Opossum's Expansion in North America.

Modern-day population structure is often influenced by historical demographic processes (Eckert et al., 2008). A firm understanding of how modern opossum populations are related will inform the comparisons made in my first three chapters. I used double digest restriction-site associated DNA sequencing to obtain 352 unlinked single nucleotide polymorphisms (SNPs) from 75 *D. virginiana*. I used the Bayesian clustering method STRUCTURE (Pritchard et al., 2000) to identify populations and test 19 phylogeographic scenarios with approximate Bayesian computation (Cornuet et al., 2014). Four populations were identified: a Yucatan Peninsula cluster, a Mesoamerican cluster, and two temperate clusters that co-occurred except in the southeastern U.S. The most strongly supported scenarios support the hypothesis that a single ancestral lineage migrated into temperate North America.

Summary

My dissertation research leveraged stable isotope analysis and reduced-representation genome sequencing to identify biological patterns in the Virginia opossum's range expansion.

By using museum collections to sample across the opossum's distribution, I empirically tested assumptions made by biologists about generalist mammals. Taken together, my chapters demonstrate that a mammal species range expansion was facilitated by its generalist behavior and agricultural modifications to the landscape. In the future, identifying an unknown barrier between neotropical and temperate populations may help shed additional light on factors influencing a generalist species' distribution.

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Chapter I

Stable Isotope Values Suggest Opossums (*Didelphis virginiana*) do not seasonally molt

Abstract

The Virginia opossum has spread north into temperate North America over the centuries, raising questions regarding how opossums are adapting to survive harsh winters. Very few marsupial species exhibit winter adaptations such as torpor or seasonal molting (Leeson and Wallis, 1986; Ruf and Geiser, 2014). In this study carbon and nitrogen stable isotope values were used to test evidence of seasonal molting. To evaluate whether biannual or annual molting occurs, guard hairs were serially cut from captive opossums and examined for stable isotope values to determine if a known diet shift early in life was detected along the hair shaft. To evaluate incomplete biannual molting, isotope values were compared between wild opossums trapped in the winter and summer. Evidence for a shift in isotope values in captive opossums and the absence of any difference between winter and summer opossum isotope values suggest that the opossum's seasonal change in pelage composition is minimal, supporting continuous shedding rather than seasonal molting.

Introduction

A mammal's coat typically includes short underhairs for insulation and longer, stiffer guard hairs that keep moisture away from the underhair and skin (Noback, 1951). Molting strategies vary across mammals, with limited phylogenetic conservatism (Ling, 1970). Seasonal or biannual molting is an adaptation exhibited in some mammal species that experience environments that challenge thermoregulation enough to outweigh the energetic costs of molting. Biannual molting allows for seasonally appropriate insulation to better regulate a mammal's metabolic rate, and in some cases also improves camouflage. Alternative strategies include annual molting, incomplete biannual molting (additional winter hair is grown and subsequently shed), and continuous shedding (replacement hair growth as hair is lost; Beltran et al., 2018).

The Virginia opossum (*Didelphis virginiana*, henceforth opossum) is a generalist marsupial omnivore found across much of North America (Gardner and Sunquist, 2003). It has an average lifespan of less than two years in the wild, extended to four years in captivity (McRuer and Jones, 2009). The opossum has been expanding northward for over a century in both the Great Lakes region and New England (Godin, 1977; Gardner and Sunquist, 2003; Walsh and Tucker, 2018). The opossum does not hibernate or exhibit torpor, and extremities can become necrotic or lost due to frostbite (Figure 1-1; Brocke, 1970; Walsh et al., 2017). Whether opossums seasonally molt for a heavier winter coat is unknown (Hinds and Loudon, 1997). Here, I leverage stable isotope analysis to conduct a pilot study using captive opossums and museum specimens to test two hypotheses regarding seasonal molting in opossums.

Wildlife biologists and ecologists are increasingly turning to stable isotope analysis to pursue questions related to diet and foraging. For elements that have multiple stable isotopes (e.g. N^{14}, N^{15}), the light isotope (N^{14}) is often preferentially incorporated into organismal tissue, including hair, as it is more common and typically enzymatically preferred (Sulzman, 2007). For numerous elements, stable isotope values change in a predictable way according to an animal's diet. Nitrogen ($\delta^{15}N$) isotope values increase by 2-5‰ between a consumer and its prey, allowing for inference of an animal's trophic level via $\delta^{15}N$ values. Because of variation in their photosynthetic enzymes' preferences for different carbon isotopes, C_4 plants have a significantly higher carbon ($\delta^{13}C$) isotope value than C_3 plants, and this difference is maintained through their respective food chains (Kelly, 2000). While not naturally found at high biomasses across large regions of temperate North America (Still et al., 2003), C_4 plants include corn (*Zea mays*), a common component of livestock feed and human foods in North America in the forms of corn meal and corn syrup (Jahren and Kraft, 2008). Biologist have leveraged the variation in $\delta^{13}C$ values between corn and the C_3 plants which dominate over much of temperate North America to determine whether North American mammals are exploiting anthropogenic resources (e.g. Newsome et al., 2010).

To determine whether opossums seasonally molt, I conducted two studies. First, I conducted an analysis of stable isotope composition along guard-hair shafts of two captive opossums with known changes in diet during captivity. If opossums do not shed biannually or annually, I expected that a known shift in diet when they were juveniles would be reflected in stable isotope values along the hair shaft. Second, the isotope values of wild-caught opossums were compared among three seasons. Gastrointestinal dissections of opossums suggest they are more carnivorous in the winter (Stieglitz and Klimstra, 1962; Table A1.1). If opossums shed seasonally or grow additional hair for the winter, I predicted that stable isotope values would vary between seasons, given evidence of seasonal variation in diet (Stieglitz and Klimstra, 1962).

Methods

Sampling Guard Hair

Guard hairs from the base of the right hind leg were donated in July 2016 from one female and one male held as education animals at the Ohio Wildlife Center (OWC, USA) due to malimprinting (Table 1-1). Hair was cut and collected by wildlife staff who routinely handled the opossums (Ohio Division of Wildlife Permit 18-60, USA). To evaluate changes in diet over time, serial samples were taken in approximately 10 mm increments along the length of a shaft of hair, from the base to the tip of the hair shaft (Figure 1-2). The tips of hair represented the oldest section of hair, whereas the base reflected the most recent hair growth and the most recent diet of the opossums. Four serial segments were taken from the male. An additional segment was taken from the female because she had longer hair. Entire guard hairs were also processed to compare the serial cuts to whole bulk samples.

For the second analysis, guard hairs were sampled from museum specimens collected in the northern part of their range (Illinois, Indiana, Iowa, Massachusetts, Michigan, New York, North Dakota, and Ohio) to compare δ^{13}C and δ^{15}N values from opossums collected in different seasons (specimens listed in Table A1.2). Samples included museum specimens collected in the fall (October – November, N = 16), winter (December – March, N = 16), and summer (June – September, N = 10). Seasons were designated based on shifts in the feeding ecology and behavior of opossums at the northern edge of their range. As temperatures begin to cool in October, opossums forage to bulk up for winter. From December through March, opossum foraging is frequently hindered by harsh winter conditions. By June, the breeding season is concluding for most individuals and invertebrate prey are abundant (Brocke, 1970; Gardner and Sunquist, 2003).

Stable Isotope Analysis

All hair samples were treated with a 2:1 ratio of chloroform – methanol to remove lipids and dried under a fume hood (Sergiel et al., 2017). For each sample, 0.5 – 1.0 mg of hair was weighed with a Mettler AE 240 balance (Toledo, OH) and placed in a tin capsule (Costech, Valencia, USA) for stable isotope analysis of δ^{13}C and δ^{15}N values conducted by the University of New Mexico Center for Stable Isotopes (UNM-CSI, USA). To analyze organic substrates, UNM-CSI uses a Delta V mass spectrometer (Thermo Scientific, Waltham, USA) with a Conflo IV interface 4010 elemental analyzer (Costech, Valencia, USA). For museum specimens collected before 2017, $\delta^{13}C$ values were adjusted to account for the decrease in atmospheric ^{13}C through time with a linear adjustment of -0.005‰ per year between 1925 and 1961 and -0.022‰ per year after 1961 (Chamberlain et al., 2005).

Captive Opossums Predictions

At the time of sampling, the captive female opossum was estimated to be approximately two years old whereas the male was estimated to be approximately one year old. Both individuals were orphaned and delivered to the OWC hospital between 10 and 11 weeks old (aged by veterinarian staff based on development and weight) and later transferred to the OWC education center when deemed non-releasable (Table 1-1). Before arriving at the education center at 23 weeks old, the female was trained for handling with horse meat (Table 1-1). If opossums do not shed annually or biannually, then $\delta^{15}N$ values should decrease in association with a decrease in meat in the diet once at the education center. To statistically evaluate the female's change in $\delta^{15}N$, I used a one sample t-test to compare her earliest hair $\delta^{15}N$ value (tips) to her three latest hair segments (base, 10-20mm, and 20-30mm).

When both opossums arrived at the education center, the amount of corn in their diet increased based on changes in standard diets between the hospital and education center. At the hospital, opossums were fed a mixture of approximately 32 g cat chow, 47 g vegetables, 32 g fruit, 7 g baby food, 19 g yogurt, and protein (boiled egg or small fish twice a week). Once in long-term captivity, to prevent or mitigate nutritional disorders including secondary hyperparathyroidism, both opossums were fed a steady, daily diet of approximately 32 g cat chow, 20 meal worms, dime-sized meatball, and a 64 g calcium-rich smoothie blended with 19 g yogurt, 51 g fruit, and 19 g corn. Thus, corn kernels and meal worms fed on grain meal (i.e. the C_4 plants corn and sorghum) were added to their diet at approximately 20 weeks and 23 weeks old when they were transferred to the education center. If opossums do not shed annually or biannually, one would expect to see an increase in $\delta^{13}C$ values associated with the rise in C_4 plants in their diet. If opossums shed annually or biannually, I would expect consistent stable isotope values across the hair shaft. To statistically evaluate the change in $\delta^{13}C$, I used one sample t-tests to compare each opossum's earliest hair $\delta^{13}C$ value (tips) to its three most recent hair segments (base, 10-20mm, and 20-30mm).

Museum Specimen Predictions

Museum specimens collected in the fall, winter, and summer were compared to assess whether a biannual or incomplete biannual molt could be detected from stable isotope values with the assumption that opossum's alter foraging strategies seasonally (Stieglitz and Klimstra, 1962). Differences in $\delta^{13}C$ values between seasons were evaluated using a one-way ANOVA in SPSS version 26.0 (IBM Corp. 2019), and differences in the non-normally distributed $\delta^{15}N$ values were evaluated using a Kruskal-Wallis (K-W) test in SPSS.

Results

Captive Opossums

The female opossum that was trained for handling with horse meat until she was 23 weeks old (Table 1-1) had a significantly higher $\delta^{15}N$ value (7.514‰) at the distal tips of the hair compared to the newer growth (mean = 7.018‰, $p = 0.017$, $t = -7.684$; Figure 1-3). When the entire hair was compared between the opossums, the female had a significantly higher $\delta^{15}N$ value than the male (7.133‰ and 6.238‰ respectively; t-test $p = 0.014$; Figure 1-3).

Both individuals exhibited a significant increase in $\delta^{13}C$ values between the tips of hair and the more proximal hair sections (Figure 1-4). The female's tips had a $\delta^{13}C$ value of -18.603‰, while the proximal 30 mm of hair had a mean of -17.973‰ ($p = 0.041$, $t = 4.812$). The male's tips had a $\delta^{13}C$ value of -16.477‰ and the proximal 30 mm of hair had a mean of -15.837‰ ($p = 0.010$, $t = 10.017$). When the entire hair was compared between the opossums, the male had a significantly higher $\delta^{13}C$ value (-15.892‰ and -18.000‰ respectively; t-test $p= 0.047$; Figure 1-4).

Museum Specimens

$\delta^{15}N$ values from hair did not vary significantly among museum specimens collected in different seasons (K-W $H = 0.685$, $p = 0.710$; Table 1-2). Likewise, $\delta^{13}C$ values from hair were not significantly different among museum specimens collected in different seasons (ANOVA $F = 1.838$, $p = 0.173$; Table 1-2).

Discussion

The stable isotopes of the captive opossums reveal that changes in diet at a juvenile life stage are reflected along the hair shaft, suggesting that opossums do not experience a biannual or annual molt. With an average lifespan of less than two years and an evolutionary history rooted in the Neotropics (Gardner and Sunquist, 2003), it is not surprising that captive *D. virginiana* do not show signs of seasonal molting. Molting can be influenced by a combination of photoperiod, temperature, and precipitation (Ling, 1972). While both opossums were kept in an outdoor enclosure and would have experienced conditions similar to wild opossums in Ohio, the definitive molting strategy of *D. virginiana* cannot be fully established from these limited results. I was fortunate to find two captive opossums whose diets had been adjusted due to differences in animal husbandry practices between the two captive locations, and these preliminary results can bolster additional research on captive opossums. Both opossums were sampled in the summer, and may experience continuous shedding or incomplete biannual molting, in which they grow additional hair for winter and shed this hair in the spring (Beltran et al., 2018). By examining hair samples from wild-caught opossums from museum collections, I found no difference in stable isotope values among fall, winter, and summer guard hairs. These results suggest that the seasonal change in pelage composition is minimal, supporting continuous shedding rather than biannual molting in opossums. Alternatively, the opossum's diet may be consistent across seasons, but this is not supported by gastrointestinal dissections (Stieglitz and Klimstra, 1962).

The opossum's apparent lack of biannual molting may be seen as an evolutionary limitation to coping with extreme winter weather, in addition to not hibernating and having naked, fleshy paws and tail susceptible to frostbite (Gardner and Sunquist, 2003). However, molting may be too energetically demanding for a mammal with a relatively low metabolic rate (Brocke, 1970). Other marsupials are seemingly better adapted to low temperatures than *D. virginiana*, including multiple species of Australian *Antechinus* that exhibit seasonal molting (Leeson and Wallis, 1986), as well as other didelphids, including *Marmosa microtarsus* and *Thylamys elegans* that exhibit daily torpor (Ruf and Geiser, 2014).

The northward expansion of *D. virginiana* offers a system in which to evaluate less conspicuous adaptations to temperate winter in a marsupial species. Mammal hair in the tropics is typically limited to 20 mm in length (Davenport, 2012), but the opossums I sampled from Ohio had guard hair between 40 and 50 mm long. Future research on Virginia opossums should include comparing isotope values in winter and summer pelage from the same individuals collected in the wild. As a species with a broad distribution encompassing both the Neotropics and temperate North America, the opossum provides an opportunity to evaluate the intraspecific variation in adaptations of hair growth.

Tables

Table 1-1 Dates in which orphaned Virginia opossums (*Didelphis virginiana*) were delivered to a wildlife hospital, transferred to an education center, and sampled, along with diet shifts experienced due to the transfer that would have impacted their nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) isotope values.

Opossum	Hospital arrival date	Transfer date	Sampling date	Diet shift related to $\delta^{15}N$	Diet shift related to $\delta^{13}C$
Female	5 August 2014 (10 wks old)	3 Nov 2014 (23 wks old)	July 2016 (2 yrs old)	Trained w/meatballs before transfer	Increase in corn products in diet at education center
Male	24 June 2015 (11 wks old)	26 Aug 2015 (20 wks old)	July 2016 (1 yr old)		Increase in corn products in diet at education center

Table 1-2 Mean nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) isotope values and standard deviations from guard hairs of Virginia opossums (*Didelphis virginiana*) museum specimens. Museum specimens collected in the fall (October – November), winter (December – March) and summer (June – September) from Illinois, Indiana, Iowa, Massachusetts, Michigan, New York, North Dakota, and Ohio were sampled to evaluate seasonal differences.

Season	**N**	**$\delta^{15}N$**	**$\delta^{13}C$**
Fall	16	8.12‰ ± 1.44	-22.33‰ ± 0.85
Winter	16	8.31‰ ± 1.83	-22.45‰ ± 1.02
Summer	10	8.47‰ ± 1.73	-21.74‰ ± 1.03

Figures

Figure 1-1 The manus (on the left) and pes (on the right) of a Virginia opossum (*Didelphis virginiana*) collected at the northern edge of its range in North Dakota. The pes is blackened from necrosis due to frostbite (Walsh et al. 2017).

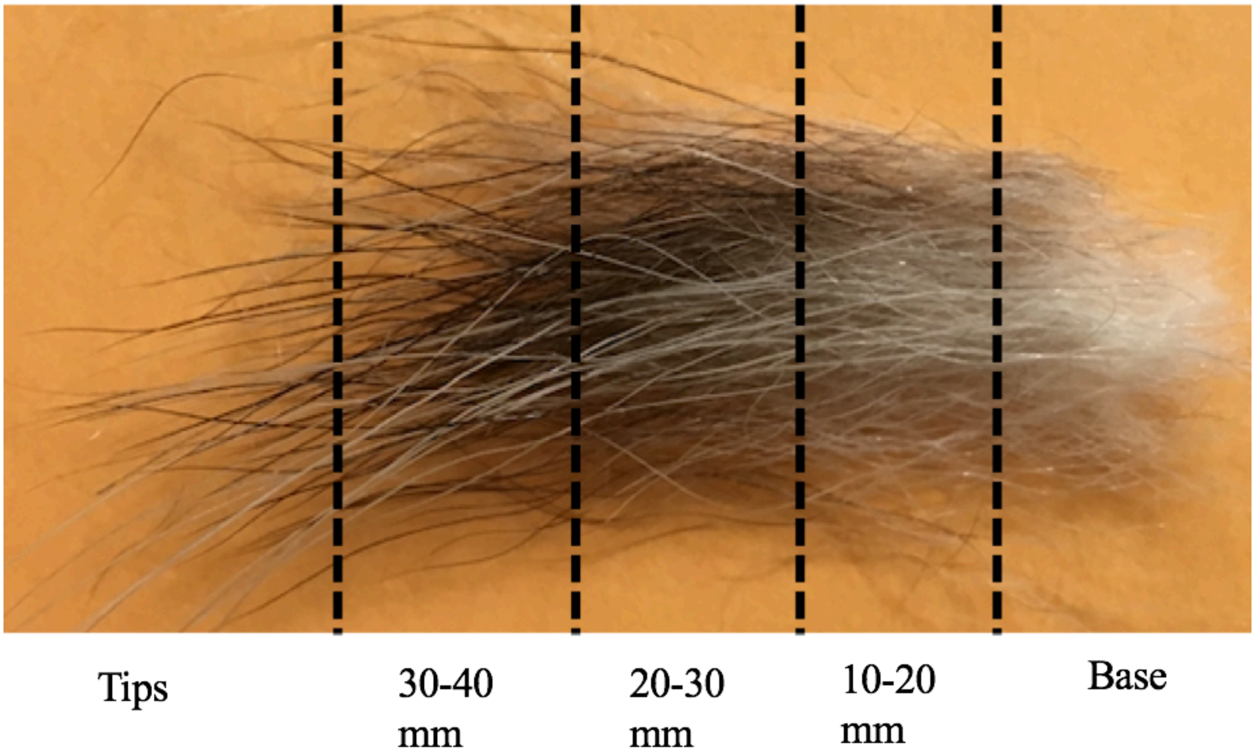

Figure 1-2 Virginia opossum (*Didelphis virginiana*) hair sample collected from captive female at the Ohio Wildlife Center education center. Dashed lines demarcate the serial sampled collected along the guard hair shaft for analysis of her diet through time.

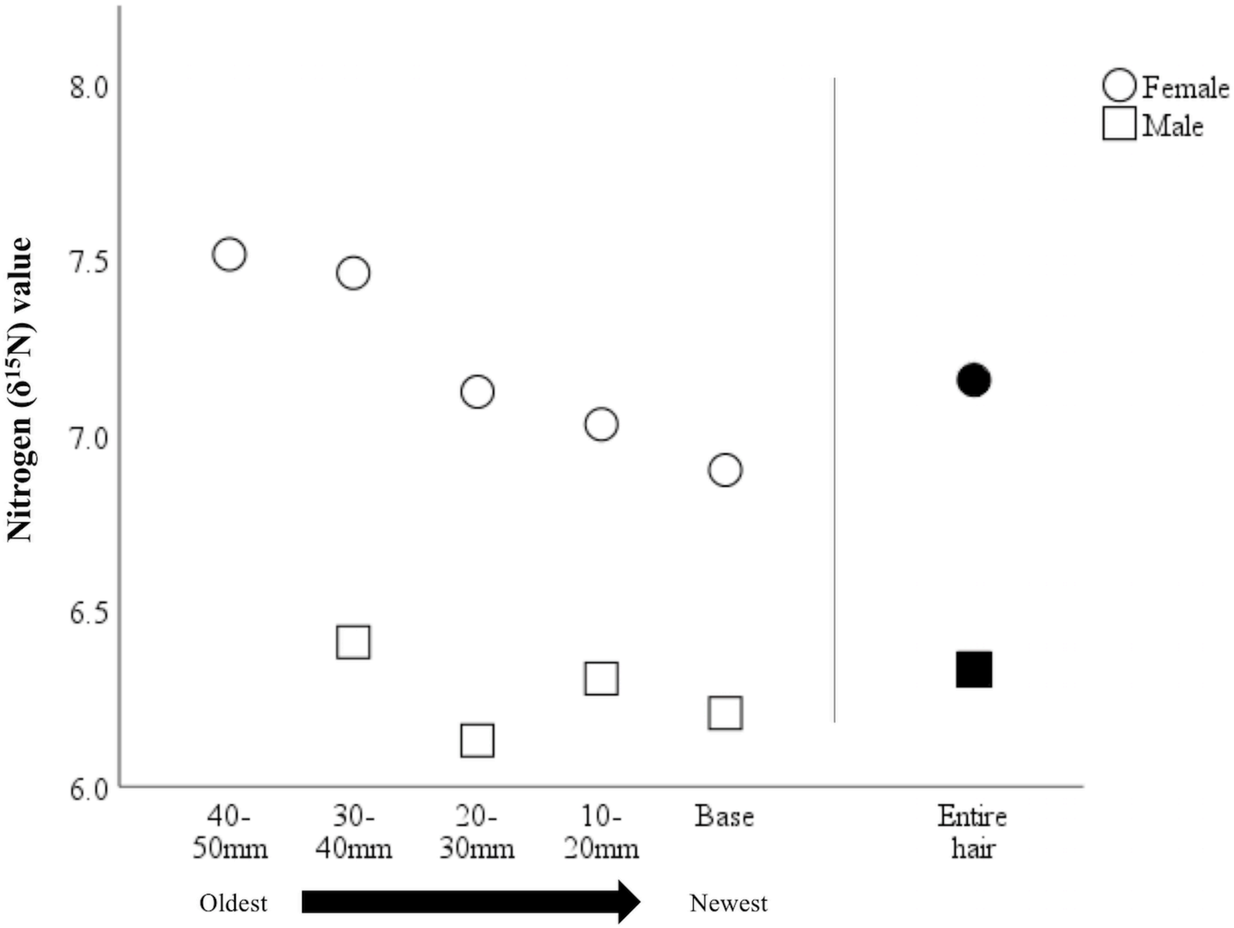

Figure 1-3 Trends over time of nitrogen ($\delta^{15}N$) isotope values from hair for two captive Virginia opossums (*Didelphis virginiana*), sampled in 10-mm increments to evaluate whether changes in diet during ontogeny are recorded in guard hair. High nitrogen ($\delta^{15}N$) values in the oldest part of the hair accurately reflects the female's juvenile diet high in meat. Because the female's hair is longer, the tips of her hair are represented in the serial sample 40 – 50 mm.

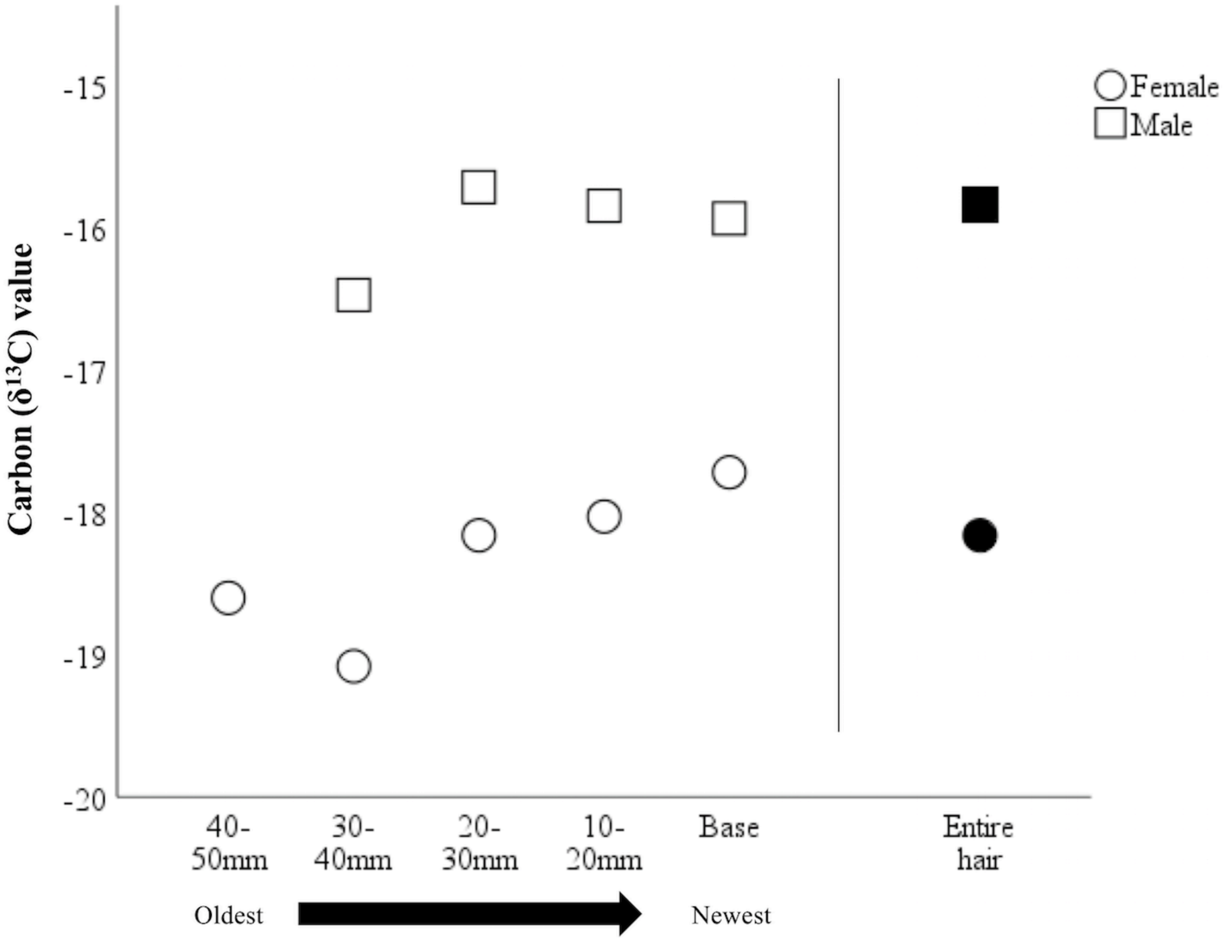

Figure 1-4 Trends over time of carbon (δ^{13}C) isotope values from hair for two captive Virginia opossums (*Didelphis virginiana*), sampled in 10-mm increments to evaluate whether changes in diet during ontogeny are recorded in guard hair. The increase in δ^{13}C values in newer hair (20-30 mm – base) for both individuals is consistent with the increased C_4 plant-based diet offered at the education center. Because the female's hair is longer, the tips of her hair are represented in the serial sample 40 – 50 mm.

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Appendix A1

Table A1.1 Seasonal volumetric percentage of food found in digestive tracts of Virginia opossums (*Didelphis virginiana*) in Illinois (Stieglitz and Klimstra, 1962).

Season	**Insects**	**Birds**	**Mammals**	**Plants**	**Reptiles and Amphibians**
Winter	2	30	60	6.5	0.5
Summer	5.5	12	17.5	47	13

Table A1.2 Data from Virginia opossum (*Didelphis virginiana*) guard hair samples including catalog number, season collected, state collected, and nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) isotope values. Samples are organized by season. Institution acronyms are as follows, alphabetically: Cornell Museum of Vertebrates (CUMV), Field Museum of Natural History (FMNH), Harvard University Museum of Comparative Zoology (MCZ), New York State Museum (NYSM), University of Michigan Museum of Zoology (UMMZ), National Museum of Natural History Smithsonian Institution (USNM), and University of Washington Burke Museum.

Sample ID	Season	State	$\delta^{15}N$ (‰)	$\delta^{13}C$ (‰)
CUMV 11489	Fall	New York	7.81	-21.69
CUMV 14863	Fall	New York	7.38	-23.58
CUMV 1974	Fall	New York	8.96	-21.95
CUMV 4970	Fall	New York	9.2	-22.83
CUMV 5148	Fall	New York	7	-22.23
CUMV 7800	Fall	New York	7.95	-21.73
FMNH 25169	Fall	Illinois	11.79	-21.55
FMNH 59027	Fall	Illinois	9.22	-21.42
MCZ 59947	Fall	Massachusetts	7.26	-22.58
MCZ 61601	Fall	Rhode Island	7.84	-20.33
NYSM 7056	Fall	New York	6.75	-22.8
UMMZ 111219	Fall	Michigan	8.72	-22.62
UMMZ 56587	Fall	Michigan	9.06	-22.36
UMMZ 81511	Fall	Michigan	8.69	-22.95
UWBM 44493	Fall	Ohio	5.72	-23.22
UWBM 44494	Fall	Ohio	6.56	-23.41
FMNH 156717	Winter	Illinois	7.69	-22.75
FMNH 198901	Winter	New York	8.15	-20.87
MCZ 62096	Winter	Massachusetts	7.27	-23.51
NYSM 7057	Winter	New York	8.65	-22.32
NYSM 7060	Winter	New York	7.1	-22.32
NYSM 7061	Winter	New York	6.75	-22.55
NYSM 7062	Winter	New York	6.53	-23.02
NYSM 7064	Winter	New York	7.89	-22.99
UMMZ 107655	Winter	Ohio	8.61	-22.04
UMMZ 177153	Winter	Michigan	8.52	-22.56
UMMZ 178776	Winter	North Dakota	8.24	-22.5
UMMZ 74603	Winter	Michigan	9.09	-22.51
UMMZ 83550	Winter	Iowa	9.73	-20.93
USNM 347673	Winter	Indiana	5.83	-22.49
UWBM 44495	Winter	Ohio	13.98	-24.99
UWBM 44497	Winter	Ohio	8.92	-20.93

CUMV 13800	Summer	New York	9.13	-19.66
CUMV 13801	Summer	New York	7.91	-22.38
CUMV 9287	Summer	New York	6.69	-23.22
FMNH 90495	Summer	Illinois	10.13	-21.66
MCZ 61602	Summer	Massachusetts	7.77	-21.08
MCZ 62102	Summer	Massachusetts	8.73	-22.24
MCZ 62199	Summer	Massachusetts	5.24	-22.03
MZ 11627	Summer	North Dakota	11.42	-20.81
UMMZ 58811	Summer	Michigan	9.31	-21.55
UMMZ 75104	Summer	New York	8.37	-22.73

Chapter II

Isotopic Niche Breadth Varies Across the Range of a Generalist Mesopredator

Abstract

Although generalist species are becoming increasingly abundant and widespread, little is known about their response to environmental variation that they encounter across their range. For example, the generalist mammal's flexible diet is cited to help explain recent range expansions, but no study has directly examined this claim. Here, I used stable isotopes of carbon and nitrogen of the Virginia opossum (*Didelphis virginiana*), a Type A generalist, to examine an extension of MacArthur's habitat heterogeneity hypothesis for a single generalist species. If a generalist's diet reflects local food diversity and abundance, then more heterogeneous landscapes should result in broader niches. I used stable isotope analysis, landcover indices, and WordClim data to further evaluate how the opossum's use of its environment varies across ancestral regions, expansion fronts, and regions of human-facilitated introductions. Niche breadth varied across its range, especially between expansion fronts. I found a positive relationship between landcover diversity and isotopic niche breadth. WorldClim variables linked to aridity and C_4 plant abundance were most strongly associated with nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) values, respectively. My results reveal that a generalist's stable isotope signature reflects its local environment, demonstrating their flexible diet is captured with stable isotopes and supporting the "generalist habitat heterogeneity hypothesis."

Introduction

Scientists have long sought to understand how species are distributed and the underlying processes controlling species ranges. In 1917, the boundaries of an individual species' range were credited to physiological limitations to the environment (Grinnell, 1917), and a few years later, Charles Elton defined a species' niche as its biotic position with predator and prey and other interactions (Elton, 1927). To better quantify a species' niche, Hutchinson proposed an n-dimensional hypervolume where the dimensions are the environmental conditions and resources that define a niche (Hutchinson, 1957). This method of drawing niches informed the development of analyzing the isotopic niche (Bearhop et al., 2004), typically depicted as the range of variation in two stable isotopes that characterize a population and reflect its environment and diet. An individual's nitrogen ($\delta^{15}N$) isotope value is positively correlated with its trophic level (and the aridity of its habitat) while an individual's carbon ($\delta^{13}C$) isotope value increases with a diet of C_4 plants or a diet of herbivores that consume C_4 plants (Kelly, 2000).

With the extirpation of apex predators in much of North America, generalist mesopredators (smaller-bodied, non-apex predators) are becoming more predominant in communities, in some cases expanding their range and entering new communities (Prugh et al., 2009). Because of the broad range of their diet, generalists can occupy multiple trophic levels within a food web, often making it difficult to predict their impacts on a community or how climate change will impact their populations (e.g. Brocke, 1970; Prugh et al., 2009). The literature examining diet variation across a generalist's geographic range is limited. Two studies evaluating generalist species across their ranges found that variation in diet was linked to local habitat including relative food abundance and human land-use (Behrens and Lafferty, 2012; Terraube and Arroyo, 2011), while a third found dietary breadth constricted in high-latitude winters (Soe et al., 2017). When the stable isotopic compositions of 12 South American bird species were analyzed across their ranges, isotopic niche breadth was not phylogenetically conserved, but species with broader geographic ranges had larger isotopic niches (Rader et al., 2017).

Despite the growing importance of generalist mesopredators in communities (Prugh et al., 2009), little is known about isotopic niche patterns across a generalist's range. On-going range expansions of widespread mesopredators offer natural experiments to investigate isotopic niche variation across a variety of habitats and range locations. The broad geographic range (Gardner and Sunquist, 2003) and northward expansion of the Virginia opossum, *Didelphis virginiana* (Walsh and Tucker, 2018) provide an opportunity to evaluate how a generalist's isotopic niche changes with its environment and at the margins of its range.

The Virginia opossum (henceforth, opossum) is a generalist marsupial found from Central America to the Midwestern United States and southeastern Canada. Opossums found on the Pacific Coast are a result of multiple introductions of the species (Gardner and Sunquist, 2003). The northward expansion of opossums is well documented in museum records (Walsh and Tucker, 2018), and their current northern margins occur west of the Great Lakes in North Dakota (Walsh et al., 2017) and east of the Great Lakes into Maine. Anecdotally, the opossum's expansion in New England appears to be static since arriving in Maine in the 1950s (C. Mosby, pers. comm. 2015).

The opossum is a habitat generalist that frequents wetland and hardwood habitats, but it can also be found in grasslands, along forest edges, and in agricultural and suburban settings (Gardner and Sunquist, 2003; Beatty et al., 2014). The opossum avoids desert habitats and especially dense forest. Stomach dissections of opossums reveal that individuals are generalist omnivores, but they are also considered mesopredators because of their medium size and trophic levels. Their broad diet includes invertebrates, small amphibians and mammals, snakes, birds and their eggs, green vegetation, mushrooms (reviewed by Gardner and Sunquist, 2003; Table 2-1), and feces (Brocke, 1970).

Geographic variation in the opossum's diet has largely been overlooked because of its generalist habits, and there are no diet studies for this species from Mesoamerica (Gardner and Sunquist, 2003). However, a handful of regional studies suggest there may be a geographic pattern (Table 2-1; Table A2.1). In five out of six studies that examined year-round digestive tract contents of opossums from northern latitudes, the largest percentage of food consumed, based on volume, was vertebrate in nature (Dearborn, 1932; Hamilton, 1958; Stieglitz and Klimstra, 1962; Knudsen and Hale, 1970; Blumenthal and Kirkland, 1976; Whitaker et al., 1977). This is in contrast to Texas, where almost half of the opossum's diet was composed of plant matter (Wood, 1954). The diet of the opossum at the northern edge of its range has received considerably more attention from researchers (Table 2-1) in part because of the limitations they face when foraging in winter. With fleshy paws and a low-hanging body, opossums are ill-equipped to forage in snow (Brocke, 1970). Numerous biologists have speculated that during a harsh winter, opossums may rely more heavily on opportunistic scavenging of road kill, cannibalism and coprophagy (Brocke, 1970), or easily obtained anthropogenic resources including trash and pet food (Kanda, 2005).

I tested the hypothesis that the isotopic niche breadth of the opossum varies across its geographic range because a generalist's stable isotope values will reflect local prey variety and habitat heterogeneity. In addition, I tested the prediction that opossum $\delta^{15}N$ values are higher in colder habitats because preliminary data suggest increased carnivory in more northern states (Table 2-1). Finally, I tested the prediction that opossum $\delta^{13}C$ values are correlated with variables that predict C_4 plant abundance including landcover type, growing season temperature, and mean annual temperature (Cotton et al., 2016).

Methods

Sample Collection

To examine how the opossum's isotopic niche varies across its range, I defined six regions of interest based on the timing of the opossum's expansion into temperate North America. Mesoamerica and the Gulf Coast were established as ancestral regions based on Pleistocene fossil records (Graham and Lundelius, 2010). The Midwest and Northeast were established as recent northward-expansion regions, and the Central Valley and California Coast were established as two regions occupied due to human-facilitated introductions (Figure 2-1). The Central Valley served as the more agricultural region in California, with much of its grassland cleared for exotic plants and agriculture (Olmstead and Rhode, 2017). Because samples spanned 100 years, each region's isotope values were evaluated for correlations with the year in which samples were collected.

Between 20 and 42 individuals were sampled from adult museum skin specimens for each region for a total of 153 specimens sampled (69 females, 75 males, 9 unknown). In addition to museum skins, one dead-on-arrival opossum was sampled from the Ohio Wildlife Center (Ohio Division of Wildlife Permit 18-60). Table A2.2 provides catalog number, year, location, and sex of each specimen sampled.

Stable Isotope Analysis

For each specimen, approximately three whole guard hairs were treated with a 2:1 ratio of chloroform – methanol to remove lipids, dried under a fume hood at ambient temperature, diced, and weighed with a Mettler AE 240 balance (Toledo, OH) to place 0.5-1.0 mg in a tin capsule (Sergiel et al., 2017). Duplicate capsules for each sample were sent to the University of New Mexico Center for Stable Isotopes (UNM-CSI) for stable isotope analysis of $\delta^{13}C$ and $\delta^{15}N$. To analyze organic substrates, UNM-CSI uses a Thermo Scientific (Waltham, MA) Delta V mass spectrometer with a Conflo IV interface, Costech (Valencia, CA) 4010 elemental analyzer, and a high-temperature conversion elemental analyzer. Analytical error across 56 runs of the UNM-CSI protein standard (casein) was 0.1099 standard deviations (SD) for $\delta^{15}N$ and 0.0543 SD for $\delta^{13}C$.

The museum specimens evaluated for this research were collected across 100 years. Because atmospheric carbon isotope ratios have decreased over the past century due to the influx of greenhouse gases derived from the burning of fossil fuels, I adjusted for changes in atmospheric composition (Suess-corrected) with a linear correction of minus 0.005‰ per year between 1917 and 1961 and minus 0.022‰ per year after 1961 (Chamberlain et al., 2005). I verified that there was no sex-based difference in $\delta^{13}C$ or $\delta^{15}N$ values in my data using a Mann–Whitney U–test, allowing me to analyze samples collectively for each region.

Isotopic Niche Evaluation

The opossums' isotopic niche breadth was calculated for each of the six regions using the Bayesian ellipse method. Ellipses can be thought of as standard deviations for bivariate datasets – in this case $\delta^{13}C$ and $\delta^{15}N$ values. By implementing Bayesian inference techniques in the R package SIBER v.2.1.3 (Jackson et al., 2011) when calculating the standard ellipse area (SEA_B), sampling error and uncertainty can be accounted for in the isotopic niche.

Because analyses can be partially confounded by sampling regions of unequal geographic area (Stein et al., 2014), two methods were implemented to evaluate the impact that geographic area might have on my calculated isotopic niche breadth. First, the area of each of the six regions was estimated in imageJ by tracing the polygon create by collection points in a given region (Figure 2-1) and evaluated for correlation with SEA_B. For a finer-scale approach, SEA_B was calculated in SIBER v.2.1.3 (Jackson et al., 2011) for eight subsamples consisting of single or adjoining counties in which at least four opossums were sampled and assessed for correlation with the total land area of the subsamples available from county census records (n = 43; Table A2.2).

To evaluate whether habitat heterogeneity impacts isotopic niche breadth, landcover data from the U.S., Canada, Mexico, Guatemala, Belize, Nicaragua, and El Salvador were downloaded from https://data.terrapop.org/terraclip#. This landcover data from 2000 assigned each geolocated grid cell of 1 km resolution one of 22 landcover types (e.g. mixed leaf tree cover, herbaceous cover, broadleaved evergreen tree cover, mosaic cropland and tree cover; Fritz et al., 2003). While the majority of samples were collected before 2000, landcover change between the 22 landcover types across my geographic scale remains low, perhaps with the exception of urban sprawl captured as "artificial surfaces" (Brown et al., 2014). Landcover type for individual opossums were extracted and included 11 unique landcover types (see Table A2.2, Figure A2.1). The heterogeneity of the six regions was evaluated using four habitat-diversity measurements: Simpson's Diversity and Evenness Indexes and Shannon's Diversity and Evenness Indexes. Diversity indexes evaluate the composition of a community, taking into account relative abundance, while evenness indexes evaluate the proportional representation of species. In my application of Simpson's and Shannon's equations, landcover types were treated as analogous to different species (Lausch and Herzog, 2002; Schindler et al., 2008; Figure A2.1). These diversity measurements (Table 2-2) were used to evaluate whether habitat heterogeneity of a region is positively correlated with its isotopic niche breadth.

Isotopes and Environmental Variables

To investigate differences in opossum stable isotope composition across habitat types, six landcover types were evaluated (each with $n \geq 7$ opossum samples). ANOVA was used for the normally distributed $\delta^{15}N$ values and the Kruskal-Wallis test was employed for the non-normally distributed $\delta^{13}C$ values (n = 133).

To investigate the influence that abiotic variables have on opossum isotope composition, 10 climate variables were extracted from the WorldClim dataset (Hijmans et al., 2005) at 10 minutes resolution using the raster package in R (Hijmans, 2015). For detailed instructions see https://www.worldclim.org/formats1. WorldClim variables are averaged measurements from 1970-2000, and these data are estimated climate variables, especially for opossums collected outside of the 30-year WorldClim window. WorldClim variables were selected to evaluate the prediction that opossums in colder areas with more seasonality would have higher $\delta^{15}N$ values and the prediction that opossum $\delta^{13}C$ values would be correlated with climate variables associated with C_4 plant abundance. The ten variables included annual precipitation (AP), mean annual temperature (MAT), minimum temperature of coldest month (MNT), mean temperature of driest quarter (MTDQ), mean temperature of wettest quarter (MTWQ), maximum temperature of warmest month (MXT), precipitation in coldest quarter (PCQ), precipitation in driest quarter (PDQ), precipitation in wettest quarter (PWQ), and temperature annual range (TAR). The correlation between each climate variable and $\delta^{13}C$ and $\delta^{15}N$ values for each opossum was evaluated using Spearman's rank correlation analysis because the climate variables were not normally distributed. In addition, for each stable isotope, the importance of these ten variables was analyzed using conditional forest (CF) analysis with the party package in R (Strobl et al., 2008). CF analysis is a classification and regression-tree machine-learning method that allows for assessment of non-parametric data and accounts for complex interactions including correlations among independent variables (Strobl et al., 2008). For each isotope, 1000 trees were built using randomly selected WorldClim variables and aggregated. The number of variables to include in each tree was determined based on the forest that yielded the smallest root mean square error measured with the 'caret' R package (Kuhn 2008; Strobl et al. 2008).

Results

Isotopic Niche Evaluation

From a sample of 153 opossums collected between 1917 and 2017 (Table A2.2), $\delta^{15}N$ values ranged from 4.35 to 13.98‰ ($\pm$0.16 SD), and Suess-corrected $\delta^{13}C$ values ranged from -24.99 to -16.23‰ ($\pm$0.20 SD; Figure A2.2). No region's isotope values were significantly correlated with the year in which they were collected (Spearman's $p \geq 0.06$). The isotopic niche breadth (SEA_B) varied across the opossum's geographic range, with the largest SEA_B observed in Mesoamerica and the Midwest, followed by the Gulf Coast (Figure 2-2). The smallest SEA_B occurred in California's agricultural Central Valley, California's coast, and the Northeast (Figure 2-2). Based on an analysis of 43 opossums from eight distinct county clusters, there was no correlation between SEA_B and county geographic area (Pearson's r = -0.490, p = 0.218; Table A2.3). The correlation coefficient increased but remained non-significant when the six regions' SEA_B were evaluated for correlation with geographic area (Pearson's r = 0.778, p = 0.069; Table 2-2). SEA_B was significantly positively correlated with Simpson's and Shannon's Diversity Indexes (Pearson's $r \geq 0.869$, $p \leq 0.025$; Table 2-2) and with Shannon's Evenness Index (Pearson's $r \geq 0.984$, $p < 0.001$; Table 2-2).

Isotopes and Environmental Variables

In comparing stable isotopes from six landcover types encompassing 133 opossum samples, there was no significant difference in $\delta^{15}N$ values among landcover types (ANOVA p = 0.300). There were significant differences in $\delta^{13}C$ values among landcover types (K-W p =

0.021; Figure A2.3). Herbaceous cover had significantly higher $\delta^{13}C$ values compared to broadleaved evergreen and broadleaved deciduous cover (Dunn's $p \leq 0.029$). Similarly, cultivated areas also had significantly enriched $\delta^{13}C$ values compared to broadleaved evergreen and broadleaved deciduous cover, along with needle-leaved evergreen cover (*Dunn's* $p \leq 0.046$).

Multiple climate variables were significantly correlated with stable isotope values (*Spearman's* $p \leq 0.027$). Precipitation in the driest quarter, annual precipitation, precipitation in the coldest quarter, and temperature annual range were negatively correlated with $\delta^{15}N$ values. Minimum temperature was positively correlated with $\delta^{15}N$ values (Table 2-3). Precipitation in the coldest quarter was negatively correlated with $\delta^{13}C$ values, while mean temperature in the wettest quarter, maximum temperature, and mean annual temperature were positively correlated with $\delta^{13}C$ values (Table 2-3).

In the conditional forest analysis, the 10 WorldClim variables selected explained 19.63% of the variance in $\delta^{15}N$ data and 13.53% of the variance in $\delta^{13}C$ data. The WorldClim variable with the highest importance for nitrogen isotopic composition of opossum guard hairs was annual precipitation, followed by precipitation in the driest quarter, mean temperature in the wettest quarter and temperature annual range (Figure 2-3). The WorldClim variable with the highest importance for carbon isotopic composition of opossum guard hairs was precipitation in the coldest quarter, followed by mean temperature in the wettest quarter, maximum temperature, and mean annual temperature (Figure 2-4).

Discussion

I used the opossum, a species experiencing a range expansion, as a system to study variation in stable isotope values and niche breadth across a broad spatial scale. Based on the observation that other generalist species' diets can reflect their local habitat and abundance of food sources (Terraube and Arroyo, 2011; Behrens and Lafferty, 2012;), my analysis supported the hypothesis that the isotopic niche breadth varies across the opossum's range (Figure 2-2). Temporal expansions and contractions of isotopic niches have been observed from the Holocene through the modern-day samples of rodents in the Great Basin (Terry, 2017; Terry et al., 2017). My results demonstrate that variation in niche breadth can also occur spatially, as populations span different environmental and climatic conditions.

I found a positive relationship between habitat diversity and isotopic niche breadth (Table 2-2), suggesting that more heterogenous landscapes result in broader isotopic niches for opossums. Habitat heterogeneity is known to increase with spatial grain of analysis (Stein et al., 2014); by conducting my analysis over a broad spatial range I was able to assess this relationship with isotopic variation for the taxon of interest. The scale of my analysis is comparable with the area required to identify signals of genetic structure in the opossum (Walsh and Tucker, 2018).

The habitat heterogeneity hypothesis states that heterogenous and complex landscapes increase the number of niches and therefore are expected to accommodate more species (MacArthur, 1972). Here, I suggest an extension for single generalist species: the 'generalist habitat heterogeneity hypothesis' wherein more heterogenous landscapes result in the broadening of a generalist species' niche. My results, along with data from other generalist species (e.g. Sacks et al., 2008; Staudacher et al., 2017), support the expected expansion of a generalist's niche in a heterogeneous landscape. To further understand isotopic niche trends in generalist mammals, I suggest sampling mesopredator mammals across various degrees of habitat heterogeneity to bolster statistical power and compare results across species.

Isotope patterns

The hypothesis that opossum $\delta^{15}N$ values would be enriched in colder habitats was not supported. Instead, $\delta^{15}N$ values are primarily correlated with precipitation patterns: areas with lower precipitation had higher $\delta^{15}N$ values. Aridity is known to make the nitrogen cycle less efficient, leading to nitrogen enrichment in soils and vegetation, but this signal can be lost in localized assessments due to other processes that influence baseline $\delta^{15}N$ values in microhabitats (Pardo and Nadelhoffer, 2010). My ability to detect a relationship between opossum nitrogen isotopic composition with precipitation across a large geographic scale is consistent with a study on two heteromyid rodent species in western North America (Smiley et al., 2016), which found a similar relationship despite the noise contributed by microhabitat processes (Pardo and Nadelhoffer, 2010). Opossum $\delta^{15}N$ values were higher in areas with less seasonality (those with smaller temperature ranges) and habitats with higher minimum temperatures. The higher $\delta^{15}N$ values in warmer areas may be partially driven by the presence of year-round insect populations. My original prediction was based on the available literature of opossum diets (Table 2-1) and observations of cannibalism and coprophagy during northern winters (Brocke, 1970). My results highlight the importance of natural history studies across a species' range, including areas of Central America, and raise questions regarding reliance on digestive tract dissections to evaluate the diet of generalist mammals. These dissection methods can be biased toward materials that require longer digestive time, yield no information when tracts are empty, and only provide a snapshot of the animal's last meal (Tieszen et al., 1983).

The prediction that opossum $\delta^{13}C$ values would reflect the local abundance of C_4 plants was supported. As predicted, opossums living in herbaceous and cultivated landcover types, which are often dominated by C_4 plants, had higher $\delta^{13}C$ values than opossums found in predominantly C_3 forest landcover types (Figure A2.3). Additionally, opossums feeding from the understory in these forests may have lower $\delta^{13}C$ values because of the canopy effect, which leads to ^{13}C-depleted underlying vegetation (van der Merwe and Medina, 1991). In general, the WorldClim variables that had the highest importance for $\delta^{13}C$ values supported the hypothesis that $\delta^{13}C$ values are influenced by the same variables that predict the relative abundance of C_3 and C_4 plant growth (e.g., growing season conditions). Areas with less rainfall and warmer temperature, climatic conditions that are more conducive for C_4 grasses than for C_3 vegetation (Terri and Stowe, 1976; Kelly, 2000), had higher $\delta^{13}C$ values. Previously, precipitation was found to be negatively correlated with plant $\delta^{13}C$ values, regardless of their photosynthetic pathway. However, temperature had no relationship with plant $\delta^{13}C$ values (Lee et al., 2005). Interestingly, even though opossums are omnivorous generalists, the climate patterns that best predict $\delta^{13}C$ values across their range are consistent with similarly broad spatial studies of large obligate grazers and small herbivores (Cotton et al., 2016; Smiley et al., 2016).

Conditional forest analyses showed WorldClim variables to have more explanatory power for nitrogen isotopic composition than for carbon isotopic composition; however explanatory power of climate variables was low (< 20%) for both isotopes. This may in part be due to sampling from across multiple climatic regimes in North America which could amplify the noise of isotopic signals caused by local ecosystem processes and ecophysiological differences among plant species (Lajtha and Marshall, 1994; Pardo and Nadelhoffer, 2010; Beck et al., 2018). This may also be due to the strong influence that human land-use (e.g. urbanization and agriculture) has on mammal communities, especially generalists (Rowe and Terry, 2014). The isotopic composition of a consumer is a combination of an animal's foraging behavior and the isoscape in which it is foraging (Yeakel et al., 2016), so it is reasonable for climate not to explain all the variance detected in a nomadic generalist species (Gardner and Sunquist, 2003). Given that opossums consume a wide variety of diet items from multiple trophic levels, it is not surprising their diverse, omnivorous diet does not closely reflect underlying vegetation (which may or may not have a strong relationship with climate variables).

Comparison with Other Species

A number of sampled opossums overlapped in location with other mammalian mesopredators with published isotopic data. In Chicago, Illinois' Cook County, opossums had $\delta^{13}C$ and $\delta^{15}N$ values that overlapped with those of coyotes (*Canis latrans*; Newsome et al., 2015). However, in California's Kern County and adjoining counties including Los Angeles, only two opossums had both overlapping $\delta^{13}C$ and $\delta^{15}N$ values with San Joaquin kit foxes (*Vulpes macrotis mutica*). Most opossums collected had lower $\delta^{13}C$ values than the kit foxes (Newsome et al., 2010). Coyotes are generalist mesopredators that consume from multiple trophic levels (Bekoff and Gese, 2003) while kit foxes prefer arid landscapes and are more carnivorous than other North American foxes (Cypher, 2003). Although the opossums and kit foxes were sampled in adjacent counties, the non-overlapping dietary niches may be driven by the kit fox's preferences for arid landscapes. My research did not directly evaluate this hypothesis, and I invite further research comparing mesopredator niches.

Range Expansion

The two regions with expansion fronts, the Midwest and Northeast, had different isotopic niche breadths – the Midwest represented one of the largest niches while the Northeast had the smallest niche (Figure 2-2). This difference may be due to the comparative homogeneity of the habitat and climate in the Northeast (Table A2.2). In addition to encompassing all habitats found in the Northeast, the Midwest included both herbaceous and shrub landcovers (Figure A2.1). The more heterogenous habitats in the Midwest (Table A2.2) that include herbaceous and shrub cover may include a greater variety of C_3:C_4 plant ratios, resulting in a broader distribution of $\delta^{13}C$ values measured in Midwestern opossums. Similarly, the range of annual precipitation in the

Midwest is greater (592 mm range) than in the Northeast (362 mm range). This larger range of precipitation may drive greater variation in $\delta^{15}N$ values in the Midwest.

The opossum is a diet and habitat generalist, and its stable isotope signature appears to generally reflect its local habitat. With their ability to consume resources across a variety of trophic levels, little should slow their continued expansion northward. This is seemingly the case in the upper Midwest, where opossums are becoming more frequent in North Dakota (Walsh et al., 2017) and genetic evidence suggests their population benefits from agricultural land, a widespread landcover type in the Midwest (Walsh and Tucker, 2018; Figure 2-1). Anecdotal data indicate that the northward expansion of opossums in the Northeast has stalled near Portland, Maine in New England (C. Mosby, pers. comm. 2015). The relatively low habitat diversity indexes in the Northeast (Table 2-2), along with the comparatively inhospitable dense coniferous forests in northern Maine, may partially explain the static range margin in Maine. More heterogenous landscapes, as identified in the Midwest, typically serve as better refugia from harsh conditions that opossums would face at the northern edge of their range (Milling et al., 2018). The contrasting results from the range expansions in the Midwest and Northeast highlight the importance of understanding how local habitat and climate influence the ecology of a generalist species.

Conclusion

Stable isotope analyses of a widespread generalist mammal indicate that their isotope values are impacted by both landcover type and climatic factors in their habitat. Because opossums have a wide, flexible diet (Gardner and Sunquist, 2003), their stable isotope values should mirror the prey abundance and isotopic variation of the dietary resources in the habitat in which they forage. This is congruent with my results, and the constricted isotopic niche of opossums in the Northeast may be a symptom of an expanding population encountering a barrier.

Tables

Table 2-1 Composition of year-round digestive tracts of Virginia opossums (*Didelphis virginiana*) based on volumetric percentage (%) in U.S.A states ordered from north to south. Omitted from this table are non-insect invertebrates representing 0 to 16.3% of food consumed.

	Mean Annual Volumetric % Food Consumed					
U.S.A. state	Insects	Birds	Mammals	Plant	Herptile	Reference
Wisconsin	10	24	41	6	12	Knudsen & Hale 1970
New York	7.9	7.2	22.6	22.2	14.9	Hamilton 1958
Michigan	16.7	25.3	23.2	30.4	8	Dearborn 1932
Pennsylvania	9	4.5	26	37	0.5	Blumenthal & Kirkland 1976
Indiana	11.7	21.3	22.2	19	2.3	Whitaker et al. 1977
Illinois	6.3	14.5	48.7	23.8	4.6	Stieglitz & Klimstra 1962
Texas	25	3.8	14.9	44.8	7.4	Wood 1954

Table 2-2 Virginia opossum (*Didelphis virginiana*) regional values for Bayesian standard ellipse area (SEA_B) and habitat diversity measurements calculated using 2000 global landcover types (Fritz et al. 2003; D = diversity index, E = evenness index). Regional geographic area was measured using imageJ (units = pixels). Pearson's correlation coefficients for SEA_B are in the last row. Significant correlations are in bold, * indicate $p < 0.01$, ** indicate $p < 0.001$.

Region	SEA_B	Simpson's D	Simpson's E	Shannon's D	Shannon's E	Geographic area
California Coast	4.89	0.60	0.58	1.00	0.72	2 428
California Valley	6.79	0.63	0.49	1.20	0.75	2 767
Mesoamerica	11.12	0.82	0.65	1.68	0.86	43 458
Gulf Coast	9.74	0.78	0.53	1.67	0.81	32 226
Midwest	11.89	0.84	0.62	1.79	0.86	23 225
Northeast	3.05	0.61	0.41	1.25	0.70	9 618
Pearson's correlation	-	**0.951***	0.768	**0.890**	**0.984****	0.778

Table 2-3 Spearman's correlation coefficients (r) for $\delta^{15}N$ and $\delta^{13}C$ values from Virginia opossum (*Didelphis virginiana*) guard hair with 10 WorldClim variables; P = precipitation, Q = quarter, T = temperature. Bold values are significant at $p<0.05$, *$p<0.01$, **$p<0.001$.

Variable	$\delta^{15}N$ r	$\delta^{13}C$ r
Annual P (AP)	**-0.308****	-0.025
Mean Annual T (MAT)	0.132	**0.292****
Minimum T (MNT)	**0.24***	0.122
Mean T Driest Q (MTDQ)	0.118	0.036
Mean T Wettest Q (MTWQ)	0.146	**0.308****
Maximum T (MXT)	0.044	**0.305****
P Coldest Q (PCQ)	**-0.179**	**-0.249***
P Driest Q (PDQ)	**-0.331****	0.096
P Wettest Q (PWQ)	-0.156	-0.097
T Annual Range (TAR)	**-0.167**	-0.03

Figures

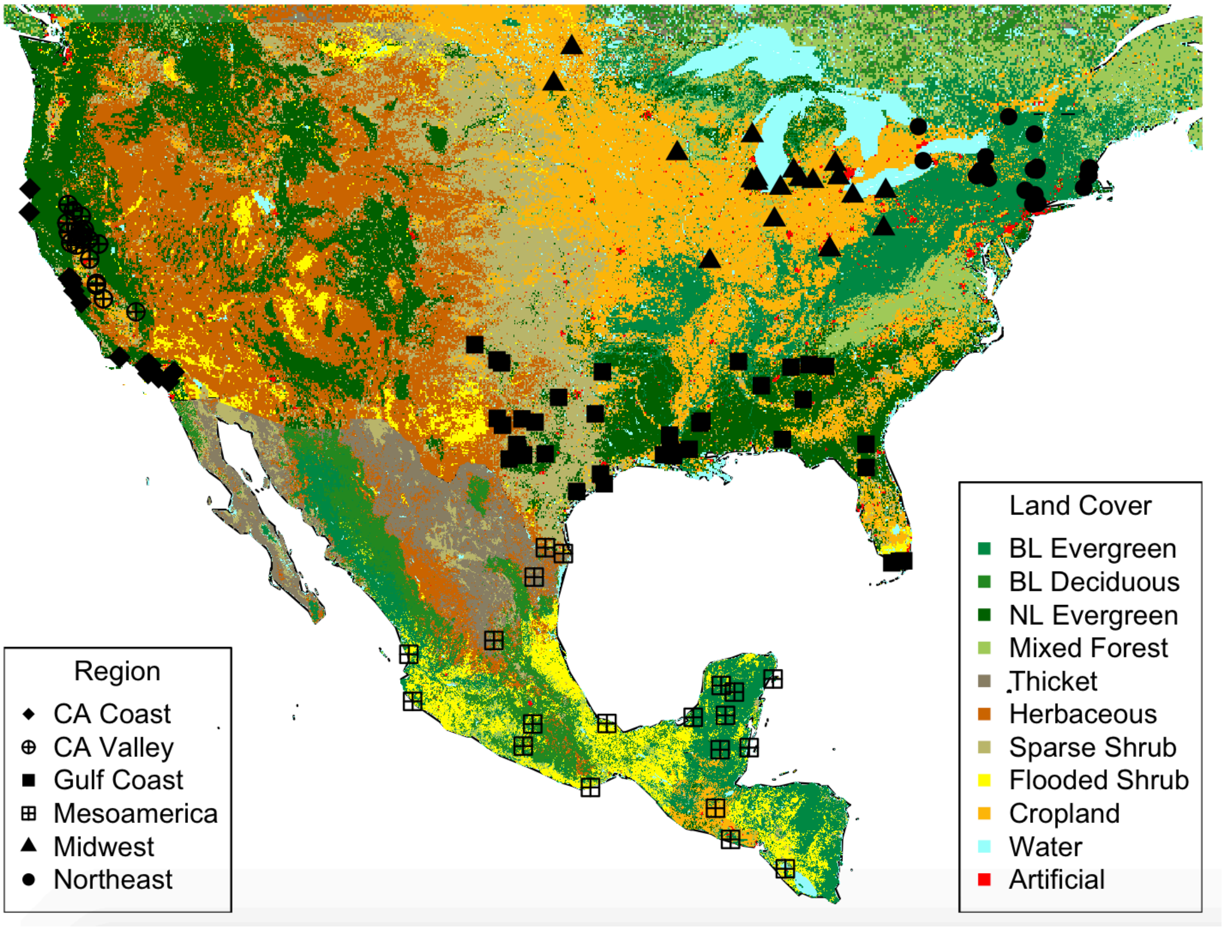

Figure 2-1 Virginia opossum (*Didelphis virginiana*) samples analyzed for stable isotope values from six regions. Individual samples are plotted onto the 2000 global landcover map (Fritz et al. 2003).

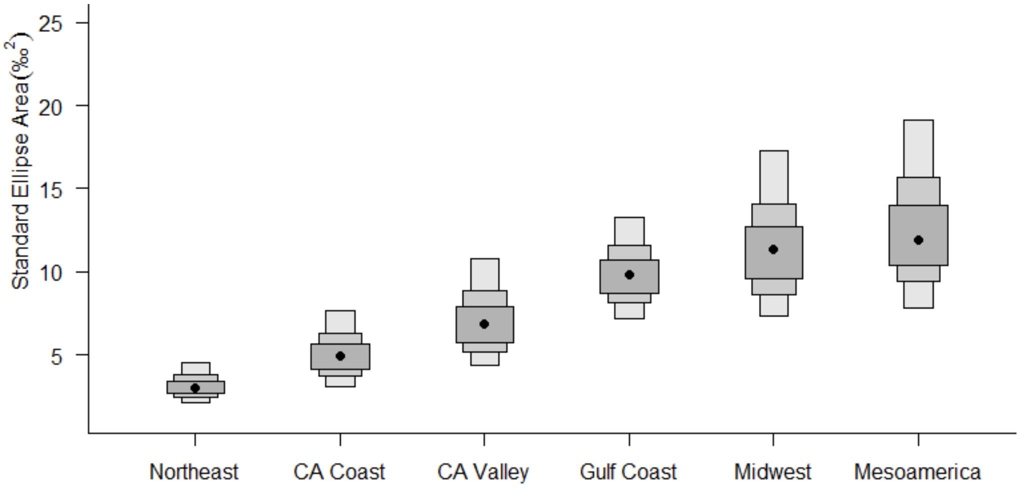

Figure 2-2 Isotopic niche breadth of Virginia opossums (*Didelphis virginiana*) in six regions calculated as the Bayesian standard ellipse area (SEA$_B$). Regions are arranged, left to right, from smallest to largest isotopic niche breadth. Black dots indicate the mode of the region's niche breadth and shaded boxes represent 50%, 75%, and 95% credible ellipse intervals, respectively.

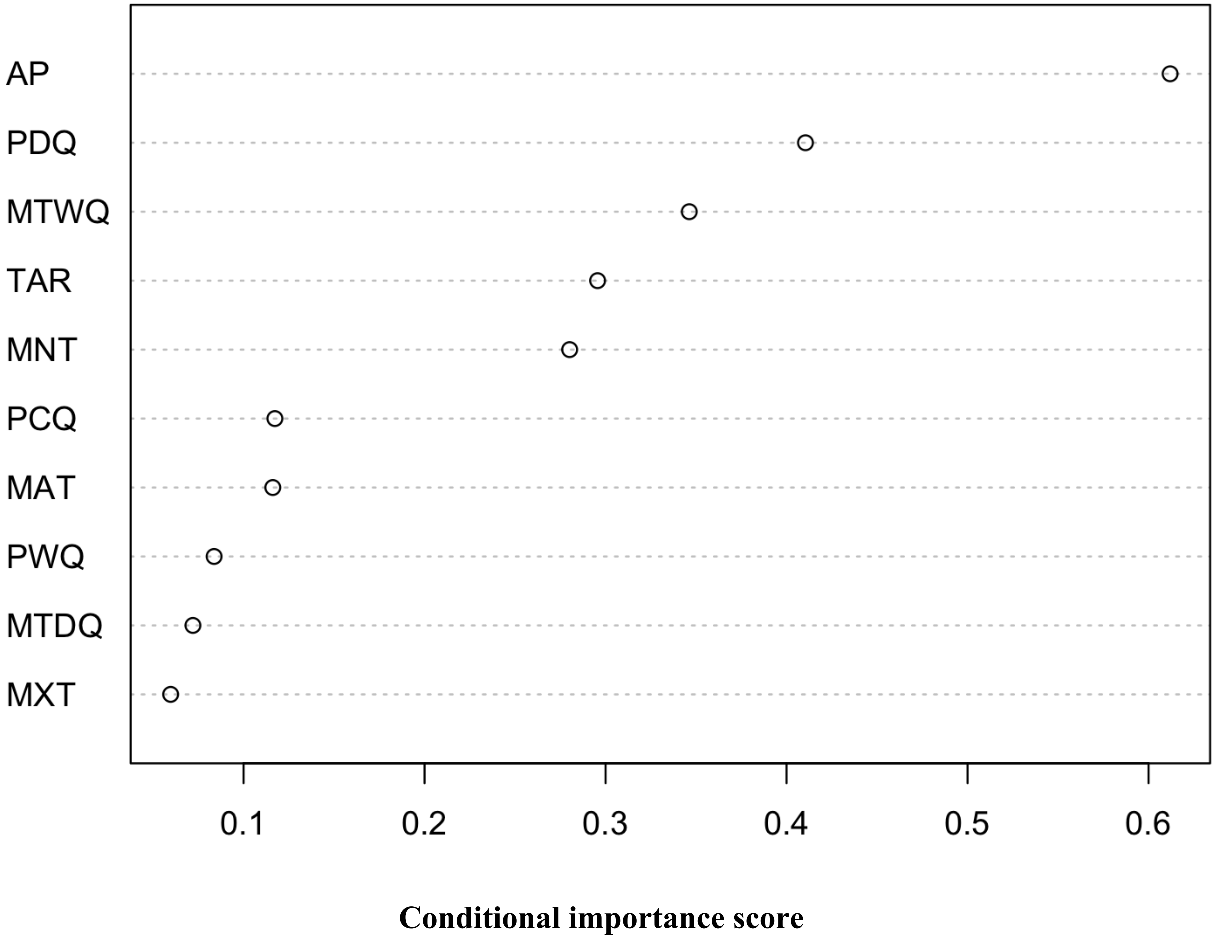

Figure 2-3 Conditional forest analysis variable importance for nitrogen ($\delta^{15}N$) isotope values of the Virginia opossum (*Didelphis virginiana*). WorldClim variables include annual precipitation (AP), precipitation in the driest quarter (PDQ), mean temperature in the wettest quarter (MTWQ), temperature annual range (TAR), minimum temperature in the coldest month (MNT), precipitation in the coldest quarter (PCQ), mean annual temperature (MAT), precipitation in the wettest quarter (PWQ), mean temperature in the driest quarter (MTDQ), and maximum temperature in the warmest month (MXT).

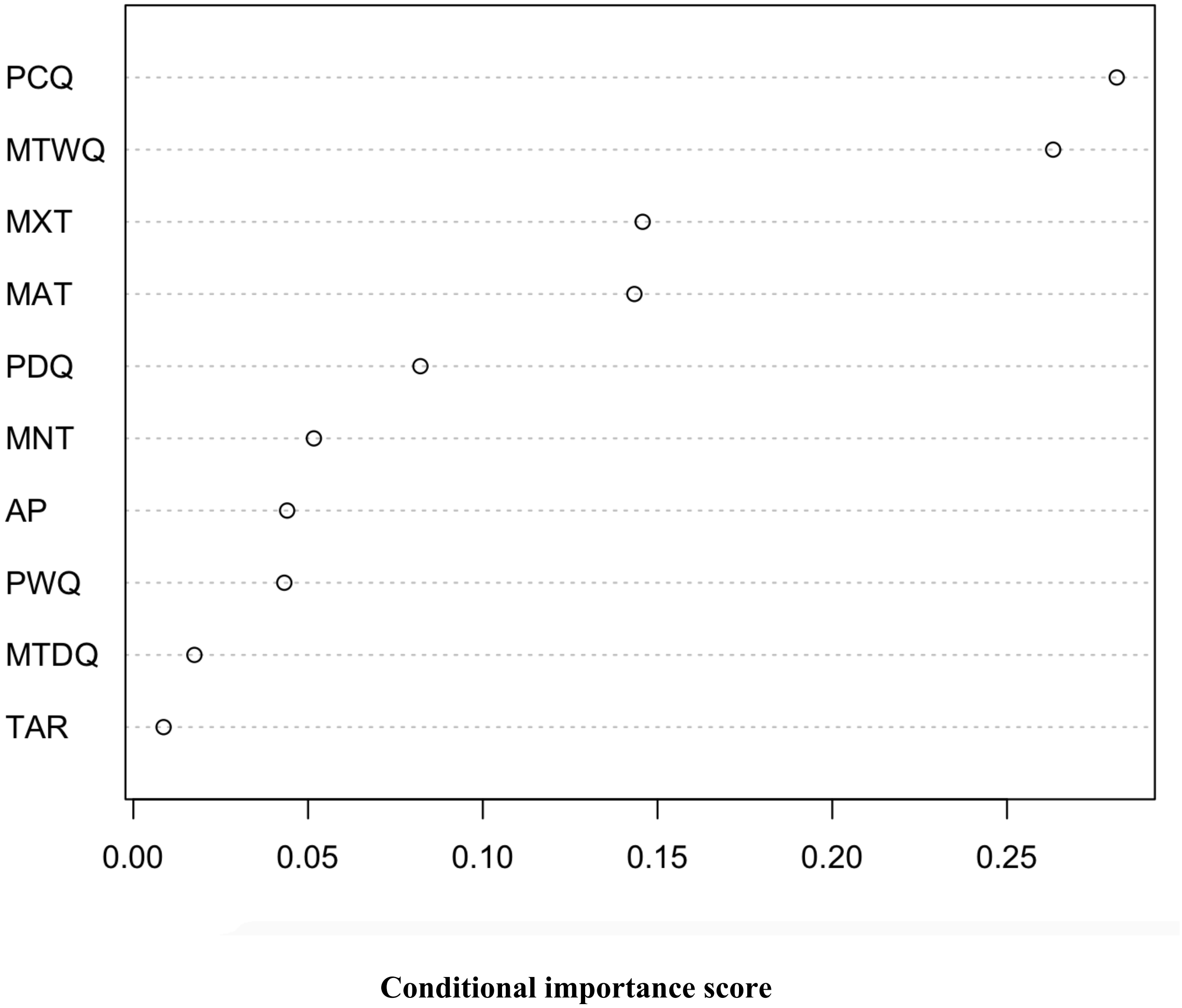

Figure 2-4 Conditional forest analysis variable importance for carbon ($\delta^{13}C$) isotope values of the Virginia opossum (*Didelphis virginiana*). WorldClim variables include precipitation in the coldest quarter (PCQ), mean temperature in the wettest quarter (MTWQ), maximum temperature in the warmest month (MXT), mean annual temperature (MAT), precipitation in the driest quarter (PDQ), minimum temperature in the coldest month (MNT), annual precipitation (AP), precipitation in the wettest quarter (PWQ), mean temperature in the driest quarter (MTDQ), and temperature annual range (TAR).

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Appendix A2

Table A2.1 Seasonal composition of digestive tracts of Virginia opossums (*Didelphis virginiana*).

STATE	Season	Author	Insects	Birds	Mammals	Plant	Herps	Other invert	Garbage	Measurement
IL	Winter	Stieglitz & Klimstra 1962	2	30	60	6.5	0.5	1.5		Volume %
	Summer		5.5	12	17.5	47	13	0.5		
NY	Winter	Hamilton 1958	25.0		63.9	44.4	16.7	25.0		% frequency
	Spring		27.5		40.2	45.7	19.6	34.5		
	Summer		66.7		27.3	38.4	36.3	13.1		
	Fall		41.1		43.8	42.5	19.6	24.6		
PA	Winter	Blumenthal & Kirkland 1976	11	4.5	30	39	0	11	2	Volume %
	Spring		6	3	24	37	1	18.5	0	
	Summer		12	10	25	37	0	8.5	0	
	Fall		7	3.5	25	38	0	13.5	0	
WI	Winter	Knudsen & Hale 1970	2	10	35	1	0		7	Volume %
	Spring		1	9	14	6	8	11	3	
	Summer		1	34	6		15	8	1	
	Fall		2	1	17	7	7	10		

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Table A2.2 Collection data from Virginia opossum (*Didelphis virginiana*) guard hair samples including catalog number, region, country, state, year, and land cover type. Samples are organized by region. Institution acronyms are as follows, alphabetically: Angelo State Natural History Collections (ASNHC), UC Santa Barbara Cheadle Center for Biodiversity and Ecological Restoration (CCBER), Washington State University Charles R. Conner Museum (CRCM), California State University Chico Vertebrate Museum (CSUC), California State University Long Beach (CSULB), Cornell Museum of Vertebrates (CUMV), Field Museum of Natural History (FMNH), Humboldt State University Vertebrate Museum (HSU), Natural History Museum of Los Angeles County (LACM), Louisiana State University Museum of Natural Science (LSU), Harvard University Museum of Comparative Zoology (MCZ), University of New Mexico Museum of Southwestern Biology (MSB), Michigan State University Natural Science Collections (MSU), UC Berkeley Museum of Vertebrate Zoology (MVZ), New York State Museum (NYSM), Sam Noble Oklahoma Museum of Natural History (OMNH), Ohio Wildlife Center (OWC), Royal Ontario Museum (ROM), Museum of Texas Tech University (TTU), University of Alaska Museum (UAM), University of Michigan Museum of Zoology (UMMZ), National Museum of Natural History Smithsonian Institution (USNM), and University of Washington Burke Museum (UWBM).

Catalog No.	**Region**	**Country**	**State**	**County**	**Year**	**Cover Type (Fritz et al. 2003)**
CCBER 1593	CACoast	USA	California	Santa Barbara	1953	Tree Cover, Needle-leaved, Evergreen
CCBER 2574	CACoast	USA	California	Santa Barbara	1963	Artificial Surfaces and Associated Areas
CSULB 121	CAcoast	USA	California	Orange	1951	Tree Cover, Needle-leaved, Evergreen
HSU 1244	CACoast	USA	California	Santa Clara	1969	Artificial Surfaces and Associated Areas
HSU 1245	CACoast	USA	California	Monterey	1970	Tree Cover, Needle-leaved, Evergreen
HSU 1336	CACoast	USA	California	Los Angeles	1976	Sparse Herbaceous or sparse Shrub Cover
HSU 2149	CACoast	USA	California	Del Norte	1985	Tree Cover, Needle-leaved, Evergreen
HSU 2857	CACoast	USA	California	Humboldt	2002	Water Bodies
LACM 40097	CACoast	USA	California	Los Angeles	1968	Artificial Surfaces and Associated Areas
LACM 8349	CACoast	USA	California	Los Angeles	1944	Artificial Surfaces and Associated Areas
LACM 91119	CACoast	USA	California	Los Angeles	1991	Artificial Surfaces and Associated Areas
MVZ 102074	CACoast	USA	California	Contra Costa	1944	Tree Cover, Needle-leaved, Evergreen
MVZ 220246	CACoast	USA	California	Alameda	2006	Artificial Surfaces and Associated Areas
MVZ 28776	CACoast	USA	California	Alameda	1918	Artificial Surfaces and Associated Areas
MVZ 47148	CACoast	USA	California	Alameda	1931	Tree Cover, Needle-leaved, Evergreen
MVZ 51974	CACoast	USA	California	Alameda	1932	Artificial Surfaces and Associated Areas
MVZ 97345	CACoast	USA	California	Alameda	1941	Artificial Surfaces and Associated Areas

UWBM 44463	CACoast	USA	California	Los Angeles	1958	Artificial Surfaces and Associated Areas
UWBM 44479	CACoast	USA	California	San Diego	1977	Tree Cover, Needle-leaved, Evergreen
UWBM 76230	CACoast	USA	California	Riverside	1964	Artificial Surfaces and Associated Areas
CSUC 1346	CAValley	USA	California	Butte/Tehama	1964	Tree Cover, Needle-leaved, Evergreen
CSUC 1661	CAValley	USA	California	Butte	1963	Tree Cover, Needle-leaved, Evergreen
CSUC 1753	CAValley	USA	California	Nevada	1966	Tree Cover, Needle-leaved, Evergreen
CSUC 1998	CAValley	USA	California	Shasta	1966	Tree Cover, Needle-leaved, Evergreen
CSUC 2180	CAValley	USA	California	Tehama	1967	Tree Cover, Needle-leaved, Evergreen
CSUC 2556	CAValley	USA	California	Butte	1965	Tree Cover, Needle-leaved, Evergreen
CSUC 2557	CAValley	USA	California	Butte	1969	Tree Cover, Needle-leaved, Evergreen
CSUC 3238	CAValley	USA	California	Shasta	1970	Tree Cover, Needle-leaved, Evergreen
CSUC 3505	CAValley	USA	California	Tehama	1970	Tree Cover, Needle-leaved, Evergreen
CSUC 3944	CAValley	USA	California	Butte	1972	Shrub Cover, closed-open, deciduous
CSUC 4578	CAValley	USA	California	Butte	1974	Shrub Cover, closed-open, deciduous
CSUC 4579	CAValley	USA	California	Shasta	1974	Tree Cover, Needle-leaved, Evergreen
CSUC 871	CAValley	USA	California	Butte	1961	Tree Cover, Needle-leaved, Evergreen
CSULB 2777	CAValley	USA	California	Stanislaus	1959	Herbaceous Cover (contains both pastures and natural)
HSU 1263	CAValley	USA	California	Stanislaus	1970	Cultivated and Managed Terrestrial Areas
LACM 40096	CAValley	USA	California	Tulare	1968	Tree Cover, Needle-leaved, Evergreen
MVZ 104560	CAValley	USA	California	Fresno	1946	Cultivated and Managed Terrestrial Areas
MVZ 106611	CAValley	USA	California	Sacramento	1945	Artificial Surfaces and Associated Areas
MVZ 138994	CAValley	USA	California	Glenn	1968	Herbaceous Cover (contains both pastures and natural)
UAM 122877	CAValley	USA	California	Sutter	2010	Herbaceous Cover (contains both pastures and natural)
ASNHC 10195	Gulf	USA	Texas	Coleman	1998	Herbaceous Cover (contains both pastures and natural)
ASNHC 10196	Gulf	USA	Texas	Randall	1992	Artificial Surfaces and Associated Areas
ASNHC 10964	Gulf	USA	Texas	Parker	1999	Herbaceous Cover (contains both pastures and natural)
ASNHC 11792	Gulf	USA	Texas	Tom	2000	Shrub Cover, closed-open, deciduous
ASNHC 12491	Gulf	USA	Texas	Brown	2002	Tree Cover, Needle-leaved, Evergreen
ASNHC 12703	Gulf	USA	Texas	Coke	1999	Shrub Cover, closed-open, deciduous

ASNHC 12847	Gulf	USA	Texas	Anderson	1997	Tree Cover, Broadleaved, Deciduous, Closed
ASNHC 13228	Gulf	USA	Texas	Lamar	2003	Herbaceous Cover (contains both pastures and natural)
ASNHC 14145	Gulf	USA	Texas	Fort	2009	Tree Cover, Needle-leaved, Evergreen
CRCM 44-1	Gulf	USA	Alabama	Etowah	1944	Herbaceous Cover (contains both pastures and natural)
LSU 20367	Gulf	USA	Mississippi	Copiah	1976	Tree Cover, Needle-leaved, Evergreen
LSU 2434	Gulf	USA	Mississippi	Lee	1947	Tree Cover, Needle-leaved, Evergreen
LSU 32187	Gulf	USA	Louisiana	Lafayette	1959	Artificial Surfaces and Associated Areas
LSU 32189	Gulf	USA	Louisiana	Acadia	1961	Cultivated and Managed Terrestrial Areas
LSU 32206	Gulf	USA	Louisiana	Lafayette	1963	Cultivated and Managed Terrestrial Areas
LSU 32225	Gulf	USA	Louisiana	Lafayette	1967	Tree Cover, Needle-leaved, Evergreen
LSU 32303	Gulf	USA	Louisiana	Lafayette	1971	Herbaceous Cover (contains both pastures and natural)
LSU 32330	Gulf	USA	Louisiana	Saint Landry	1976	Cultivated and Managed Terrestrial Areas
LSU 34298	Gulf	USA	Mississippi	Lincoln	1986	Tree Cover, Needle-leaved, Evergreen
LSU 523	Gulf	USA	Louisiana	East Baton Rouge	1937	Artificial Surfaces and Associated Areas
LSU 9569	Gulf	USA	Louisiana	Avoyelles	1964	Cultivated and Managed Terrestrial Areas
MSB 25303	Gulf	USA	Alabama	Tuscaloosa	1952	Artificial Surfaces and Associated Areas
TTU 102296	Gulf	USA	Texas	Kerr	2003	Herbaceous Cover (contains both pastures and natural)
TTU 107707	Gulf	USA	Texas	Edwards	2004	Herbaceous Cover (contains both pastures and natural)
TTU 109051	Gulf	USA	Texas	Hall	2008	Herbaceous Cover (contains both pastures and natural)
TTU 35624	Gulf	USA	Georgia	Polk	1975	Tree Cover, Needle-leaved, Evergreen
TTU 56322	Gulf	USA	Texas	Kimble	1989	Herbaceous Cover (contains both pastures and natural)
TTU 75105	Gulf	USA	Texas	Brazoria	1996	Herbaceous Cover (contains both pastures and natural)
TTU 75236	Gulf	USA	Texas	Calhoun	1996	Tree Cover, Needle-leaved, Evergreen
TTU 76988	Gulf	USA	Texas	Briscoe	1997	Herbaceous Cover (contains both pastures and natural)
TTU 77775	Gulf	USA	Texas	Kerr	1998	Tree Cover, Needle-leaved, Evergreen
TTU 77776	Gulf	USA	Texas	Kerr	1998	Tree Cover, Needle-leaved, Evergreen
TTU 98449	Gulf	USA	Texas	Kimble	2002	Tree Cover, Needle-leaved, Evergreen
UMMZ 55799	Gulf	USA	Florida	Alachua	1923	Artificial Surfaces and Associated Areas

UMMZ 57652	Gulf	USA	Georgia	Milton	1924	Tree Cover, Needle-leaved, Evergreen
UMMZ 65534	Gulf	USA	Texas	Blanco	1932	Herbaceous Cover (contains both pastures and natural)
UMMZ 81836	Gulf	USA	Alabama	Lee	1937	Herbaceous Cover (contains both pastures and natural)
UMMZ 96859	Gulf	USA	Florida	Miami-Dade	1949	Sparse Herbaceous or sparse Shrub Cover
UMMZ 98903	Gulf	USA	Florida	Monroe	1949	Regularly Flooded Shrub and/or Herbaceous Cover
USNM 223934	Gulf	USA	Georgia	Okefenoke Swamp	1917	Tree Cover, Needle-leaved, Evergreen
USNM 265004	Gulf	USA	Florida	Everglades	1938	Regularly Flooded Shrub and/or Herbaceous Cover
USNM 348115	Gulf	USA	Florida	Okaloosa	1970	Tree Cover, Needle-leaved, Evergreen
ASNHC 13677	Mesoamerica	USA	Texas	Hidalgo	2009	Tree Cover, Needle-leaved, Evergreen
ASNHC 14681	Mesoamerica	Mexico	Morelos	Tlaquiltenango	2007	Tree Cover, Broadleaved, Deciduous, Closed
LSU 15106	Mesoamerica	Mexico	San Luis		1969	Tree Cover, Broadleaved, Evergreen
MSU 23916	Mesoamerica	Mexico	Jalisco		1974	Cultivated and Managed Terrestrial Areas
MSU 8148	Mesoamerica	Mexico	Nayarit		1957	Tree Cover, Broadleaved, Evergreen
MVZ 121179	Mesoamerica	Mexico	Veracruz		1955	Tree Cover, Needle-leaved, Evergreen
MVZ 91164	Mesoamerica	Mexico	Nuevo Leon		1940	Tree Cover, Needle-leaved, Evergreen
ROM 96302	Mesoamerica	Mexico	Campeche		1989	Tree Cover, Broadleaved, Evergreen
ROM 96484	Mesoamerica	Mexico	Yucatan		1989	Tree Cover, Broadleaved, Evergreen
ROM 96971	Mesoamerica	Mexico	Campeche		1990	Tree Cover, Broadleaved, Evergreen
ROM 97763	Mesoamerica	Mexico	Quintana Roo	Isla Cozumel	1990	Water Bodies
TTU 63542	Mesoamerica	El Salvador	La Paz	San Luis Talpa	1993	Tree Cover, Mixed Leaf type
UMMZ 58019	Mesoamerica	USA	Texas	Cameron	1926	Tree Cover, Needle-leaved, Evergreen
UMMZ 61562	Mesoamerica	Mexico	Tamaulipas	Mulato	1930	Shrub Cover, closed-open, deciduous
UMMZ 62921	Mesoamerica	Belize			1931	Water Bodies
UMMZ 63558	Mesoamerica	Guatemala	Peten	Uaxactun	1931	Tree Cover, Broadleaved, Evergreen
UMMZ 81039	Mesoamerica	Mexico	Oaxaca	Santa Cruz Bay	1938	Water Bodies
UMMZ 84018	Mesoamerica	Mexico	Guerrero	Chilpancingo	1940	Tree Cover, Needle-leaved, Evergreen
UMMZ 95657	Mesoamerica	Mexico	Quintana Roo		1947	Tree Cover, Broadleaved, Evergreen
USNM 334581	Mesoamerica	Nicaragua	Managua	Chiltepe	1962	Water Bodies

USNM 564604	Mesoamerica	Guatemala	El Progreso	El Jute	1983	Cultivated and Managed Terrestrial Areas
FMNH 156717	Midwest	USA	Illinois	Cook	1995	Artificial Surfaces and Associated Areas
FMNH 25169	Midwest	USA	Illinois	Marion	1925	Artificial Surfaces and Associated Areas
FMNH 59027	Midwest	USA	Illinois	Cook	1972	Artificial Surfaces and Associated Areas
FMNH 90494	Midwest	USA	Illinois	Cook	1940	Artificial Surfaces and Associated Areas
FMNH 90495	Midwest	USA	Illinois	Cook	1942	Tree Cover, Needle-leaved, Evergreen
MZ 11627	Midwest	USA	North Dakota	Ransom	2017	Herbaceous Cover (contains both pastures and natural)
OMNH 40167	Midwest	USA	Wisconsin	Manitowac	1985	Tree Cover, Mixed Leaf type
OWC 903	Midwest	USA	Ohio	Franklin	2017	Tree Cover, Broadleaved, Deciduous, Closed
UMMZ 107655	Midwest	USA	Ohio	Lake	1939	Artificial Surfaces and Associated Areas
UMMZ 111219	Midwest	USA	Michigan	Washtenaw	1961	Artificial Surfaces and Associated Areas
UMMZ 177153	Midwest	USA	Michigan	Kalamazoo	1938	Tree Cover, Broadleaved, Deciduous, Closed
UMMZ 178776	Midwest	USA	North Dakota	Grand Forks	2017	Sparse Herbaceous or sparse Shrub Cover
UMMZ 56587	Midwest	USA	Michigan	Berrien	1925	Cultivated and Managed Terrestrial Areas
UMMZ 58811	Midwest	USA	Michigan	Genesee	1927	Cultivated and Managed Terrestrial Areas
UMMZ 74603	Midwest	USA	Michigan	Calhoun	1935	Cultivated and Managed Terrestrial Areas
UMMZ 81511	Midwest	USA	Michigan	Allegan	1938	Tree Cover, Needle-leaved, Evergreen
UMMZ 83550	Midwest	USA	Iowa	Winneshiek	1935	Cultivated and Managed Terrestrial Areas
USNM 347673	Midwest	USA	Indiana	Tippecanoe	1962	Artificial Surfaces and Associated Areas
UWBM 44493	Midwest	USA	Ohio	Clermont	1972	Tree Cover, Broadleaved, Deciduous, Closed
UWBM 44494	Midwest	USA	Ohio	Clermont	1973	Tree Cover, Broadleaved, Deciduous, Closed
UWBM 44495	Midwest	USA	Ohio	Ottawa	1973	Water Bodies
UWBM 44497	Midwest	USA	Ohio	Clermont	1976	Tree Cover, Broadleaved, Deciduous, Closed
ASNHC 5640	Northeast	USA	New York	Westchester	1984	Tree Cover, Broadleaved, Deciduous, Closed
CUMV 11489	Northeast	USA	New York	Schuyler	1940	Cultivated and Managed Terrestrial Areas
CUMV 13800	Northeast	USA	New York	Tompkins	1975	Tree Cover, Needle-leaved, Evergreen
CUMV 13801	Northeast	USA	New York	Onondaga	1975	Tree Cover, Broadleaved, Deciduous, Closed
CUMV 14863	Northeast	USA	New York	Tompkins	1990	Tree Cover, Broadleaved, Deciduous, Closed
CUMV 1974	Northeast	USA	New York	Tompkins	1939	Cultivated and Managed Terrestrial Areas

CUMV 3464	Northeast	USA	New York	Albany	1942	Tree Cover, Broadleaved, Deciduous, Closed
CUMV 4970	Northeast	USA	New York	Tioga	1949	Tree Cover, Broadleaved, Deciduous, Closed
CUMV 5148	Northeast	USA	New York	Adirondacks	1949	Tree Cover, Broadleaved, Deciduous, Closed
CUMV 7800	Northeast	USA	New York	Tompkins	1953	Tree Cover, Broadleaved, Deciduous, Closed
CUMV 9287	Northeast	USA	New York	Tompkins	1951	Water Bodies
FMNH 198901	Northeast	USA	New York	Ulster	1968	Tree Cover, Broadleaved, Deciduous, Closed
MCZ 59947	Northeast	USA	Massachusetts	Middlesex	1987	Artificial Surfaces and Associated Areas
MCZ 61601	Northeast	USA	Rhode Island	Providence	1991	Tree Cover, Needle-leaved, Evergreen
MCZ 61602	Northeast	USA	Massachusetts	Suffolk	1994	Artificial Surfaces and Associated Areas
MCZ 62096	Northeast	USA	Massachusetts	Essex	1997	Tree Cover, Broadleaved, Deciduous, Closed
MCZ 62102	Northeast	USA	Massachusetts	Essex	1997	Tree Cover, Mixed Leaf type
MCZ 62199	Northeast	USA	Massachusetts	Middlesex	1998	Artificial Surfaces and Associated Areas
NYSM 7056	Northeast	USA	New York	Albany	1937	Tree Cover, Broadleaved, Deciduous, Closed
NYSM 7057	Northeast	USA	New York	Albany	1939	Tree Cover, Broadleaved, Deciduous, Closed
NYSM 7060	Northeast	USA	New York	Albany	1942	Tree Cover, Broadleaved, Deciduous, Closed
NYSM 7061	Northeast	USA	New York	Rockland	1942	Tree Cover, Broadleaved, Deciduous, Closed
NYSM 7062	Northeast	USA	New York	Albany	1947	Tree Cover, Broadleaved, Deciduous, Closed
NYSM 7064	Northeast	USA	New York	Albany	1948	Tree Cover, Broadleaved, Deciduous, Closed
ROM 116019	Northeast	Canada	Ontario	Simcoe	2004	Cultivated and Managed Terrestrial Areas
ROM 21604	Northeast	Mexico	Ontario	Dundas	1952	Tree Cover, Broadleaved, Deciduous, Closed
ROM 85808	Northeast	Canada	Ontario	Niagara	1980	Cultivated and Managed Terrestrial Areas
UMMZ 75104	Northeast	USA	New York	Orange	1933	Tree Cover, Broadleaved, Deciduous, Closed

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Table A2.3 Virginia opossum (*Didelphis virginiana*) county or adjacent county values for Bayesian standard ellipse area (SEA_B) and geographic land area obtained from census records (units = miles^2).

County	SEA_B	geographic area
Alameda, California	0.552	739
Los Angeles, California	1.525	4058
Butte, California	4.033	962
Acadia + Lafayette + St. Landry, Louisiana	4.089	803
Cook, Illinois	7.361	523
Albany, New York	1.878	2354
Schuyler + Tompkins, New York	2.451	1636
Kerr + Kimble, TX	1.903	1586

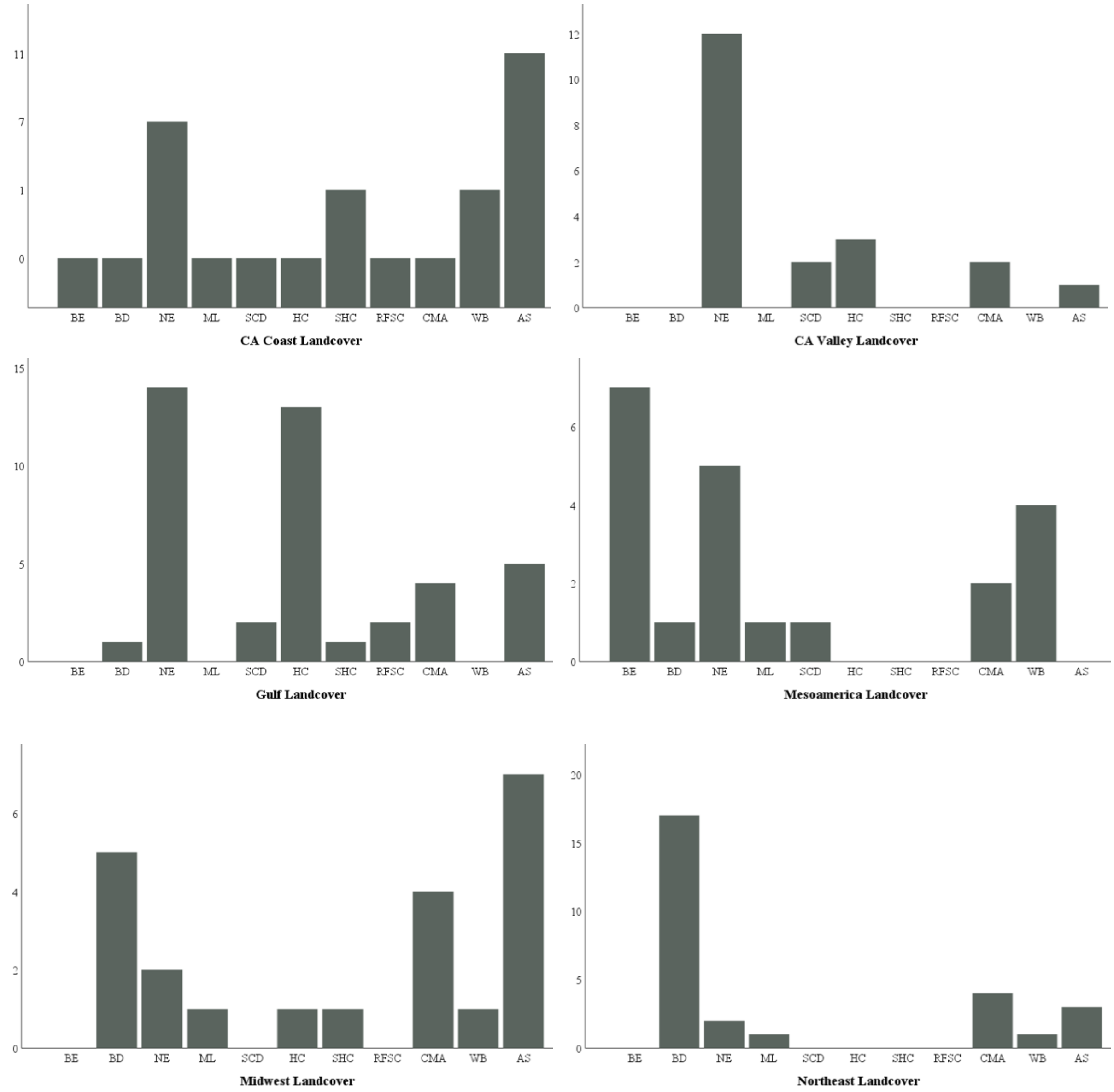

Figure A2.1 Histograms of landcover type (Fritz et al. 2003) associated with collection sites of Virginia opossums (*Didelphis virginiana*) for six regions: California (CA) coast, CA Valley, Gulf coast, Mesoamerica, Midwest, and Northeast. Landcover types are broadleaved evergreen tree cover (BE); broadleaved deciduous closed tree cover (BD); needle-leaved evergreen tree cover (NE); mixed leaf type tree cover (ML); shrub cover, closed-open, deciduous (SCD); herbaceous cover (HC); sparse herbaceous or sparse shrub cover (SHC); regularly flooded shrub and/or herbaceous cover (RFSC); cultivated and managed terrestrial areas (CMA); water body (WB); and artificial surfaces and associated areas (AS).

Fritz, S., E. Bartholomé, A. Belward, A. Hartley, H. Stibig, H. Eva, P. Mayaux, S. Bartalev, R.Latifovic, S. Kolmert, P.S. Roy, S. Agrawal, W. Bingfang, X. Wenting, M. Ledwith, J. Pekel, C. Giri, S. Mücher, E. de Badts, R. Tateishi, J.Champeaux, and P. Defourny. 2003. Harmonisation, mosaicing and production of the Global Land Cover 2000 database (Beta Version). Publication of the European Commission: EUR 20849 EN Luxembourg *Office for Official Publications of the European Communities*.

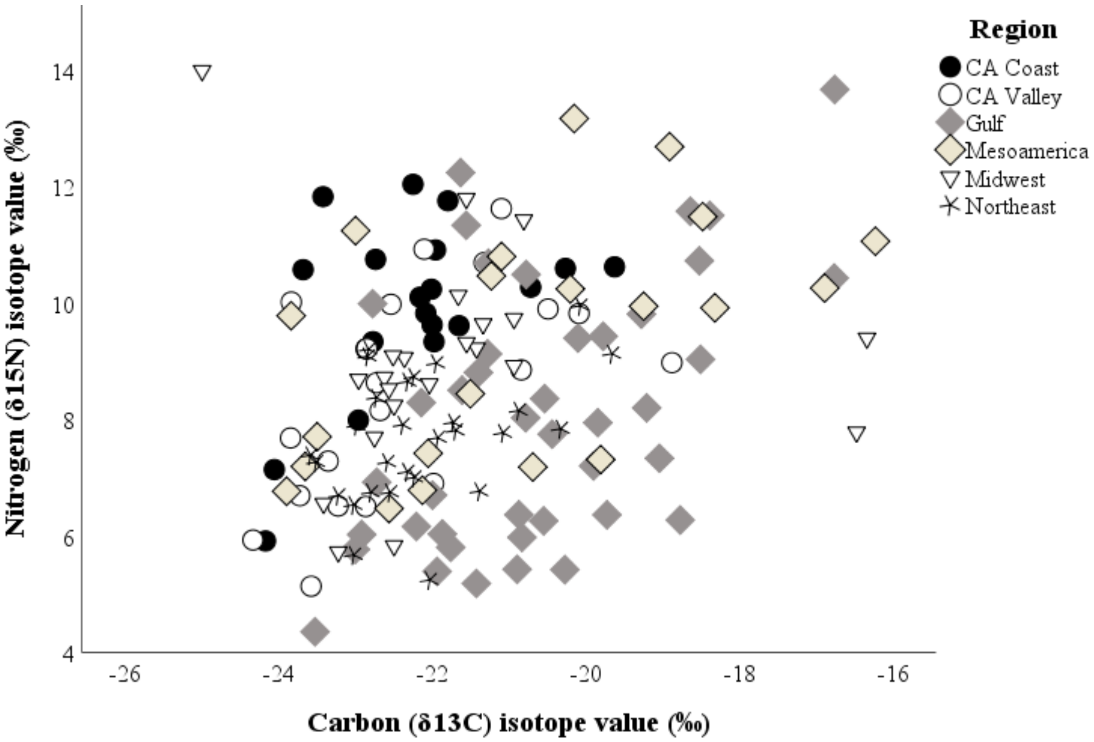

Figure A2.2 Bivariate data points of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope values for Virginia opossums (*Didelphis virginiana*) collected from six regions.

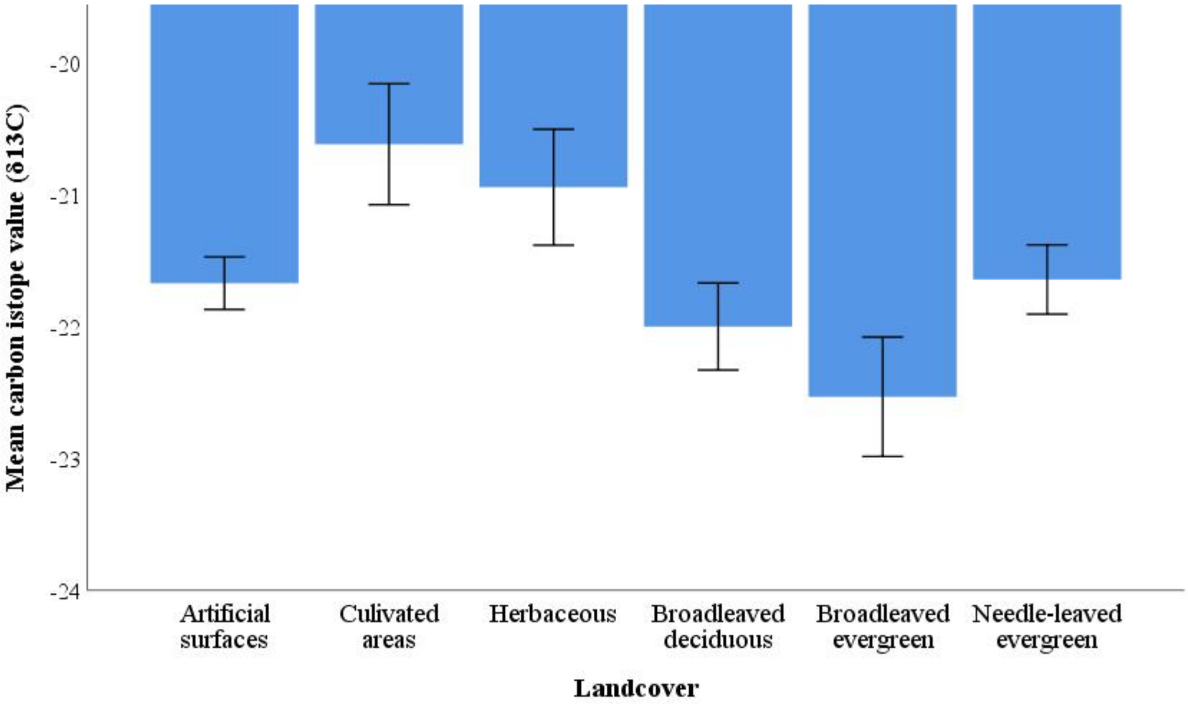

Figure A2.3 Mean opossum carbon ($\delta^{13}C$) isotope values found in six different landcover types extracted from 2000 global landcover data (Fritz et al. 2003). Error bars indicate 1 SE.

Fritz, S., E. Bartholomé, A. Belward, A. Hartley, H. Stibig, H. Eva, P. Mayaux, S. Bartalev, R.Latifovic, S. Kolmert, P.S. Roy, S. Agrawal, W. Bingfang, X. Wenting, M. Ledwith, J. Pekel, C. Giri, S. Mücher, E. de Badts, R. Tateishi, J.Champeaux, and P. Defourny. 2003. Harmonisation, mosaicing and production of the Global Land Cover 2000 database (Beta Version). Publication of the European Commission: EUR 20849 EN Luxembourg *Office for Official Publications of the European Communities*.

Chapter III

The Opossum's Diet Isn't Trash! Opossum Carbon Isotope Values Through Time Don't Match Uptick of Corn in our Foods

Abstract

The composition of North American communities is changing in large part due to climate change and anthropogenic land-use alteration (Myers et al., 2009; Parks et al., 2020). At the same time, mammalian mesopredator species have grown more abundant and expanded their ranges (Prugh et al., 2009). Their ability to consume anthropogenic refuse due to their generalist diet has been referenced to partially explain their success in altered landscapes and harsher environments as they spread north (e.g. Kanda, 2005; Walsh and Tucker, 2018). I evaluated this hypothesis using carbon isotope values ($\delta^{13}C$) of the Virginia opossum, a mesopredator undergoing a northward range expansion. $\delta^{13}C$ values increase from direct or indirect consumption of C_4 plants including corn, a common additive in North American human foods. I used $\delta^{13}C$ values of guard hairs of the Virginia opossum from two geographic expansion fronts to evaluate the proposition that the opossum's northward expansion is facilitated in part by consuming anthropogenic food resources. Opossums from the Midwestern U.S. and Northeast were evaluated together and separately to identify relationships between their $\delta^{13}C$ value and latitude or winter climate. Because opossums in the "Corn Belt" Midwest may encounter corn in either urban refuse or cornfields, I used the Northeast as a control for corn agriculture. Because corn has become a more common component of U.S. foods over the past century, I also evaluated $\delta^{13}C$ values through time to test whether the pattern of increased corn consumption through time is mirrored in northern opossums. I did not observe significant relationships between latitude or winter harshness and opossum $\delta^{13}C$ values, but variance in $\delta^{13}C$ values

increased in Midwestern opossums after 1970. $\delta^{13}C$ values compared across space and time do not indicate opossums are expanding in northern areas due to accessible food in the form of anthropogenic refuse, but bolster evidence for the influence of agricultural development on the opossum's range expansion.

Introduction

Range expansions are often conspicuous consequences of climate change or land-use alteration. In addition to documenting the environmental drivers of expansions, scientists are identifying life history traits that further facilitate expansion of particular species into novel habitats and communities (Chuang and Peterson, 2016). By pinpointing these behavioral, morphological, and ecological traits, we can better predict how species will react to changing landscapes.

Dispersal ability, growth rate, and ability to respond to physiologically harsh environments are commonly associated with a species' capability to expand its range (Therry et al., 2014; Chuang and Peterson, 2016; Krehenwinkel et al., 2016). While some of these life history traits associated with range expansions are more applicable to invertebrates, the trait of a generalist diet is common in mesopredator mammals (Chuang and Peterson, 2016). The flexibility of a broad diet is used to explain mesopredators' ability to adapt to anthropogenic land-use change, especially urbanization (Sol et al., 2013). For example, the exploitation of anthropogenic food resources has been observed in numerous mammalian mesopredators in urban areas (e.g. Contesse et al., 2004; Morey et al., 2007; Newsome et al., 2015).

Anthropogenic food in the form of refuse benefits some generalist mesopredator populations (e.g. Contesse et al., 2004; Newsome et al., 2010). This observation has been posited as an explanation for some current range expansions. However, the importance of anthropogenic food has yet to be evaluated in the context of an on-going range expansion. The Virginia opossum (*Didelphis virginiana*; henceforth referred to as opossum) serves as an ideal test case. Over the past decades, tt has expanded north in both the Midwest and Northeast (Godin, 1977; Gardner and Sunquist, 2003; Walsh et al., 2017; Figure 3-1). Demographic and genetic research indicate that opossum survival increases in human-modified landscapes, and anthropogenic food sources are hypothesized as a primary explanation (Kanda, 2005; Walsh and Tucker, 2018).

The opossum is a generalist mesopredator ranging across much of North America (Figure 3-1), where it is primarily found in mixed deciduous forest and wetlands, but also in agricultural and urban landscapes (Gardner and Sunquist, 2003). Opossums have been observed foraging under bird feeders and near dog houses (Walsh et al., 2017), and trash has been found in stomach dissections (Knudsen and Hale, 1970; Blumenthal and Kirkland, 1976).

Opossums are prone to frostbite (Gardner and Sunquiest, 2003; Walsh et al., 2017) and tend to avoid foraging outside when temperatures are below freezing (Brocke, 1970). General linear modeling demonstrated that the climate variable that best explained microsatellite diversity in midwestern opossums was the number of days with snow on the ground, followed by the number of days in which the temperature reached freezing. Genetic and demographic data from the Midwest and Massachusetts indicate that agricultural and urbanized areas support a greater number of opossums than natural habitats (Kanda et al., 2009; Beatty et al., 2016; Walsh and Tucker, 2018). A primary hypothesis behind these results is that in the winter, accessible food in the form of anthropogenic garbage reduces both foraging time and exposure to winter weather, thereby increasing winter survival and population growth.

The Midwest and Northeast (Figure 3-2) have markedly different levels of corn agriculture. The Midwest is the center of U.S. corn agriculture (Philpott, 2008), with an average of 9.4% of county land occupied by corn fields in my dataset. In contrast, the Northeast is a region at similar latitude with only 2.2% county land occupied by corn fields in my dataset (USDA Archive, 2018; Table A3.1). Because opossums have expanded northward in two areas of the United States, I can evaluate potential dietary shifts due to agriculture and urbanization within and outside the "Corn Belt" of the Midwestern U.S.

I used stable isotope analysis to evaluate the hypothesis that opossums are spreading north in part by consuming anthropogenic food resources. As tissue forms, it incorporates heavy and light isotopes (e.g. ^{13}C and ^{12}C) at a ratio that indicates various aspects of the animal's diet and environment. An individual's carbon isotope value ($\delta^{13}C$) increases with a rise in consumption of C_4 vegetation (Kelly, 2000). Because C_3 plants are the dominant primary producers across much of the eastern U.S. (Terri and Stowe, 1976; Still et al., 2003) and because of the prevalence of corn, a C_4 plant, in North American food products, processed food and meat have a significantly higher $\delta^{13}C$ value compared to the natural vegetation in the surrounding habitat, allowing scientists to explore the role of natural and human food sources in wildlife (Jahren and Kraft, 2008). Corn first became a common additive in North American foods in the 1950s when it was fed to livestock (Matsushima and Farr, 1995), and became more abundant in foods with the popularization of corn syrup in the 1970s (White, 2008).

To evaluate the hypothesis that opossums are spreading north because of human-provided food, I directly evaluated the relationship between an opossum's $\delta^{13}C$ value and the latitude at which they were collected. If opossums are increasingly reliant on human food as they move north, I expected to observe a positive relationship between $\delta^{13}C$ and latitude. Because anthropogenic refuse is hypothesized to subsidize natural food when it becomes too cold and snowy for opossums to easily forage, I also predicted a positive relationship between $\delta^{13}C$ and winter harshness (measured as days below freezing and days with snow on ground, factors selected based on population genetics modeling performances [Walsh and Tucker, 2018]). I evaluated the change in mean $\delta^{13}C$ and variance through time (1925-2017) in opossums from the Midwest and Northeast with the expectation that $\delta^{13}C$ values would increase in recent decades due to the increased corn use in the U.S. in the late 20th Century (Matsushima and Farr, 1995; White, 2008). Finally, I compared the performance of winter climate, agricultural, and human density variables using generalized linear mixed models.

Methods

Sample collection and processing

Guard hairs from adult opossums were collected from 22 individuals from midwestern states (Illinois, Indiana, Iowa, Michigan, North Dakota, Ohio, Wisconsin) and from 24 individuals from northeastern states (Massachusetts, New York, Rhode Island). The opossums sampled represented 31 different years, from 1925 to 2017 (Table A3.1). All hair samples came from museum specimens except for one wildlife rehabilitation hospital (OWC) carcass (Table A3.1). Museums that mailed samples were instructed to destructively sample guard hair from the hind leg of each specimen to match my sampling technique.

All hair samples were treated with a 2:1 ratio of chloroform – methanol to remove lipids (Sergiel et al., 2017) and were dried under a fume hood at ambient temperature before being weighed with a Mettler AE 240 balance (between 0.5 and 1.0 mg per sample; approximately three entire guard hairs) and placed in a tin capsule for stable isotope analysis of $\delta^{13}C$ values conducted by the University of New Mexico Center for Stable Isotopes (UNM-CSI). For more details, see Chapter II. Two hair samples were analyzed for each specimen at UNM-CSI, and results were averaged across the two samples for each specimen for all analyses. Outliers were identified as $\delta^{13}C$ values more than 2.2 times the interquartile range outside the 25th or 75th percentile, according to Hoaglin et al. (1986), and analyses were conducted both with and without outliers.

Suess-effect corrections

Because of the influx of greenhouse gases during the industrial era, the heavier carbon isotopes, ^{13}C and ^{14}C, have decreased in the atmosphere over the past century (Verburg, 2007). Therefore, when opossums are compared across time, the $\delta^{13}C$ value of older samples would overestimate the influence of C_4 plants, skewing the historical data in a positive direction. In order to accurately compare samples collected across the last 100 years, I adjusted samples collected prior to 2017 for changing atmospheric ^{13}C with a linear correction of -0.005‰ per year between 1917 and 1961 and -0.022‰ per year after 1961, with the oldest samples from 1917 corrected by -1.452‰ (Chamberlain et al., 2005).

Range expansion factors and $\delta^{13}C$

Three variables [latitude, number of days when temperature reached freezing (DT32), and days with at least one-inch of snow on ground (DSNW)] were each examined for their relationship with $\delta^{13}C$ in opossums in the Midwest, the Northeast, and both northern regions using the curve estimation function in SPSS version 26.0 (IBM Corp. 2019) with and without two identified outliers. The curve estimation function allows the user to simultaneously run up to 11 regression models (e.g. linear, logistic, exponential). DT32 and DSNW were selected to measure winter harshness based on their strong performances in population genetics modeling [Walsh and Tucker, 2018]). Latitude was obtained from museum records while DT32 and DSNW county data from December-March were collected and summed from the year in which each opossum was collected [National Oceanic and Atmospheric Administration (NOAA);

available from http:// www.ncdc.noaa.gov/cdo-web/search)]. Because some regression models only work with data above zero, I adjusted my dataset based on the lowest δ^{13}C value (so that each value was increased by 25.0‰ so as not to inverse the relationship).

To compare the winter harshness variables to alternative variables, generalized linear mixed models (GLMM) were run using latitude and longitude as random effects and the positively adjusted δ^{13}C + 25‰ as the dependent variable in a gamma distribution with a log link and fit with maximum likelihood in SPSS. Single covariate models were built using the following variables: DT32, DSNW, human density, year, % farmland, and % corn fields. Population size of each county was extracted from USA Census results in the year closest to each opossum's collection date and divided by county land area to acquire human density data (U.S. Census Bureau, 2018). Farmland acreage and acres of corn were acquired from United States Department of Agriculture (USDA) Census of Agriculture results in the year closest to each opossum's collection date and divided by county land area to acquire % farmland and % corn fields data (USDA NASS, 2017; USDA Archive, 2018). For independent variables that were not correlated (Pearson's $p > 0.05$), two-covariate and three-covariate GLMM models were built (see Table A3.2 for list of models). All independent variables were standardized by centering along the mean and dividing by two standard deviations with the R package "arm" (Gelman, 2008). An intercept-only GLMM served as the null model. All GLMMs were compared using ΔAIC_c (AIC_{ci} - AIC_{cMIN}; Burnham and Anderson 2002).

Changes in $\delta^{13}C$ over time

δ^{13}C values increase with a rise in consumption of C_4 plants, including corn (Suits et al., 2005). The use of corn feed for livestock became widespread in the 1950s (Matsushima and Farr, 1995), while the commercial use of corn syrup became popular in the 1970s (White, 2008). An increase in mean $\delta^{13}C$ after 1950 or 1970 in the Midwest, the center of corn agriculture (Philpott, 2008), would suggest a shift in opossum foraging strategies from native landscapes to either agricultural or urban resources. Because of its minimal corn agriculture, an increased mean $\delta^{13}C$ in the Northeast in recent decades would support the hypothesis that opossums are reliant on urban resources (e.g. anthropogenic food waste).

To evaluate if there were stepwise changes in $\delta^{13}C$ mean or variance in the Northeast or Midwest based on the increased use of corn products in human foods in the 1950s and 1970s, I examined the Northeast and Midwest separately by comparing data collected before and after 1950, and data collected before and after 1970. The Northeast pre-1950 and post-1950 $\delta^{13}C$ values were compared using an independent samples t-test in SPSS (Shapiro-Wilks $P = 0.0705$), and homogeneity of variance was evaluated with Levene's test. This was repeated to compare pre-1970 and post-1970. If the mean $\delta^{13}C$ value increased in opossums in the Northeast after the widespread industrialization of corn products in the 1950s or 1970s, this would support the hypothesis that opossums are reliant on food incidentally provided by humans. The same strategy was employed for the Midwest, but because the Midwest data were not normally distributed (Shapiro-Wilks $P = 0.004$), $\delta^{13}C$ values were compared using a Mann–Whitney U–test in SPSS.

Results

Museum sample results

Averaged across duplicate samples for each specimen, the range of Suess-corrected $\delta^{13}C$ values was -24.99‰ to -16.34‰, with an average standard deviation of 0.17 per specimen. There was no significant difference found between males ($n = 28$) and females ($n = 17$; $p = 0.164$). Two outliers were detected with especially high $\delta^{13}C$ values: one opossum collected from

Manitowac County, Wisconsin in 1985 (OMNH 40167), and one collected in Franklin County, Ohio in 2017 (OWC 903).

Range expansion factors and $\delta^{13}C$

Latitude had no significant relationship with δ^{13}C values of opossums in the Midwest or Northeast (all $p \geq 0.459$, all $R^2 \leq 0.083$; Table 3-1a). Days of snow on the ground did not have a significant relationship with δ^{13}C values of opossums in the Midwest or Northeast (all $p \geq 0.298$, all $R^2 \leq 0.109$; Table 3-1b). Days below freezing did not have a significant relationship with δ^{13}C values of opossums in the Midwest or Northeast (all $p \geq 0.296$, all $R^2 \leq 0.055$; Table 3-1c).

The best performing GLMM included year + % corn fields as covariates ($AIC_c = 66.938$, parameter estimates = 0.229 and 0.163, respectively; Table 3-2). There was one additional GLMM with substantial support, indicated by a ΔAIC_c value less than two (Burnham and Anderson 2002; Table 3-2). This strongly supported GLMM only included year as the covariate ($\Delta AIC_c = 1.555$; parameter estimate = 0.225). These models performed significantly better than the top performing model with human density included ($\Delta AIC_c = 3.436$), as well as the null model ($\Delta AIC_c = 9.047$), but the null model performed better than the GLMM with human density as the only covariate ($\Delta AIC_c = 11.737$; Table 3-2).

Differences in $\delta^{13}C$ over time

The difference in mean δ^{13}C values between time periods was not significant in either the Northeast or Midwest (all $P \geq 0.162$; Table 3-3). There was no significant change in the variance of δ^{13}C between time periods in the Northeast (all Levene's $P \geq 0.162$), but the variance was significantly greater after 1970 in the Midwest (Levene's $P = 0.013$; Figure 3-3; Table 3-3) and remained so when the two outliers were removed (Levene's $P = 0.041$; Table A3.3).

Discussion

A variety of mesopredators have recently expanded their ranges, entering new communities and becoming more conspicuous to the public (Prugh et al., 2009). Understanding the factors impacting a mesopredator's invasion of new habitats is critical to predict changes in communities. Identifying anthropogenic factors is especially important, as mesopredators are often vilified and labelled as pests for both substantiated and fallacious reasons. Understanding the relationship between humans and wildlife is crucial given that over the past 25 years, more than one million acres were converted to urban land each year in the United States (NRCS, 2013; Prugh et al. 2009). By better understanding human-mesopredator dynamics, wildlife managers can mitigate negative interactions while educating the public to better appreciate their local fauna.

Compelling genetic and demographic data from the Midwest and Northeast demonstrate that the opossum's range expansion is limited by heavy snow cover but promoted in agricultural and suburban landscapes (Kanda et al., 2009; Beatty et al., 2016; Walsh and Tucker, 2018). Analysis of genetic data of opossums collected in the Midwest support the hypothesis that the opossum's range expansion is facilitated by climate change in the form of reduced snow cover (Walsh and Tucker, 2018). Anecdotal observations of opossums foraging in suburban areas led to the hypothesis that opossums in cold regions are partially reliant on human-provided resources in the form of processed foods containing corn.

My data suggest that rather than primarily consuming household anthropogenic food waste, opossums are likely consuming native plants and animals in their environment, which often includes agricultural landscapes. The increased variation in $\delta^{13}C$ values after 1970 in the Midwest (but not in the Northeast) suggests that additional foods became abundant and novel foraging strategies in agricultural systems became available to opossums in the Midwest. In the early 1970s, the Midwest was the focal point of a major push by the USDA to increase corn and soy production (Philpott, 2008). This “get big or get out” mentality led to increased areas of agricultural cropland that might have contributed to the enlarged variance of $\delta^{13}C$ values observed in opossums from this area.

$\delta^{13}C$ values are higher in animals foraging in a food web with C_4 plants as the primary resource. By evaluating opossums from the Midwest and Northeast, where naturally occurring C_4 plants are limited outside of agriculture, I was able limit the variation in $\delta^{13}C$ that would be due to native C_4 plants. The best performing GLMM included year and % corn fields as positive predictors. This further supports the conclusion that opossum isotope values are influenced by agriculture. Perhaps, opossums have become increasingly reliant on human-modified landscapes through time. The matrix of landscapes that a single nomadic individual would encounter could include edge habitats of both agricultural and urban land-use. In addition, the two outliers highlight the individual variation in foraging that might be taking place. One sample was collected from Franklin County, Ohio in 2017, one of the most densely human-populated counties sampled. The other outlier was collected in 1985 from Manitowac County, Wisconsin, which had the 4th highest density of corn production (Table A3.1). These opossums may be outliers due to anthropogenic factors in their specific location (i.e., dense human population or dense corn crops), but opossums in similar landscapes did not have similarly high $\delta^{13}C$ values. Home range sizes vary widely within the species (Gardner and Sunquist, 2003), and the two outliers may have had especially restricted home ranges with limited natural landscape for foraging.

When opossums from six regions across their range were compared (Chapter 2), the isotopic niche width was positively correlated with habitat heterogeneity, and the niche width of the Midwest was almost four-fold larger than that of the Northeast. In this analysis, I found that opossums in cultivated landscapes had significantly higher mean $\delta^{13}C$ than opossums found in broadleaved evergreen and deciduous forests. This result is in contrast with the GLMM results – percent farmland did not explain $\delta^{13}C$ values across the Midwest and Northeast. The discrepancy in results can be explained by a difference in scale. In Chapter 2, every opossum could only be classified with one of 22 landcover types based on 2001 landcover data. "Cultivated land" was one of many landcover types driving the opossum's isotopic signal. In the current study, I extracted USDA county data to calculate percent farmland for all opossums, so that even in areas not classified as “cultivated land,” the percent farmland could be included in a GLMM. Based on GLMM results, "farmland" itself did not lead to increased $\delta^{13}C$ values. In both regions, farmland can include C_3 crops including soy, wheat, apples and even livestock pastures that would not cause an increase in carbon isotope ratios (USDA NASS, 2017).

Data collected across a century of specimens from the midwestern and northeastern United States do not support the hypothesis that the diet of opossums in northern areas has noticeably shifted toward human-provided resources in the form of refuse. However, the distribution of percent farmland sampled had a better representation of variation than the distribution of human densities sampled (Figure A3.1).

My data suggest a generalist mammal’s ability to consume human food does not appear to be a strong factor broadly influencing opossums as they expand northward. While opossums benefit from urbanization (Kanda, 2005; Walsh and Tucker, 2018), the facilitating factor of a northern urban environment may be milder microclimates in winter. While there is anecdotal evidence of opossums in garbage bins, my results suggest that if they are foraging in bins, opossums might prefer to consume unprocessed anthropogenic refuse such as vegetables and fruit scraps. Rather than primary food sources, garbage bins may serve as temporary dens for opossums. Outside of the scientific community, the perception of opossums primarily feeding on refuse has been popularized online (e.g. Mandelbaum, 2019), perpetuating the view of opossums as dirty, urban pests. My stable isotope results indicate that the perception of opossums should not be linked to refuse, but as generalist mesopredators that succeed in the face of changing landscapes.

Tables

Table 3-1 Best performing regression models for carbon ($\delta^{13}C$) isotope values of Virginia opossums (*Didelphis virginiana*) and a) latitude b) days with at least one inch of snow on the ground (DSNW) and c) days below freezing (D32). Groups examined included opossums from the Midwest (MW), Northeast (NE), and both (Combined). Two outliers from the MW were removed from MW and Combined to evaluate if significant relationships changed.

a.

Latitude vs. $\delta^{13}C$			
	Best model	***p***	***R^2***
Combined	exponential	0.565	0.008
Combined – outliers	linear	0.503	0.011
MW	logarithmic	0.634	0.012
MW – outliers	linear	0.404	0.039
NE	exponential	0.364	0.038

b.

DSNW vs. $\delta^{13}C$			
	Best model	***p***	***R^2***
Combined	exponential	0.366	0.019
Combined – outliers	exponential	0.271	0.029
MW	exponential	0.496	0.024
MW – outliers	cubic	0.128	0.292
NE	quadratic	0.298	0.109

c.

D32 vs. $\delta^{13}C$			
	Best model	***p***	***R^2***
Combined	quadratic	0.296	0.055
Combined – outliers	linear	0.268	0.029
MW	quadratic	0.584	0.055
MW – outliers	linear	0.394	0.041
NE	inverse	0.565	0.015

Table 3-2 Generalized linear mixed models for carbon ($\delta^{13}C$) isotope values of Virginia opossums (*Didelphis virginiana*) ranked by ΔAIC_c**.** Variables include year opossum was collected (year), percentage of county land occupied by corn (corn), and county human density (human). Models highlighted gray are considered to have substantial support (Burnham and Anderson, 2002), and the null intercept-only model is italicized.

Model	AIC_c	ΔAIC_c
Year + Corn	66.938	0
Year	68.493	1.555
Year + Corn + Human	70.374	3.436
Corn	74.855	7.917
Intercept-only	*75.985*	*9.047*
Human	78.675	11.737

Table 3-3 Mean carbon ($\delta^{13}C$) isotope values and standard deviation (SD) of Virginia opossums (*Didelphis virginiana*) compared before and after 1950 (1950 Division) and before and after 1970 (1970 Division). Northeast time periods were compared using a t-test, and Midwest time periods were compared using a Mann–Whitney U–test. Levene's statistic and significance are included to compare variance between time periods.

Northeast						
	Pre $\delta^{13}C$	Pre SD	Post $\delta^{13}C$	Post SD	Test significance	Levene's
1950 Division	-22.29	0.81	-21.93	1.12	0.162	2.090 $P = 0.162$
1970 Division	-22.22	0.86	-21.93	1.27	0.502	1.213 $P = 0.282$
Midwest						
1950 Division	-21.95	0.64	-21.50	2.63	0.792	3.069 $P = 0.095$
1970 Division	-22.05	0.62	-21.84	2.86	0.843	11.404 $P = 0.013$

Figures

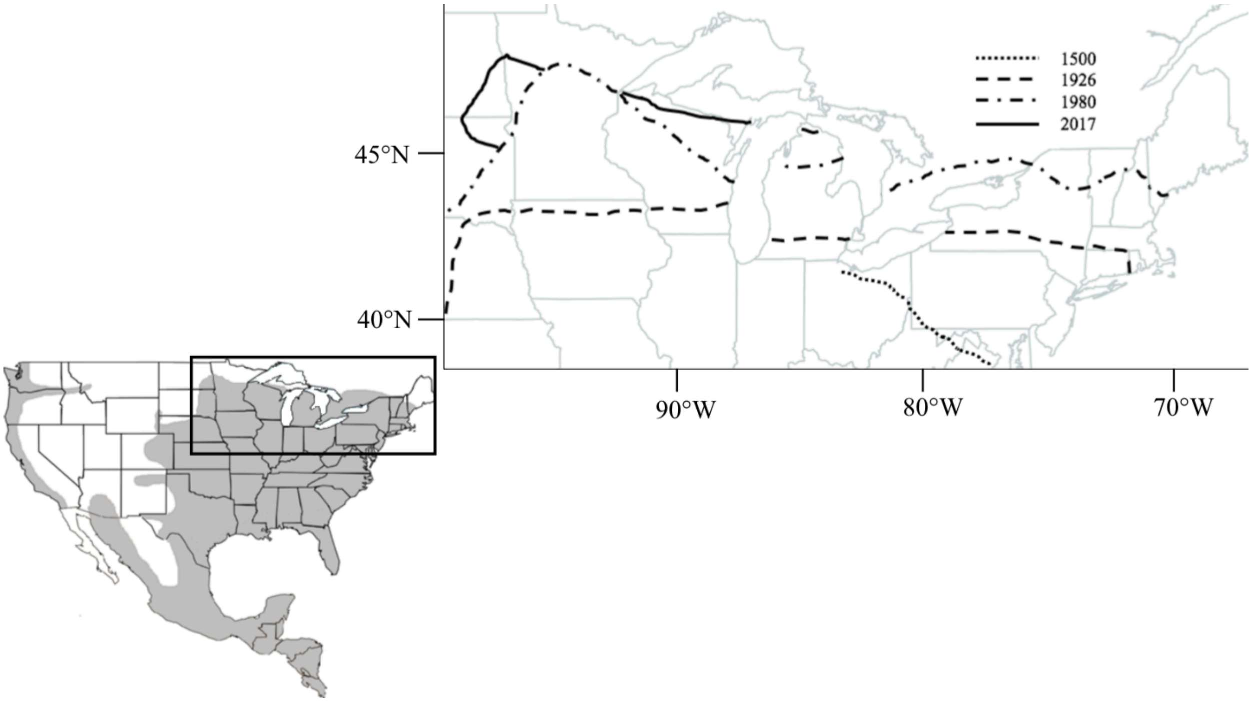

Figure 3-1 Map of Virginia opossum's (*Didelphis virginiana*) current range (shaded) with inlaid estimated northern range boundaries through time, demarcated by lines (adapted from Gardner and Sunquist, 2003).

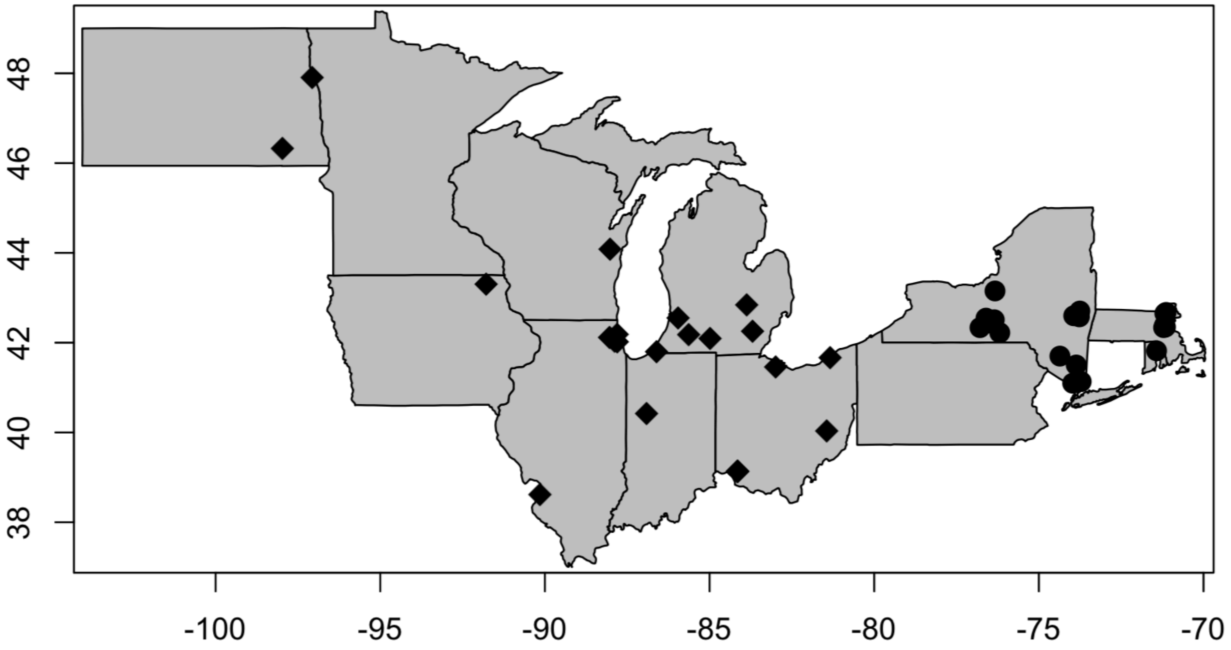

Figure 3-2 Collection locations indicated for Virginia opossums (*Didelphis virginiana*) sampled from two regions: 22 samples from the Midwestern USA demarcated with diamonds (Illinois, Indiana, Iowa, Michigan, North Dakota, Ohio, Wisconsin) and 25 samples from Northeastern USA demarcated with circles (Massachusetts, New York, Rhode Island).

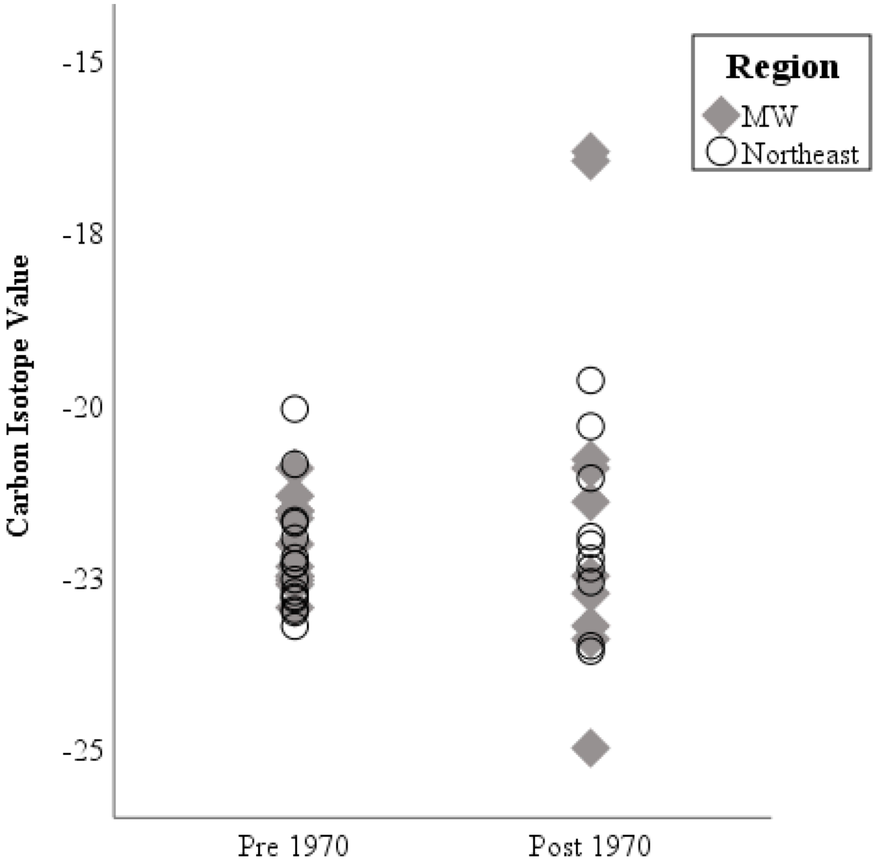

Figure 3-3 Comparison of $\delta^{13}C$ values of Virginia opossums (*Didelphis virginiana*) before and after 1970. Samples from the Midwest are gray diamonds and samples from the Northeast are white circles (see Figure 3-2 for collection locations). Variance of $\delta^{13}C$ values significantly increased in the Midwest after 1970 (*Levene's P* = 0.013).

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Appendix A3

Table A3.1 Data from destructive loans of Virginia opossum (Didelphis virginiana) guard hair samples including catalog number, year collected, state and county collected, the specimen's sex, and county data including percentage land cover composed of corn fields and humans per square mile. Samples are chronologically organized within a region. Institution acronyms are as follows, alphabetically: Angelo State Natural History Collections (ASNHC), Washington State University Charles R. Conner Museum (CRCM), Cornell Museum of Vertebrates (CUMV), Field Museum of Natural History (FMNH), Louisiana State University Museum of Natural Science (LSUMZ), Harvard University Museum of Comparative Zoology (MCZ), University of New Mexico Museum of Southwestern Biology (MSB), New York State Museum (NYSM), Sam Noble Oklahoma Museum of Natural History (OMNH), Ohio Wildlife Center (OWC), Museum of Texas Tech University (TTU), University of Michigan Museum of Zoology (UMMZ), and National Museum of Natural History Smithsonian Institution (USNM).

Catalog No.	Region	Year	State	County	Sex	County percentage corn fields	Humans per square mile
FMNH 25169	Midwest	1925	Illinois	Marion	male	11.11	62.3
UMMZ 56587	Midwest	1925	Michigan	Berrien	female	8.30	142.7
UMMZ 58811	Midwest	1927	Michigan	Genesee	male	5.13	332.2
UMMZ 74603	Midwest	1935	Michigan	Calhoun	male	9.29	133.4
UMMZ 83550	Midwest	1935	Iowa	Winneshiek	male	17.94	32.3
UMMZ 177153	Midwest	1938	Michigan	Kalamazoo	female	8.49	178.1
UMMZ 81511	Midwest	1938	Michigan	Allegan	male	8.45	50.7
UMMZ 107655	Midwest	1939	Ohio	Lake	male	3.98	220.4
FMNH 90494	Midwest	1940	Illinois	Cook	female	9.15	4299.8
FMNH 90495	Midwest	1942	Illinois	Cook	male	9.15	4299.8
UMMZ 111219	Midwest	1961	Michigan	Washtenaw	male	13.44	244.2
USNM 347673	Midwest	1962	Indiana	Tippecanoe	female	25.21	178.3
FMNH 59027	Midwest	1972	Illinois	Cook	female	2.18	5813.5
UWBM 44493	Midwest	1972	Ohio	Clermont	female	3.69	211.0
UWBM 44494	Midwest	1973	Ohio	Clermont	female	3.69	211.0
UWBM 44495	Midwest	1973	Ohio	Ottawa	female	8.78	145.5
UWBM 44497	Midwest	1976	Ohio	Clermont	male	4.40	284.3
OMNH 40167	Midwest	1985	Wisconsin	Manitowoc	male	16.09	136.5

FMNH 156717	Midwest	1995	Illinois	Cook	female	1.40	5689.8
MZ 11627	Midwest	2017	North Dakota	Ransom	male	20.78	6.3
OWC 903	Midwest	2017	Ohio	Franklin	NA	3.70	2186.9
UMMZ 178776	Midwest	2017	North Dakota	Grand Forks	male	12.04	46.6
UMMZ 75104	Northeast	1933	New York	Orange	female	2.70	NA
NYSM 7056	Northeast	1937	New York	Albany	male	2.17	423.2
CUMV 1974	Northeast	1939	New York	Tompkins	male	3.02	89.1
NYSM 7057	Northeast	1939	New York	Albany	male	2.12	423.2
CUMV 11489	Northeast	1940	New York	Schuyler	male	1.98	39.6
CUMV 3464	Northeast	1942	New York	Albany	male	2.12	423.2
NYSM 7060	Northeast	1942	New York	Albany	male	2.12	426.8
NYSM 7061	Northeast	1942	New York	Rockland	male	0.51	457.7
NYSM 7062	Northeast	1947	New York	Albany	female	2.58	457.7
NYSM 7064	Northeast	1948	New York	Albany	male	1.44	160.6
CUMV 4970	Northeast	1949	New York	Tioga	female	2.86	58.1
CUMV 5148	Northeast	1949	New York	Adirondacks	male	NA	124.5
CUMV 9287	Northeast	1951	New York	Tompkins	male	3.44	125.7
CUMV 7800	Northeast	1953	New York	Tompkins	female	3.44	124.5
FMNH 198901	Northeast	1968	New York	Ulster	Male	0.62	1709.6
CUMV 13800	Northeast	1975	New York	Tompkins	male	7.22	183.3
CUMV 13801	Northeast	1975	New York	Onondaga	female	7.52	596.3
ASNHC 5640	Northeast	1984	New York	Westchester	female	0.34	2015.3
MCZ 59947	Northeast	1987	Massachusetts	Middlesex	male	0.26	1367.6
CUMV 14863	Northeast	1990	New York	Tompkins	male	4.25	198.1
MCZ 61601	Northeast	1991	Rhode Island	Providence	female	0.15	11446.7
MCZ 61602	Northeast	1994	Massachusetts	Suffolk	female	0.00	1467.4
MCZ 62096	Northeast	1997	Massachusetts	Essex	male	0.23	1467.4
MCZ 62102	Northeast	1997	Massachusetts	Essex	NA	0.23	1792.7
MCZ 62199	Northeast	1998	Massachusetts	Middlesex	male	0.12	423.2

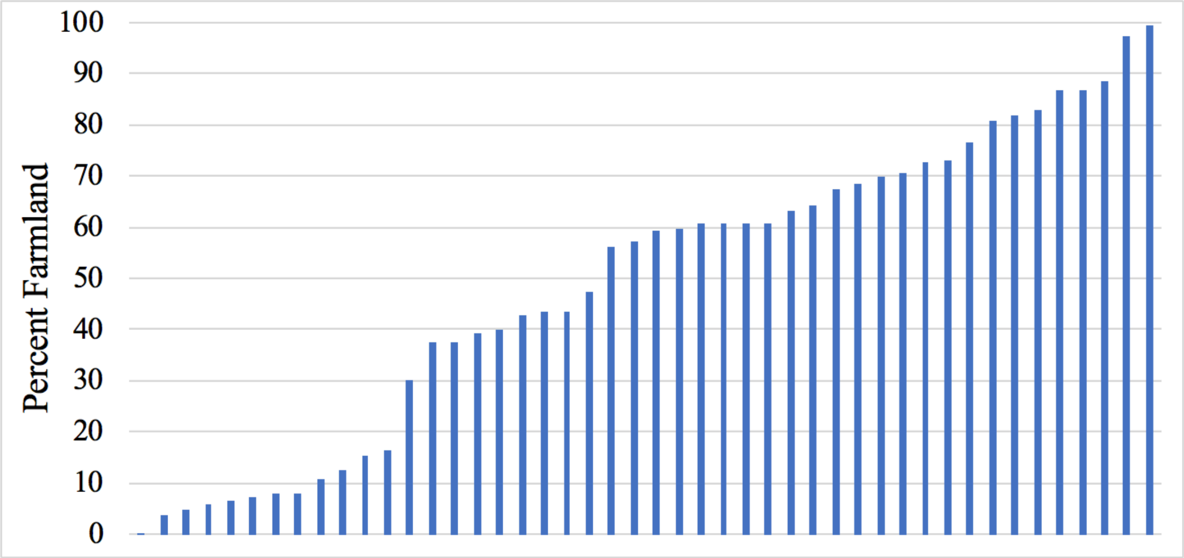
Table A3.2 Generalized linear mixed models for carbon ($\delta^{13}C$) isotope values of Virginia opossums (*Didelphis virginiana*) ranked by ΔAIC_c**.** Variables include year opossum was collected (year), percentage of county land occupied by corn (corn), days temperature reached below freezing (DT32), days with at least one-inch snow on the ground (DSNW), county human density (human), and percentage of county land occupied by agriculture (farmland). Models highlighted gray are considered to have substantial support (Burnham and Anderson, 2002), and the null intercept-only model is italicized.

Model	AICc	ΔAIC
Year + Corn	66.938	0
Year	68.493	1.555
DT32 + Year + Corn	69.118	2.18
DSNW + Year + Corn	69.495	2.557
Year + Corn + Human	70.374	3.436
Year + DSNW	70.604	3.666
Year + DT32	70.604	3.666
Human + Year	71.953	5.015
DSNW + Year + Human	73.871	6.933
DT32 + Year + Human	74.128	7.19
Corn	74.855	7.917
Corn + DT32	75.228	8.29
Farmland	75.458	8.52
Intercept-only	*75.985*	*9.047*
Human +Corn	76.214	9.276
DSNW + Farmland	77.327	10.389
DSNW + Corn	77.591	10.653
DT32	77.663	10.725
DT32 + Human + Corn	77.701	10.763
DSNW	78.199	11.261
DSNW + Human + Corn	78.59	11.652
Human	78.675	11.737
Human + DSNW	80.468	13.53
Human + DT32	80.806	13.868

Table A3.3 Mean carbon ($\delta^{13}C$) isotope, with two outliers removed, of Midwest Virginia opossums (*Didelphis virginiana*) compared before and after 1950 (1950 Division) and before and after 1970 (1970 Division). Time periods were compared using a t-test. Levene's statistic and significance are included to compare variance between time periods.

Midwest				
	Pre $\delta^{13}C$	Post $\delta^{13}C$	Test significance	Levene's
1950 Division	-21.95	-22.51	0.217	1.519 *P* = 0.234
1970 Division	-22.05	-22.5	0.415	4.518 *P* = 0.041

A.



B.

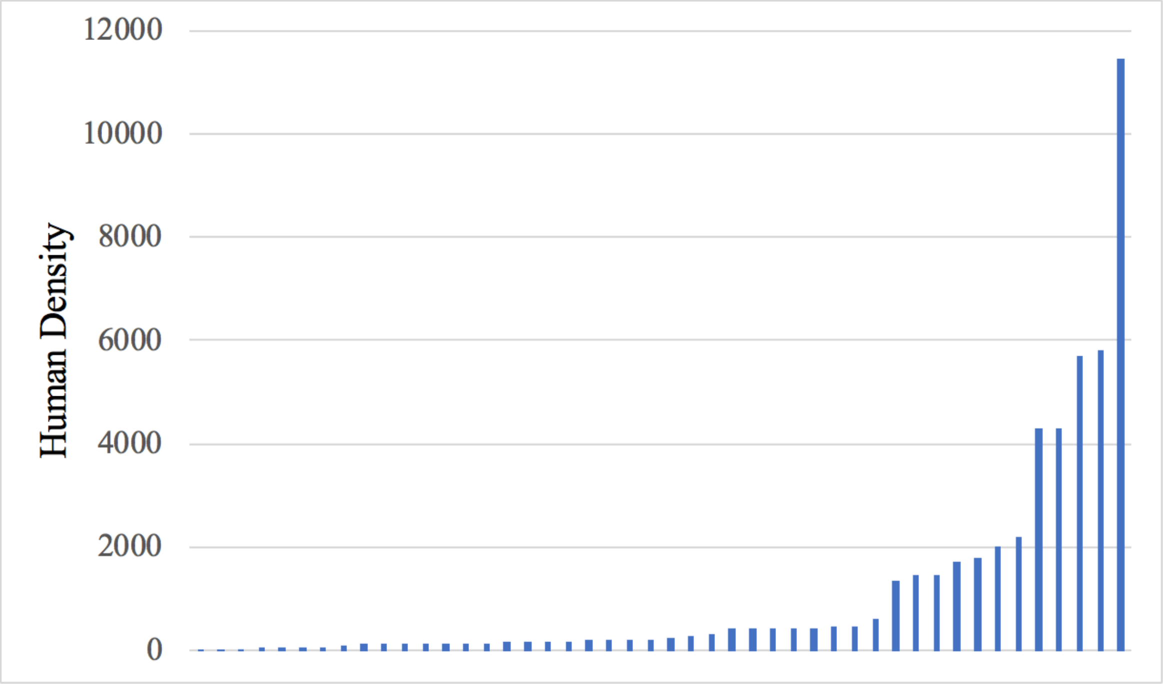


Figure A3.1 Distribution of values from county data in which opossums were collected: A) percent farmland and B) human density.

Chapter IV

Historical Context of the Virginia Opossum's Expansion in North America

Abstract

A firm understanding of historical processes can serve as a vital foundation for analyzing present-day populations (Eckert et al., 2008). For more than a century, Virginia opossums have expanded their range east and west of the Great Lakes, potentially offering two genetically distinct lineages to assess adaptation to the winter elements. Here, I genotype opossums across their range to better understand how they spread north out of Mesoamerica. Genetic markers were generated using double digest restriction-site associated DNA sequencing (ddRADseq), and 75 samples were analyzed using Bayesian clustering, discriminant analysis of principal components (DAPC), and approximate Bayesian computation (ABC) to evaluate migration patterns and relatedness across the opossum's range. Occurrence data were downloaded from VertNet to create ecological niche models (ENMs) in MaxEnt for past and future opossum populations. Both Bayesian clustering and DAPC found four genetic clusters, with the primary division separating temperate and Mesoamerican clusters. ABC simulations support a single ancestral lineage migrated into temperate North America.

Introduction

Individuals at the edge of their range can experience unique or strengthened ecological and evolutionary pressures (Excoffier et al., 2009), allowing range margins to serve as ideal natural experiments for biologists (e.g. Wellenreuther et al., 2011; Bridle et al., 2014). The majority of studies examining current range expansions fail to consider the deep-seated historical context of the populations they sample. Extant species' ranges have fluctuated throughout the Quaternary, with many species affected heavily by glaciation periods as recently as 12,000 years ago (FAUNMAP Working Group, 1996).

The modern genetic structure and distribution of a species often reflects historical geographic barriers (Eckert et al., 2008). For example, the phylogeographic analysis of two highly divergent clades of bighorn sheep (*Ovis canadensis*) suggests a single ancestral population was fractured when ice-free corridors disappeared prior to the Illinoian glaciation (Buchalski et al., 2016). In addition to dynamic glaciation corridors, a number of common geographic barriers in the eastern United States, including the Mississippi River and Appalachian Mountains, restricted gene flow during Quaternary glaciation events (Soltis et al*.,* 2006). Phylogeographic studies have illustrated that climatic events from recent glaciations strongly affected the distribution and connectivity of vertebrate populations (reviewed by Soltis et al., 2006).

The patterns observed in numerous North American taxa emphasize that contemporary range expansions would be more effectively studied in the context of historical migration events, as extant species' population genetics have been shaped by demographic processes dating back to the Quaternary (FAUNMAP Working Group, 1996). A firm understanding of historical demographic processes that separated populations also strengthens evaluations of convergent evolution. For example, because of the extensive research published on the patterns of human migration out of Africa, scientists were able to hypothesize and test for signatures of convergent evolution in adaptations to living in high altitudes such as Tibet and the Andes Mountains (Foll et al., 2014).

Ongoing range expansions allow scientists to identify putative mechanisms driving the expansion, but they also serve as potential study systems for novel ecological interactions and rapid evolution. Range expansions are often coupled with demographic growth, and the combination of drift, asymmetric gene flow, and novel selective pressures can reshape a population, both genetically and phenotypically (Excoffier et al*.,* 2009). Selectively beneficial traits to novel habitats and increased movement have been observed at the edge of expansions (Bridle et al., 2014; Brown et al., 2014). However, the genetic stock at range margins is often strongly influenced, and perhaps limited by, historical processes that shaped ancestral lineages (Eckert et al., 2008).

The Virginia opossum (*Didelphis virginiana*) is a generalist marsupial found from Costa Rica to the Midwestern United States and southeastern Canada (Gardner and Sunquist, 2003; Figure 4-1). Its genus, *Didelphis*, was part of the last wave of taxa to spread into North America during the Great American Interchange. The earliest *D. virginiana* fossils date back 0.8 million years from Mexico, Florida, and Texas (Graham and Lundelius, 2010). Observational data indicate *D. virginiana* reached Ohio by the 1600s, first entered Michigan in the 1820s, and arrived in southern Maine by the 1950s (Dice, 1927; McManus, 1974). A population genetics study of Midwestern opossum microsatellites identified two genetically distinct populations, one on either side of Lake Michigan, suggesting that the northern United States was colonized by multiple distinct lineages. This genetic data also indicated that *D. virginiana* populations are negatively affected by snow on the ground and positively impacted by anthropogenic land use change including agriculture (Walsh and Tucker, 2018).

Opossums found in California, Oregon, Washington, and small parts of Idaho and Montana are a result of multiple introductions of the species (Gardner and Sunquist, 2003). Opossums were introduced to Los Angeles, California from an unknown origin around 1890 and expanded to Ventura County by the 1920s. Opossums from Tennessee were brought to San Jose, California for food but escaped from enclosures in the 1910s. Opossums brought from South Carolina for a fur farm escaped in Visalia, California at an unknown date (Krause and Krause, 2006). Opossums were introduced in Oregon by the 1920s and Washington by the 1940s, both due to pet releases (Witmer and Lewis, 2001).

Here, I identify historical patterns of the Virginia opossum's expansion out of Mesoamerica using ddRADseq. A large pool of biallelic SNPs maximizes accuracy and provides more resolution in genetic assessments than traditional mitochondrial or microsatellite markers (Macher et al., 2015). To best evaluate northern populations for adaptations to temperate winter conditions, it is beneficial to establish whether the Midwest and Northeast were colonized by distinct lineages or by a single ancestral lineage. I hypothesize that the colonization of temperate North America was the result of a single expansion of *D. virginiana* out of Mesoamerica, with subsequent divergence events in the northern populations. If this is the case, then opossums from northern populations will share a relatively recent common ancestor, and populations from the Midwest and Northeast will coalesce with each other before coalescing with more southern populations. Alternatively, colonization of temperate North America is the result of multiple expansions of *D. virginiana* out of Mesoamerica, and distinct populations in temperate North America will be not be more closely related to each other than to populations in Mesoamerica. The Virginia opossum's early arrival in Florida may have been the result of an early route near the mouth of the Mississippi River during periods of low sea level (i.e. Cullingham et al., 2008). If *D. virginiana* spread north both along the Atlantic coast and through continental Mexico, then Northeastern populations will coalesce with a Mesoamerican lineage before coalescing with a Great Plains lineage.

Methods

DNA Extraction

Tissue samples of Virginia opossums from throughout their range (Figure 4-1) were processed to extract DNA. Tissue samples were acquired from museum institutions, fur trappers, and state wildlife biologists. Tissue samples from ear, muscle, heart, kidney, and liver were processed with a QIAGEN DNEasy kit following QIAGEN protocol with modifications. Samples were incubated between 4 and 24 hours, samples were centrifuged for six minutes to spin down Buffer AW2, and water sat on the spin column for 10 minutes to elute.

In addition to obtaining frozen tissue and skin samples from 15 museum institutions, I collaborated with approximately 15 fur trappers and wildlife biologists to acquire and accession tissue samples in the University of Michigan Museum of Zoology. To solicit collaborations, emails were sent to state fur trapping societies and state wildlife biologists providing an overview of the research and request for samples of ear from opossums. Collaborators were mailed a sampling kit including gloves, microcentrifuge tubes with dimethyl sulfoxide saturated with NaCl, alcohol wipes, scalpels, sampling instructions, and return postage.

I supplemented tissue samples of Virginia opossums with museum specimens from 13 institutions. To determine the optimum museum sample to request, I used University of Michigan's Mammal Teaching Collection (MTC) specimens to destructively sample bone (MTC 32), toe pad (MTC 10), and skin near the sutures on the abdomen (MTC 10). I processed samples using an ancient DNA extraction protocol (Moraes-Barros and Morgante, 2007; Campos and Gilbert, 2012; Fulton et al., 2012). A 2% agarose gel of the three samples' DNA yields indicated the best sampling location for opossums was abdominal skin (Figure A4.1A).

In addition, I modified and verified a protocol for extraction of DNA from museum specimens using University of Michigan specimens. The protocol requires you wipe a new weigh boat with an alcohol prep pad, place skin on dried boat, use forceps to anchor the skin, and scrape any hair and top layer of epithelial cells off the skin sample. As much hair should be removed from the skin as possible. Place the sample in a microcentrifuge tube, submerge with ddH_2O, and place in the refrigerator (4°C) for 24 hours. Replace ddH_2O and return to the refrigerator 24 hours. Remove the water, add TE solution (Tris 10mM and EDTA 1mM), and return the sample to the refrigerator for 24 hours. Rinse the sample with 70% ethanol, followed by ddH_2O. Hydrate the sample in TE solution for 24 hours in the refrigerator. Holding the sample over a sterile weigh boat, rinse it with 0.5M EDTA and pat dry with Kimwipe.

The QIAGEN DNeasy Blood & Tissue Kit protocol calls for tissue to be cut into small pieces prior to digestion. To evaluate the optimum method of cutting tissue, three skin samples from MTC 12 were rehydrated (as described above). One sample was placed in a tube with ceramic beads, a second sample was placed in a tube with metal beads, and both tubes were shaken for 60 seconds by the benchtop FastPrep homogenizer (MP Biomedicals, Solon, Ohio). A third sample was manually cut with a scalpel into small pieces. DNA yields from the three techniques were compared using gel electrophoresis (Figure A4.1B).

A total of 173 opossums were processed for DNA, including second attempts from eight specimens yielding low DNA concentrations but that had enough material for a second extraction. Samples were analyzed for DNA concentration using a Qubit 3 Fluorometer with an HS assay (Invitrogen, Waltham, MA). Excluding reads that yielded “Sample too low” results, DNA concentrations ranged from 0.063ng/uL – 678ng/uL.

ddRadseq Library Preparation

Genetic markers were generated using double digest restriction-site associated DNA sequencing (ddRADseq), a method that uses restriction enzyme sites to generate a large set of single nucleotide polymorphisms (SNPs) from throughout their genome. Five restriction enzyme pairs were evaluated to identify the ideal enzyme pair to use for *D. virginiana*: (*EcoRI* + *MspI*; *EcoRI* + *MseI; NlaIII+MluCI; SphI* + *MluCI;* and *SphI* + *MseI*; Peterson et al. 2012). Three opossum samples were selected to test restriction enzyme pairs: TTU 43180 (Texas frozen tissue), MZ 11630 (Vermont ear tissue), and MVZ 114842 (Nebraska rehydrated skin). Samples were digested with each enzyme pair (10 U per enzyme) for 37°C for three hours. Digested samples were visualized using gel electrophoresis (Figure A4.2) and submitted to the UM Advanced Genomics Core for QC analysis (Figure A4.3), and *NlaIII+MluCI* was the restriction enzyme pair selected based on DNA yield and distribution of digested DNA lengths.

Samples with the highest DNA concentrations and geographically rare samples with at least 200 ng were processed using the Peterson et al. (2012) protocol to prepare three ddRADseq libraries. Briefly, samples were digested, cleaned, ligated with flex adapters in three separate sublibraries (16 samples per sublibrary), cleaned, size selected for 200-350 base pairs, amplified with a unique PCR primer for each sublibrary, cleaned, and submitted to the UM Advanced Genomics Core for sequencing (Table A4.1). Because of equipment turnover, the first library was genotyped using an Illumina HiSeq platform while the second and third libraries were genotyped using a NovaSeq platform. Collectively, a total of 118 opossums were submitted for sequencing (Figure A4.4).

Genotyping

Each library was demultiplexed using Step 1 in iPyrad (v 0.7.28; Eaton and Overcast, 2020). Libraries 1 and 2 were processed separately in iPyrad to evaluate sample quality, and samples with fewer than 1000 recovered loci were removed. The remaining samples were pooled into a single folder with Library 3 samples to call SNPs in iPyrad (N = 94). Low-quality reads and poor-quality alignments were filtered during the process of SNP calling (iPyrad Steps 2-7). Briefly, for each opossum sample, iPyrad Step 2 removes library adapters and reads with a Phred score below 33 or more than five ambiguous sites. Step 3 merges overlapping paired reads, de novo clusters reads based on at least 85% similarity, and aligns clustered reads. Step 4 estimates heterozygosity and sequencing error rate for diploids, which Step 5 uses to estimate consensus allele sequences. Step 5 requires a depth between five and 10000 to reduce repetitive regions, no more than 10% ambiguous bases, and no more than 8% heterozygous bases to remove poor alignments. Step 6 pools samples together to cluster reads based on at least 85% similarity. Finally, Step 7 removes loci found in fewer than four opossums, with more than 20% bases called as SNPs, with more than eight indels, or more than 50% shared polymorphic sites. Samples with fewer than 5000 loci recovered were removed from the data (N = 15), and *Vcftools* was used to filter out indels, SNPs unique to one sample, and SNPs with depths less than 9 (Danecek et al., 2011; Table A4.2). For downstream analyses in which linkage may be a problem (e.g. STRUCTURE, DAPC, DIYABC), one SNP per locus was selected based on the least missing data and highest coverage. VCF files were converted to other file formats using PGDSpider 2.1.1.5 (Lischer and Excoffier, 2012).

Identifying non-D. virginiana samples

Because *D. virginiana* is sympatric with *D. marsupialis* in Mexico and Central America, and the two species are morphologically similar (Voss and Jansa, 2009), a phylogeny was built to identify and remove samples that were not *D. virginiana* from downstream analyses. A maximum-likelihood phylogeny was generated for 79 samples using a GTRGAMMA model and 100 ML searches in Randomized Axelerated Maximum Likelihood (RAxML; Stamatakis, 2014). *Didelphis aurita* and *D. albiventris* were used as an outgroup to root the 79-sample tree. The tree was visualized using Tree Graph 2 v2.15 (Stöver and Müller, 2010), and branches with bootstrap values below 50 were collapsed into polytomies. Two samples formed a sister group with the outlier species, and they were removed from further analyses (Figure A4.5) for a final dataset of 75 *D. virginiana* samples with 352 polymorphic loci.

Identifying populations with STRUCTURE and DAPC

To identify putative populations, the Bayesian clustering method, STRUCTURE 2.3.4 (Pritchard et al., 2000), was used with an admixture model to identify the most probable number of clusters (K) and assign individuals to clusters. Trials were run for $K = 1–10$ for 20 iterations with a burn-in of 10,000 and 200,000 runs in each trial, and ΔK was calculated to identify the optimum number of clusters (Evanno et al., 2005). Individuals were assigned to clusters based on the top-performing replicate's membership coefficient (Q) outputs (Jakobsson and Rosenberg, 2007). Each resulting cluster with $N > 10$ was examined for substructure using the same STRUCTURE methods. For example, the 64 samples assigned to "cluster one" were extracted from the original dataset and analyzed in STRUCTURE to determine K within "cluster one" based on ΔK (Evanno et al., 2005) and assigned to subclusters based on Q outputs (Jakobsson and Rosenberg, 2007). STRUCTURE bar plots were generated using the "conStruct" R package (Bradburd, 2019).

In addition to Bayesian methods, the 75 *D. virginiana* were also analyzed for population clusters using Discriminant Analysis of Principal Components (DAPC) in the "adegenet" R package (Jombart, 2008). Briefly, the program first uses a clustering algorithm to maximize differences between K groups (K maximum set to 20). BIC is calculated by the program to determine the optimal K. The program then runs a discriminant analysis of principal components to maximize allelic differences between groups. In this step, I used the optimization procedure provided by 'adegenet' to determinate that the optimal number of PCs to retain was 40.

Approximate Bayesian Computation

I used approximate Bayesian computation (ABC) to compare different migration hypotheses with the software DIYABC v. 2.1.0 (Cornuet et al., 2014). A vcf file with 352 SNPs and population assignments based on STRUCTURE results was converted to a DIYABC-compatible file using the python script vcf2DIYABC.py (available at https://github.com/loire/vcf2DIYABC.py). I estimated the posterior probability of 19 migration scenarios (Figures A4.6 and A4.7), with priors set to uniform distributions. Priors for effective population sizes ranged from 1 to 10000, priors for generation time ranged from 5 to 10000, and priors for admixture rate ranged from .001 to 0.999. Simulations were run for 900,000 generations and summary statistics were generated for: mean gene diversity across polymorphic loci, pairwise F_{ST} variance, mean pairwise F_{ST}, pairwise Nei's distance variance, and mean pairwise Nei's distances. Posterior probabilities of each scenario were calculated by direct estimation and logistic regression and plotted (Figure A4.8) to select the most competitive models (Figure 4-2). The six best performing models were rerun with the same settings, except simulations were run for 9,000,000 generations. Posterior probabilities of the six scenarios were calculated by direct estimation and logistic regression considering 500 and 60,000 closest simulated datasets, respectively. The six best performing models were also run with population assignments based on DAPC results for 600,000 generations. Posterior probabilities of the six scenarios were calculated by direct observation and logistic regression considering 500 and 6,000 closest simulated datasets, respectively.

Ecological Niche Modelling

D. virginiana records were downloaded from VertNet and georeferences were added for specimens that were missing latitude and longitude coordinates but had locality data at the city or county level. Samples collected for this study and for Walsh and Tucker (2018) were added to the dataset. All specimens from known introductions on the West Coast were removed for the final occurrence dataset. Environmental layers were downloaded from the WorldClim database of bioclimate variables averaged from 1970-2000, as well as those calculated for 2020-2050 (CMIP5 HE model) using the raster package in R (Hijmans, 2015). Bioclimate layers (CCSM 4.0 models) for the last glacial maximum (22,000 years ago) and mid-Holocene (6000 years ago) were directly downloaded from WorldClim. All raster files were cropped to Central America through southern Canada and converted to asci files for maximum entropy modelling (MaxENT) environmental layers.

Two subsets of occurrence data were used for ecological niche modeling. A conservative distribution of opossums collected until 2000 (N = 5433), and a liberal distribution of all opossums collected through 2019 (N = 5837). For each occurrence dataset, ecological niche models (ENMs) were generated with MaxEnt version 3.4.1 for current distributions (Phillipps and Dudik, 2008). Using the 2000 occurrence dataset, ENMs were also generated for distributions in 2050, the mid-Holocene, and last glacial maximum. All ENMs were generated by removing duplicate presence records, randomly splitting samples into 25% testing and 75% training samples and bootstrapping 10 replicates.

Results

SNPs generated

Out of 116 opossums submitted for ddRAD sequencing, 37 samples had fewer than 500000 reads, while 79 samples had between 581240 and 40 million reads produced from the IlluminaSeq and NovaSeq platforms. After iPyrad filtering, the 79 high-quality samples had between 5693 and 2166682 loci. Additional filtering in *vcftools* yielded 553 loci with 2524

SNPs. When other *Didelphis* species were removed from the dataset, a total of 352 polymorphic loci remained with 1932 SNPs.

RAxML

The phylogeny generated with RAxML placed a Honduras sample (MSB 45756) and an El Salvador sample (TTU TK 34853) as sister to *D. aurita* and *D. albiventris*, while the remaining 75 specimens formed a monophyletic group (Figure A4.5). While two groups of Mesoamerican samples were strongly supported as monophyletic groups with recent ancestors, deeper divergence events were not supported (Figure A4.5).

STRUCTURE and DAPC

The first Bayesian clustering step implemented in STRUCTURE identified two genetic clusters (Figure A4.9): cluster one (N = 64) was composed of all U.S. and Canada (temperate) opossums except for the New Mexico specimen (MSB 70022) and cluster two (N = 11) was composed of MSB 70022 and all Mesoamerican samples. When cluster one was evaluated for substructure, two clusters were identified, both with broad geographic distributions (N = 40 and 24; Figure 4-3 and Figure 4-4A). When cluster two was evaluated for substructure, two clusters were identified. One cluster was composed of three specimens from the Yucatan Peninsula, and the second cluster was composed of eight samples broadly distributed across Mesoamerica (Figure 4-3 and 4-4B)

In my DAPC analysis, the retained 40 principal components explained 88% of variance, with three retained eigenvalues of 2573.05, 1771.39, and 272.32. DAPC identified four clusters (Figure 4-5A). Unlike the STRUCTURE results, two Mesoamerican specimens were assigned to Cluster 1, which was composed primarily of temperate North American opossums (Figure 4-5B).

The first eigenvalue separated the primarily temperate Clusters 1 and 2 from the Mesoamerican Clusters 3 and 4, while the second eigenvalue differentiated Clusters 3 and 4.

Approximate Bayesian Computation

The ABC simulation most strongly supported by logistic regression of posterior probabilities was Scenario 4 (logistic regression: 0.9523), while the simulation most strongly supported by direct regression of posterior probabilities was Scenario 6 (direct regression: 0.5440; Table 4-1). Both scenarios place the two temperate clusters as a monophyletic group in which population one was derived from population two. Scenario 4 placed the two Mesoamerican clusters as a monophyletic group, while Scenario 6 placed the broadly distributed Mesoamerican cluster as sister to the temperate clusters. Both scenarios were similarly supported when DAPC population assignments were used.

Ecological Niche Models

For ENMs created with 2000 and 2019 occurrence data, the following variables contributed the most to MaxENT models, arranged from highest contribution: annual mean temperature, annual precipitation, minimum temperature in the coldest month, precipitation in the warmest quarter, and temperature seasonality (Table 4-2).

MaxENT results indicate Central America, much of Mexico, and the Gulf Coast were habitable for opossums during the last glacial maximum (Figure 4-6). Three *D. virginiana* fossils dating to the Pleistocene were found in habitable areas, while a fourth was found in Texas just north of the predicted range margin. By 6000 years ago, opossums are predicted to have extended into much of their current range (Figure 4-7). 2050 projections suggest habitat in

Minnesota, the Dakotas, and Ontario will become more conducive for opossums. Additionally, opossums are likely to spread into Québec and the Maritime provinces (Figure 4-8).

Discussion

The maximum likelihood phylogeny (Figure A4.5) placed the Honduras sample, MSB 45756, and El Salvador sample, TTU TK 34853, as sister to *Didelphis aurita.* MSB 45756 was collected in 1981 and identified as *D.v. yucatanensis*, a relatively small subspecies with dark pelage and a tail at least 80% the length of its body (Gardner, 1973). TTU TK 34853 was collected in 1990 and identified as *D. v. californica*, a medium-sized subspecies with dark pelage and a tail 80-90% of its body length (Gardner, 1973). Voss and Jansa (2009) found that *D. virginiana* is the most divergent species within *Didelphis*. Based on the sympatric geographic range of other didelphids, the MSB and TTU specimens are likely misidentified *D. marsupialis*, a species with an especially prehensile tails that is slightly smaller than most *D. virginiana* (Voss and Jansa, 2009). Because *Didelphis* species are morphologically similar to one another, the potential misidentification of two Central American opossum samples supports my choice to use known allopatric species as outgroups to find misidentified specimens.

Bayesian clustering in STRUCTURE identified two geographically distinct clusters: all temperate opossums were in Cluster 1, while all Mesoamerican opossums were in cluster 2 (Figure 4-3). Further substructure was found in both geographic areas: there was no discernible pattern in the two temperate clusters, but three opossums from the Yucatan Peninsula were genetically distinct from 8 opossums found broadly distributed across Mesoamerica (Figure 4-3). This Yucatan cluster was further supported by DAPC results (Figure 4-5). Similar to STRUCTURE, DAPC also found four clusters, and the two temperate clusters were closely related. The DAPC cluster 1 included two Mesoamerican samples, suggesting some polymorphic sites distinguish these samples from the other Mesoamerican opossums. The different cluster assignments resulting from STRUCTURE and DAPC are likely due to differences between the Bayesian algorithm, which treats every SNP with equal weight, and the DAPC algorithm, which seeks to maximize differences between clusters (Evanno et al., 2005; Jombart, 2008). However, both analyses suggest the two temperate clusters were closely related.

The sympatric distribution of both clusters in temperate North America suggests incomplete lineage sorting (Figure 4-3). This could be a result of a rapid wave of expansion from the Gulf Coast as the Laurentide ice sheets retreated. Alternatively, because temperate North America has two genetic clusters, this may indicate two waves of expansion that experienced recent admixture. Out of 19 phylogeographic scenarios tested, ABC simulations support a single wave of expansion into temperate North America, followed by recent divergence. The most strongly supported scenarios both include temperate population 1 as derived from population 2. The two viable models posit that the Yucatan and Mesoamerica opossums coalesce before coalescing with the temperate opossum clusters, or the Yucatan opossums are basal (Figure 4-2). Due to the limited tissue collections of Mesoamerican opossums available in the U.S., my analysis is constrained by the small sample sizes of opossums in their southern range. These preliminary results should be further investigated.

The ABC analysis supports the hypothesis that opossums entered temperate North America from a single, ancestral lineage. During the last glacial maximum, habitable areas in the Yucatan Peninsula were divided, which may help explain the genetic distance between three of the Yucatan samples and other Mexican samples (Figure 4-3; Figure 4-6). The Rio Grande or Sierra Madre Oriental may serve as a modern-day barrier between Mesoamerica and temperate North America opossum populations, and these putative geographic barriers should be studied further.

None of the analyses revealed distinct lineages on either side of the Great Lakes, although population 1 is more abundant than population 2 in both the Midwest and Northeast (Figure 4-3). DIYABC simulations suggests population 1 is derived from population 2. One potential explanation of this pattern is a vicariance event followed by secondary contact. The estimated habitat conditions for opossums during the last glacial maximum (Figure 4-6) suggest opossums would have colonized the Gulf coast on either side of the Mississippi, but reduced habitat conditions near the southern border of Mississippi and Alabama may represent a permeable barrier near the Mississippi River. Temperate population 2 is not represented in the southeast (Figure 4-3). The two populations may represent two different routes across the Mississippi River: cluster 1 crossed the Mississippi near the Gulf Coast, while cluster 2 crossed the Mississippi further north. Alternatively, these two clusters are a result of incomplete lineage sorting. Opossums from introduced states (California, Utah, and Washington), represent both temperate clusters. Interestingly, both opossums in the San Jose, California region belong to the same cluster as Tennessee opossums, the documented source of their introduction (Krause and Krause, 2006).

In the next 30 years, opossums are expected to reach as far north as the Maritime provinces (Figure 4-8). Interestingly, the 2019 heat map does not indicate that North Dakota or most of Minnesota are currently habitable, despite the occurrence of opossums beginning in the 1990s (Walsh et al., 2017). This is likely a reflection of the variables used in ENMs and limited sampling in the region. Bioclimatic data does not capture the microhabitat details that make areas conducive to opossums, including rivers and marshland (Gardner and Sunquist, 2003; Walsh et al., 2017). Additionally, it does not reflect landscape changes resulting from agriculture and urban sprawl that may be facilitating the opossum's continued expansion northward (Kanda et al., 2009; Walsh and Tucker, 2018).

Four subspecies of opossums are delineated based on size, coat pelage, tail color, and tail length. *D. v. yucatanensis* is limited to the Yucatan Peninsula (Mexican states: Campeche, Quintana Roo, Yucatan). *D. v. californica* is distributed across the rest of the opossum's Mexican range, extending into the southern edge of Texas. *D. v. pigra* is limited to the Gulf Coast (all of Florida, southern Georgia, and Gulf Coast edges of Alabama, Louisiana, Mississippi, and Texas). The remaining opossum range is occupied by *D. v. virginiana* (Figure 4-1). Preliminary results indicate there is support for *D.v. californica* and *D.v. yucatanensis*, but no support for *D. v. pigra* as genetically distinct from northern opossums. However, additional samples from these regions are required for more definitive conclusions.

While phylogeographic studies using ddRADseq to examine other species have yielded strong phylogenetic and geographic patterns (e.g. Lam et al., 2018; Leydet et al., 2018; Berv et al., 2019), this is not the case for opossums north of Mexico. Older studies on other widespread mesopredators that used alternative genetic markers may shed light on this. Raccoon (*Procyon lotor*) mitochondrial data identified three lineages that did not appear to be historically separated by the Mississippi River or Appalachian Mountains. The authors suggest the lineages were a result of sea level fluctuations that caused repeat migration events from refugia in Florida, followed by admixture (Cullingham et al., 2008). Mitochondrial and microsatellite sequencing of striped skunks (*Mephitis mephitis*) indicate four lineages that resulted from pulsed expansions as glacial sheets fluctuated, followed by secondary contact to complicate the genetic composition across their range. Despite this, genetic data suggest the Mississippi River served as a significant barrier to movement (Barton and Wisely, 2012).

I hypothesized that opossums colonized temperate North America in a single expansion event, with subsequent divergence events in the northern populations. My clustering results and ABC similuations support the prediction that there was a single, large expansion wave out of

Mesoamerica. The geographically sympatric subclusters in temperate North America do not support a significant divergence event taking place to geographically separate contemporary northern populations. Instead, similar to other widespread mesopredators in North America, the opossum’s relatively recent expansion across temperate North America lacks a strong genetic footprint.

Tables

Table 4-1 Posterior probability estimates (direct and logistic) for the best six phylogeographic scenarios tested in DIYABC (see Fig. 4-2). The best models are highlighted in bold font.

Scenario	Direct regression	Logistic Regression
1	0.0520 [0.0000,0.2466]	0.0256 [0.0000,0.8206]
2	0.0340 [0.0000,0.1929]	0.0000 [0.0000,0.8095]
3	0.0740 [0.0000,0.3035]	0.0221 [0.0000,0.8196]
4	0.1860 [0.0000,0.5271]	**0.9523 [0.9124,0.9922]**
5	0.11 [0.0000,0.3843]	0.0000 [0.0000,0.8095]
6	**0.544 [0.1074,0.9806]**	0.0000 [0.0000,0.8357]

Table 4-2 Highest contributed WorldClim bioclimate variables in ENMs created using opossum occurrence data through 2000 and 2019. Contributions are calculated in MaxENT by the regularized gains attributed to a given variable for each iteration of training.

Bioclimate variable	**%contribution (2000)**	**% contribution (2019)**
Annual mean temperature	34.1	27.5
Annual precipitation	19.8	18.8
Minimum temp in coldest month	16.2	17.5
Minimum temp in coldest month	11.3	13
Temperature seasonality	8.1	8.6

Figures

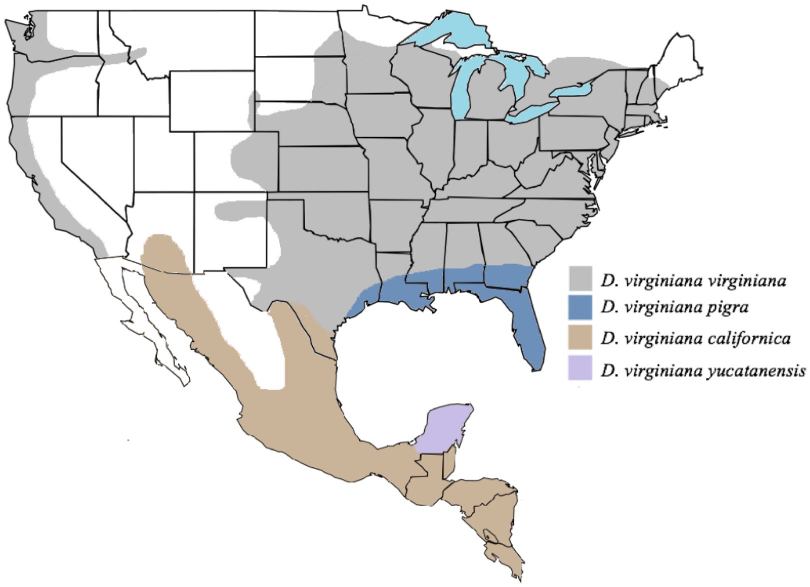


Figure 4-1 Map of contemporary Virginia opossum (*Didelphis virginiana*) range including putative subspecies (adapted from Gardner, 1973).

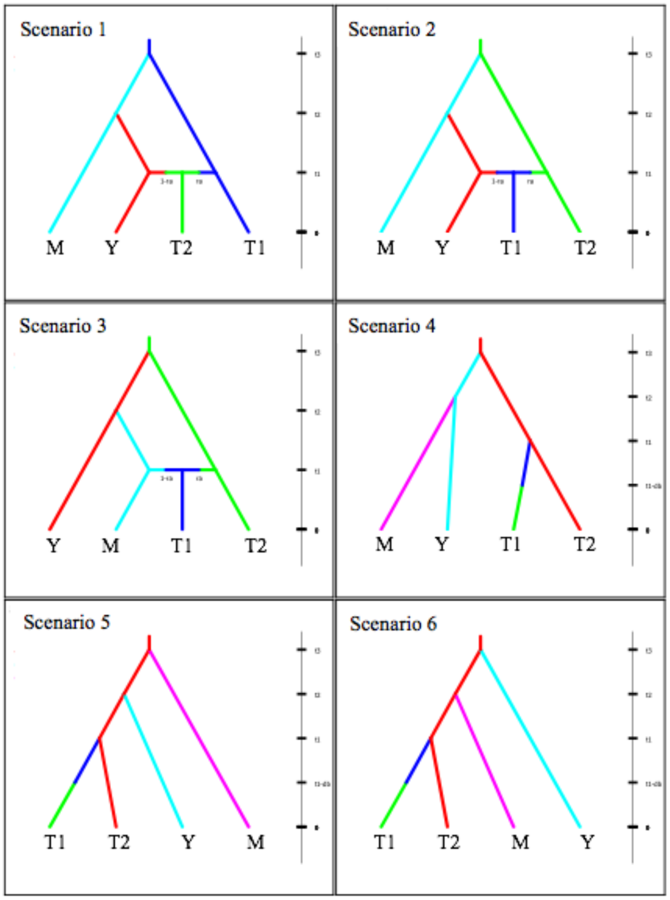


Figure 4-2 The six best phylogeographic scenarios tested using approximate Bayesian computation simulations in DIYABC (see A4 for all scenarios). The four populations were identified in STRUCTURE: M = Mesoamerica (cluster 2.2), Y = Yucatan Peninsula (cluster 2.1), T1 = Temperate cluster 1.1, and T2 = Temperate cluster 1.2. The most well-supported scenarios are Scenario 4 and Scenario 6.

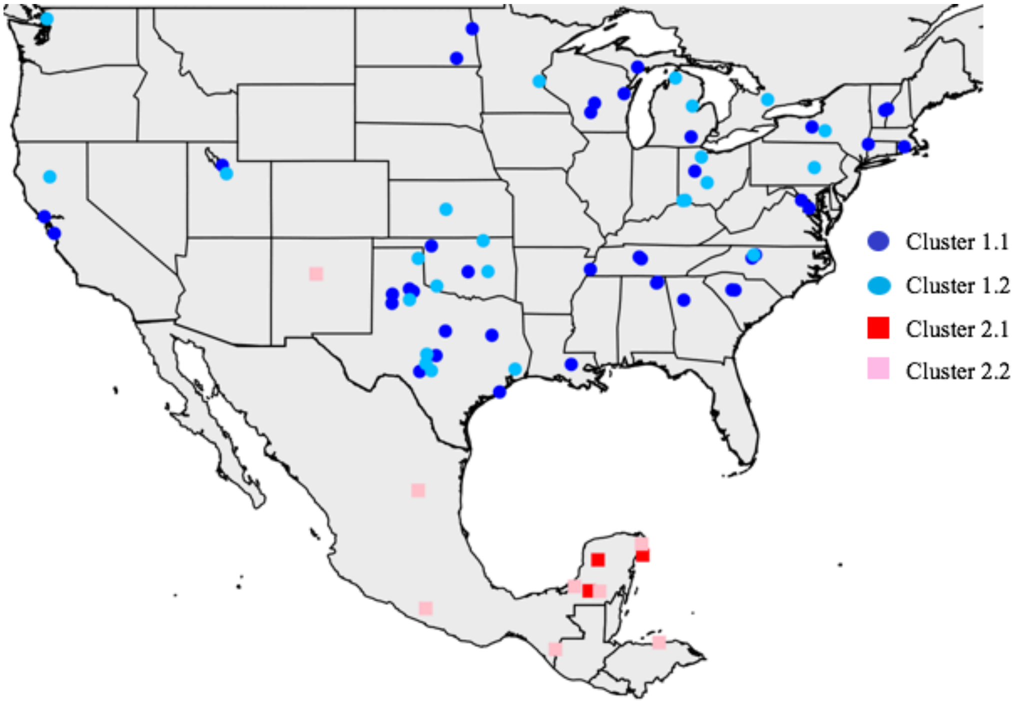


Figure 4-3 Map of opossum STRUCTURE results, including substructure within each cluster.

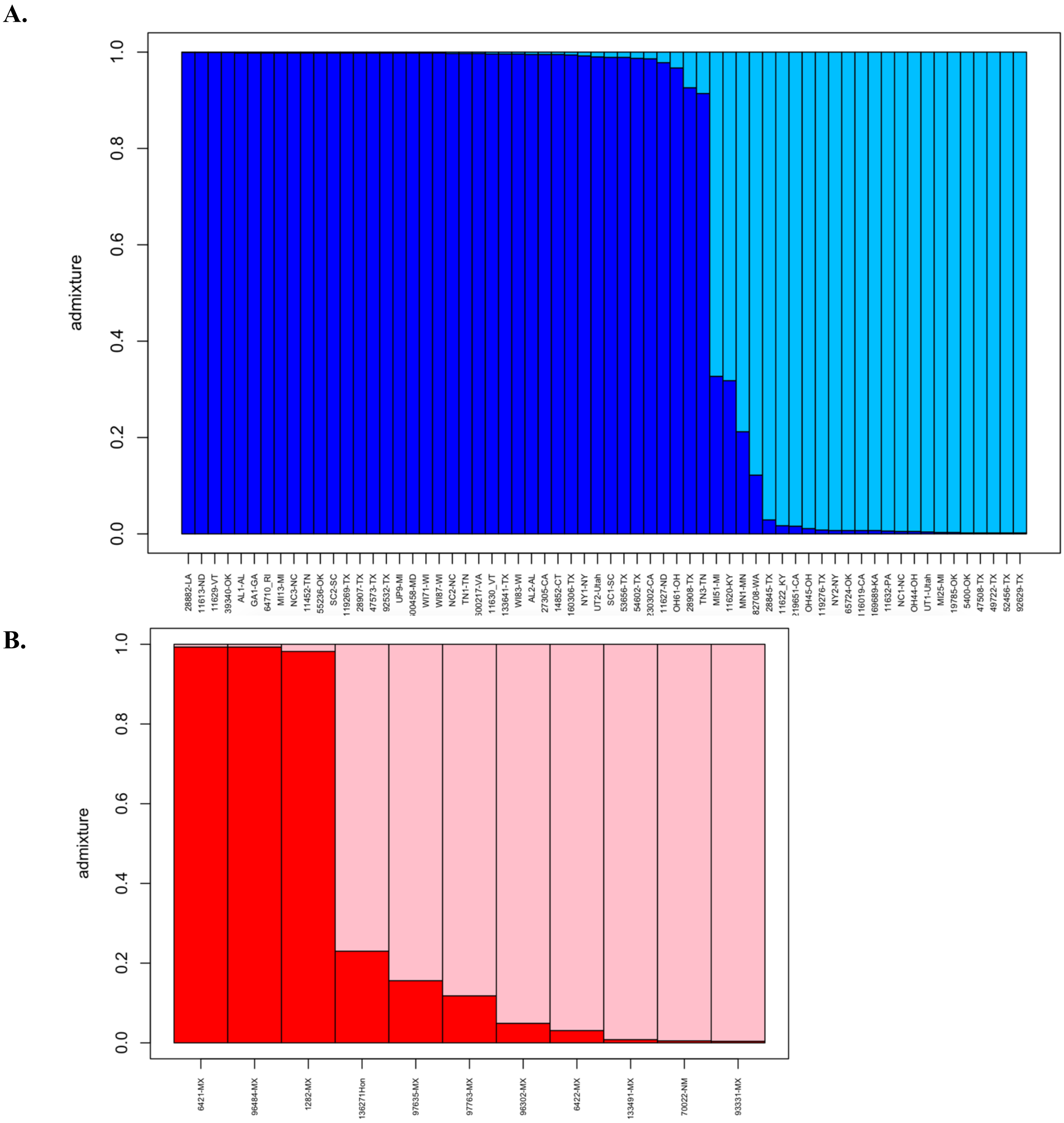


Figure 4-4 Individual cluster assignment bar plots for the substructure of A) cluster 1 (N = 64) and B) cluster 2 (N = 11). Geographic distribution of individuals is available in Figure 4-3.

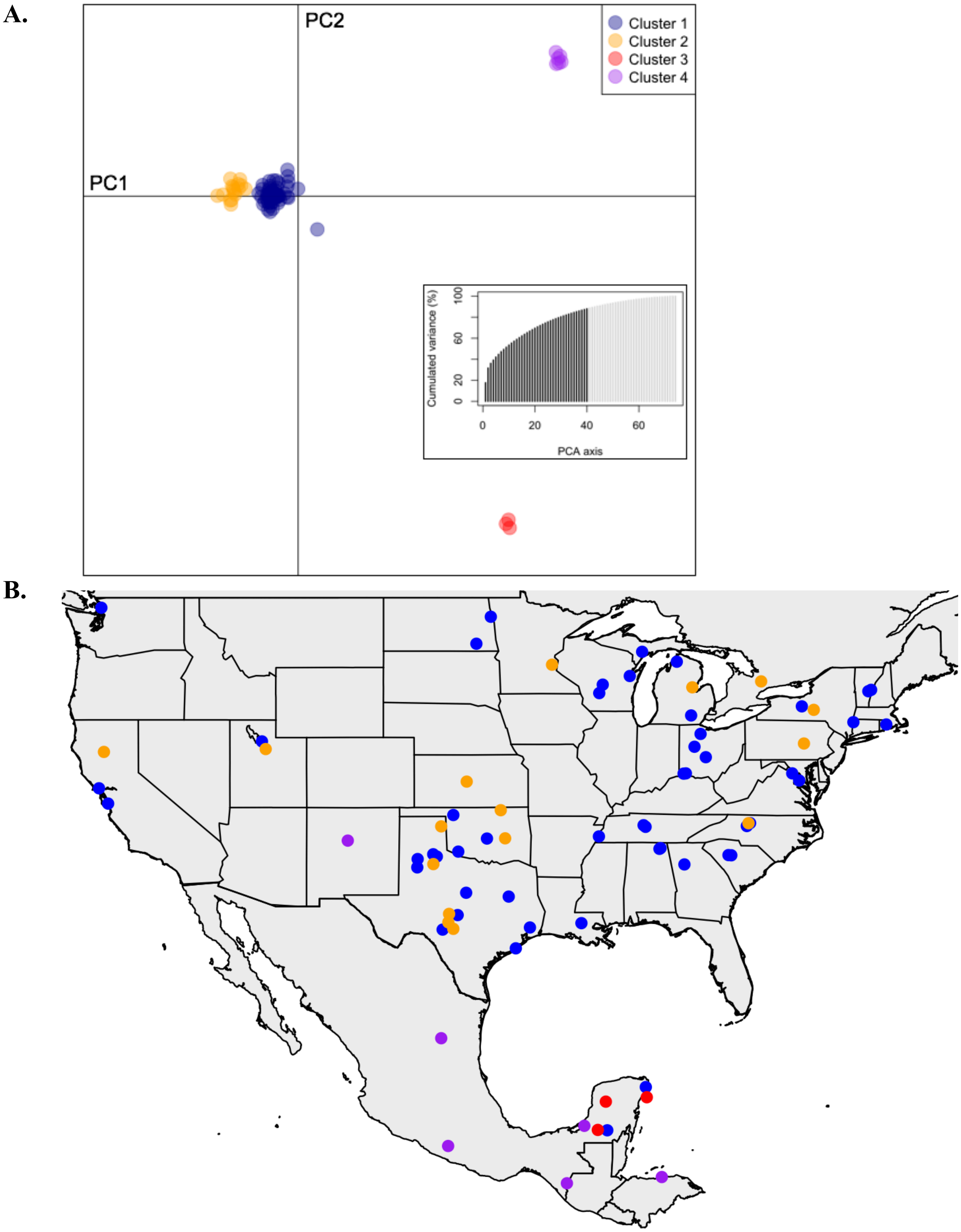


Figure 4-5 Results of Discriminant Analysis of Principal Components (DAPC) using 325 SNPs from 75 *D. virginiana* presented as A) a scatterplot on the first PCs with an inset graph of variation explained by the 40 PCs used and B) a map of DAPC cluster assignments.

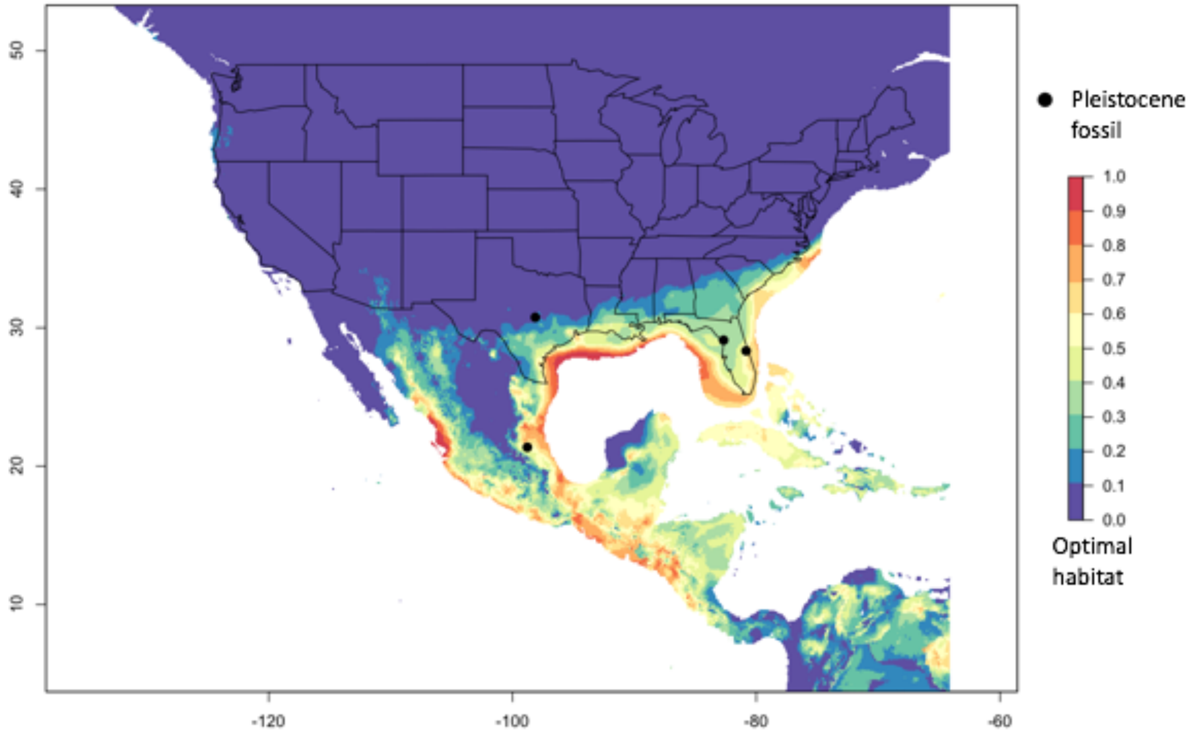


Figure 4-6 Predicted habitat conditions for *Didelphis virginiana* during the last glacial maximum, based on 2000 occurrence data and WorldClim bioclimate variables analyzed in MaxENT for 10 bootstrapped replicates. Warmer colors indicate more optimal habitat conditions. Four Pleistocene fossils are plotted as black circles.

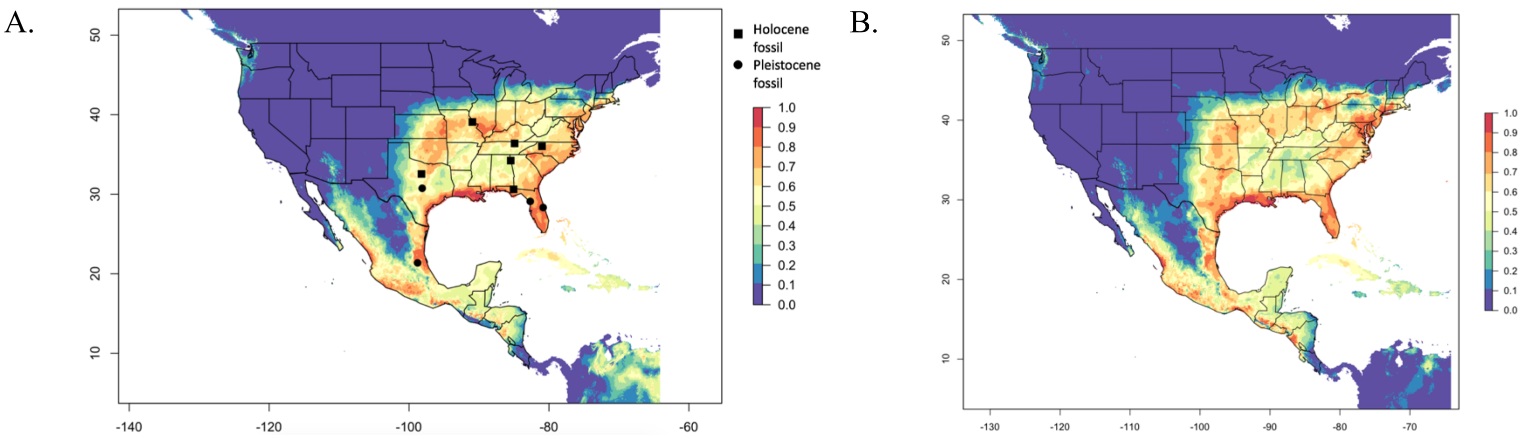


Figure 4-7 Ecological niche models for *Didelphis virginiana* based on 2000 occurrence data and WorldClim bioclimate variables analyzed in MaxENT for 10 bootstrapped replicates, with warmer colors indicate more optimal habitat conditions. a) Predicted habitat conditions approximately 6,000 years ago with Pleistocene fossils plotted as circles and early Holocene fossils plotted as squares. b) Habitat conditions for the year 2000.

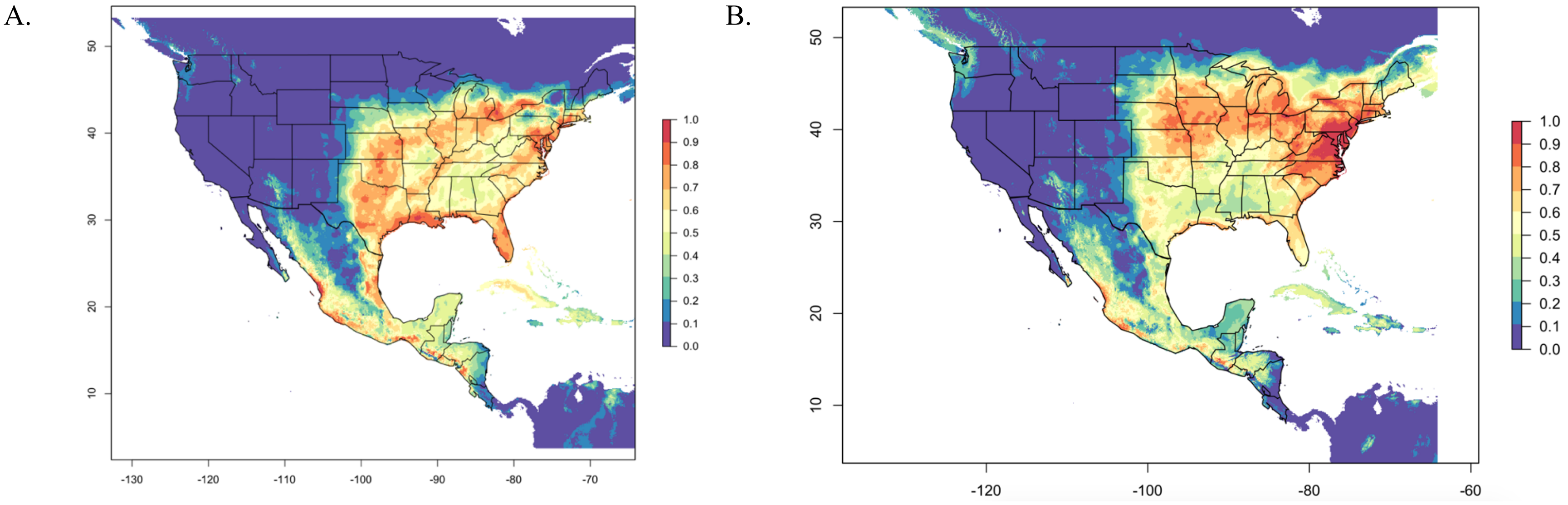


Figure 4-8 Ecological niche models for *Didelphis virginiana* based on occurrence data and WorldClim bioclimate variables analyzed in MaxENT for 10 bootstrapped replicates, with warmer colors indicate more optimal habitat conditions. a) Habitat conditions based on 2019 occurrence data and b) projected 2050 habitat conditions based on 2000 occurrence data.

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Appendix A4

Table A4.1 Oligonucleotide sequences unique to individual flex adapters and PCR primers used to identify specimens for ddRADseq.

Flex Adapter	Bar Code
1	GCATG
2	AACCA
3	CGATC
4	TCGAT
5	TGCAT
6	CAACC
7	GGTTG
8	AAGGA
9	AGCTA
10	ACACA
11	AATTA
12	ACGGT
13	ACTGG
14	ACTTC
15	ATACG
16	ATGAG

PCR Primer	Bar Code
PCR2 Index 1	ATCACG
PCR2 Index 2	CGATGT
PCR2 Index 3	TTAGGC

Table A4.2 Opossum (*Didelphis*) samples included in maximum-likelihood phylogeny and population clustering analyses (DAPC and STRUCTURE). Institution abbreviations include Angelo State Natural History Collections (ASNHC), California Academy of Sciences (CAS), Louisiana State University Museum of Natural Science (LSUMZ), Harvard University Museum of Comparative Zoology (MCZ), University of New Mexico Museum of Southwestern Biology (MSB), U.C. Berkeley Museum of Vertebrate Zoology (MVZ), Sam Noble Oklahoma Museum of Natural History (OMNH), Royal Ontario Museum (ROM), Museum of Texas Tech University (TTU), University of Michigan Museum of Zoology (UMMZ), National Museum of Natural History Smithsonian Institution (USNM), University of Washington Burke Museum (UWBM), and Yale Peabody Museum (YPM).

Institution	Catalog No.	Sex	Year	Country	State/Province	County	DNA (ng/uL)	DAPC	Structure
ASNHC	1282	F	1984	Mexico	Quintana Roo		41.2	DAPC3	Cluster2.1
ASNHC	6421	F	1987	Mexico	Campeche		25.4	DAPC3	Cluster2.1
ASNHC	6422	M	1987	Mexico	Campeche		11.5	DAPC1	Cluster2.2
CAS	27305	M	2003	USA	California	Marin	1.91	DAPC1	Cluster1.1
LSUMZ	28882	M	1984	USA	Louisiana	East Baton Rouge	210	DAPC1	Cluster1.1
MCZ	64710	M	2004	USA	Rhode Island	Bristol	86	DAPC1	Cluster1.1
MSB	45756	M	1981	Honduras			60	*marsupialis*	*marsupialis*
MSB	70022	M	1992	USA	New Mexico	Bernalillo	36.9	DAPC4	Cluster2.2
MVZ	219651	F	2007	USA	California	Tehama	13.6	DAPC2	Cluster1.2
MVZ	230302	M	2010	USA	California	Santa Clara	6.16	DAPC1	Cluster1.1
OMNH	39340	M	2010	USA	Oklahoma	Woodward	65.4	DAPC1	Cluster1.1
OMNH	55236	M	2013	USA	Oklahoma	Cleveland	83.2	DAPC1	Cluster1.1
OMNH	65724	F	2016	USA	Oklahoma	Tillman	54.8	DAPC1	Cluster1.2
OMNH	OCGR 11452	M	2002	USA	Tennessee	Shelby	37.8	DAPC1	Cluster1.1
OMNH	OCGR 5400	F	2008	USA	Oklahoma	Osage	19	DAPC2	Cluster1.2
ROM	96302	M	1989	Mexico	Campeche		137	DAPC4	Cluster2.2
ROM	96484	F	1989	Mexico	Yucatan		18	DAPC3	Cluster2.1
ROM	97635	M	1990	Mexico	Chiapas		19.9	DAPC4	Cluster2.2
ROM	97763	F	1990	Mexico	Quintana Roo	Isla Cozumel	7.38	DAPC1	Cluster2.2
ROM	116019	F	2004	Canada	Ontario		90.6	DAPC2	Cluster1.2

TTU	TK 119269	M	2004	USA	Texas	Edwards	181	DAPC1	Cluster1.1
TTU	TK 119276	F	2004	USA	Texas	Menard	68.6	DAPC2	Cluster1.2
TTU	TK 133491	M	2005	Mexico	Nuevo León		24.9	DAPC4	Cluster2.2
TTU	TK 133641	M	2005	USA	Texas	Mason	26.9	DAPC1	Cluster1.1
TTU	TK 136271	M	2004	Honduras	Colón		22.2	DAPC4	Cluster2.2
TTU	TK 160306	M	2008	USA	Texas	Hall	27.9	DAPC1	Cluster1.1
TTU	TK 169689	F	2011	USA	Kansas	Barton	98.6	DAPC2	Cluster1.2
TTU	TK 19785	F	1982	USA	Oklahoma	Hughes	5.39	DAPC2	Cluster1.2
TTU	TK 28845	F	1985	USA	Texas	Hardin	678	DAPC1	Cluster1.2
TTU	TK 28907	M	1985	USA	Texas	Comanche	55.8	DAPC1	Cluster1.1
TTU	TK 28908	F	1985	USA	Texas	Hale	17.4	DAPC1	Cluster1.1
TTU	TK 34853	M	1990	El Salvador	LA PAZ		225	*marsupialis*	*marsupialis*
TTU	TK 47508	F	1994	USA	Texas	Dickens	148	DAPC2	Cluster1.2
TTU	TK 47573	M	1996	USA	Texas	Lubbock	24	DAPC1	Cluster1.1
TTU	TK 49722	F	1996	USA	Texas	Kimble	9.9	DAPC2	Cluster1.2
TTU	TK 52456	F	1996	USA	Texas	Hemphill	20.2	DAPC2	Cluster1.2
TTU	TK 53656	M	1998	USA	Texas	Brazoria	223	DAPC1	Cluster1.1
TTU	TK 54602	M	1997	USA	Texas	Briscoe	32.8	DAPC1	Cluster1.1
TTU	TK 92532	M	2001	USA	Texas	Anderson	62.8	DAPC1	Cluster1.1
TTU	TK 92629	F	2001	USA	Texas	Kerr	25.2	DAPC2	Cluster1.2
TTU	TK 93331	M	2000	Mexico	Guerrero		10.4	DAPC4	Cluster2.2
UMMZ	AL 1	NA	2019	USA	Alabama	Jackson	51	DAPC1	Cluster1.1
UMMZ	AL 2	NA	2019	USA	Alabama	Jackson	45.5	DAPC1	Cluster1.1
UMMZ	GA 1	NA	2020	USA	Georgia	Fulton	48	DAPC1	Cluster1.1
UMMZ	MI 13	NA	2013	USA	Michigan	Washtenaw	36.9	DAPC1	Cluster1.1
UMMZ	MI 25	F	2013	USA	Michigan	Bay	72.2	DAPC2	Cluster1.2
UMMZ	MI 51	NA	2013	USA	Michigan	Emmet	56	DAPC1	Cluster1.2
UMMZ	MN 1	NA	2019	USA	Minnesota	Anoka	49.7	DAPC2	Cluster1.2

UMMZ	MZ 11613	M	2017	USA	North Dakota	Grand Forks	453	DAPC1	Cluster1.1
UMMZ	MZ 11620	F	2017	USA	Kentucky	Campbell	144	DAPC1	Cluster1.2
UMMZ	MZ 11622	F	2017	USA	Kentucky	Kenton	57.4	DAPC1	Cluster1.2
UMMZ	MZ 11627	M	2017	USA	North Dakota	Ransom	91.2	DAPC1	Cluster1.1
UMMZ	MZ 11629	NA	2017	USA	Vermont	Windsor	187	DAPC1	Cluster1.1
UMMZ	MZ 11630	NA	2017	USA	Vermont	Windsor	352	DAPC1	Cluster1.1
UMMZ	MZ 11632	NA	2017	USA	Pennsylvania	Schuylkill	12.6	DAPC2	Cluster1.2
UMMZ	MZ900843	M	1999	Paraguay			116	*albiventris*	*albiventris*
UMMZ	MZ900844	NA	1989	Argentina			224	*aurita*	*aurita*
UMMZ	NC 1	NA	2019	USA	North Carolina	Forsyth	48.2	DAPC2	Cluster1.2
UMMZ	NC 2	NA	2019	USA	North Carolina	Forsyth	50	DAPC1	Cluster1.1
UMMZ	NC 3	NA	2019	USA	North Carolina	Davie	43.3	DAPC1	Cluster1.1
UMMZ	New York #1	NA	2017	USA	New York	Seneca	122	DAPC1	Cluster1.1
UMMZ	New York #2	NA	2017	USA	New York	Cortland	85.6	DAPC2	Cluster1.2
UMMZ	OH 44	F	2013	USA	Ohio	Seneca	26.7	DAPC1	Cluster1.2
UMMZ	OH 45	NA	2013	USA	Ohio	Franklin	54	DAPC1	Cluster1.2
UMMZ	OH 61	NA	2013	USA	Ohio	Logan	34.6	DAPC1	Cluster1.1
UMMZ	SC 1	NA	2019	USA	South Carolina	Newberry	55	DAPC1	Cluster1.1
UMMZ	SC 2	NA	2019	USA	South Carolina	Newberry	8.19	DAPC1	Cluster1.1
UMMZ	TN 1	NA	2018	USA	Tennessee	Williamson	85.3	DAPC1	Cluster1.1
UMMZ	TN 3	NA	2018	USA	Tennessee	Williamson	2.64	DAPC1	Cluster1.1
UMMZ	UP 9	NA	2013	USA	Michigan	Delta	146	DAPC1	Cluster1.1
UMMZ	UT 1	NA	2012	USA	Utah	Utah	31.9	DAPC2	Cluster1.2
UMMZ	UT 2	NA	2019	USA	Utah	Salt Lake	45.6	DAPC1	Cluster1.1
UMMZ	WI 71	NA	2013	USA	Wisconsin	Brown	39.9	DAPC1	Cluster1.1
UMMZ	WI 83	NA	2013	USA	Wisconsin	Adams	51	DAPC1	Cluster1.1
UMMZ	WI 87	NA	2013	USA	Wisconsin	Sauk	32.7	DAPC1	Cluster1.1
USNM	600217	NA	2010	USA	Virginia	Fairfax	46.2	DAPC1	Cluster1.1

USNM	600458	NA	1993	USA	Maryland	Charles	32.8	DAPC1	Cluster1.1
UWBM	82708	NA	2010	USA	Washington	Skagit	2.53	DAPC1	Cluster1.2
YPM	14852	M	2008	USA	Connecticut	Litchfield	37.4	DAPC1	Cluster1.1

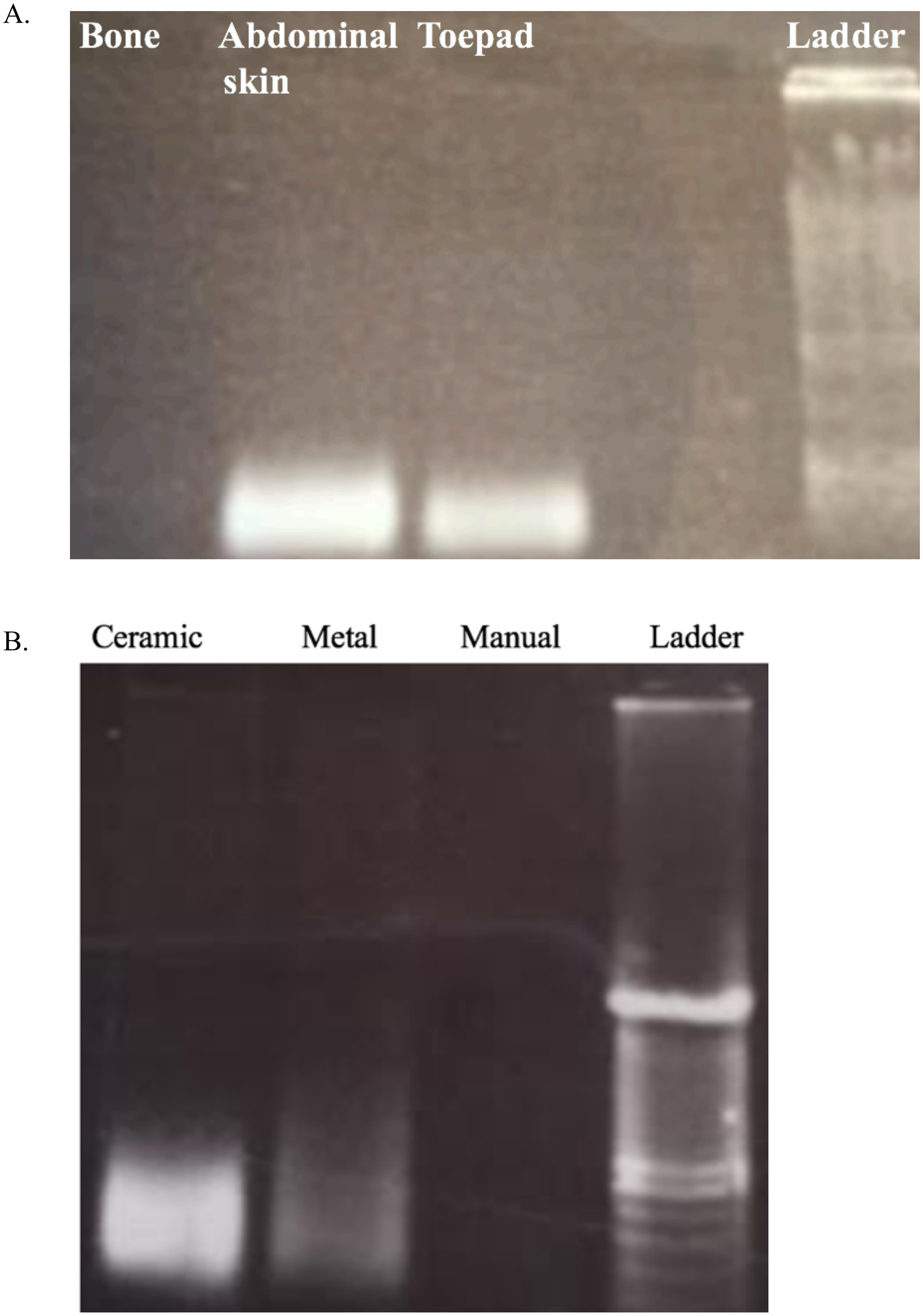

Figure A4.1 Agarose gel (2%) image of 5 ul DNA A) extracted from bone, museum skin, and toepad of Virginia opossums in the University of Michigan Mammal Teaching Collection B) museum skin sheared with ceramic beads, metal beads, or manually with a scalpel. Ladder is 100bp (Invitrogen).

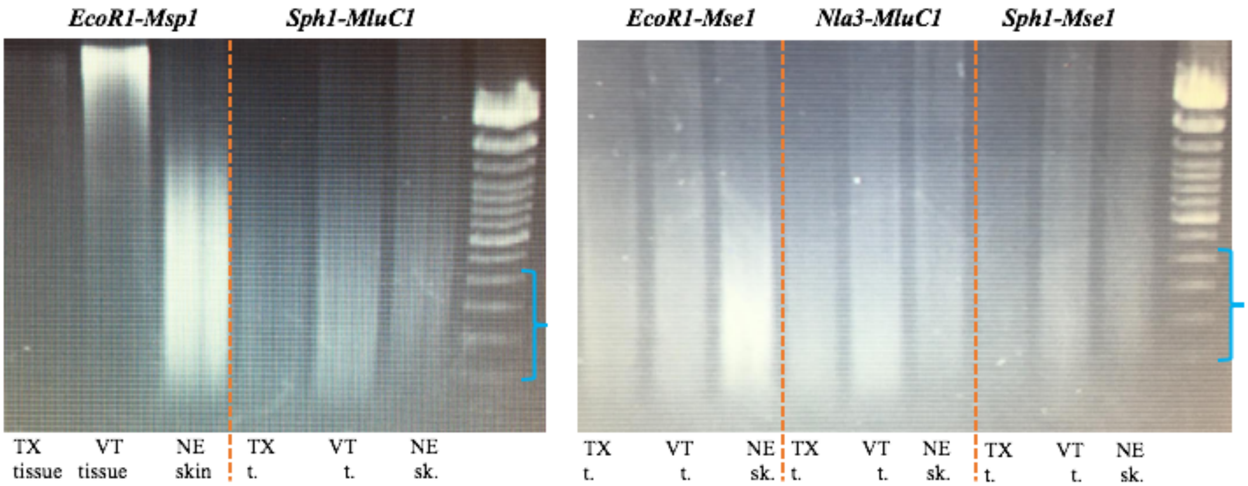

Figure A4.2 Agarose gel (2%) image of 5 ul digested DNA for 5 enzyme pairs. From left to right, the samples digested are Texas frozen tissue (TTU 43180), Vermont roadkill ear tissue (MZ 11630), and Nebraska rehydrated skin (MVZ 114842). Ladder is 100bp (Invitrogen), with 100-400bp highlighted with brackets.

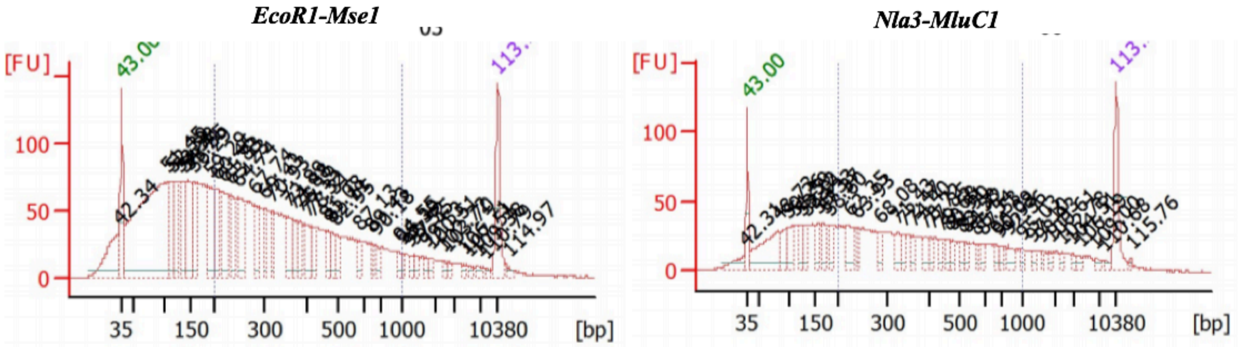

Figure A4.3 Digested sample length histograms for the top-performing restriction enzyme pairs and the rehydrated Nebraska skin (MVZ 114842).

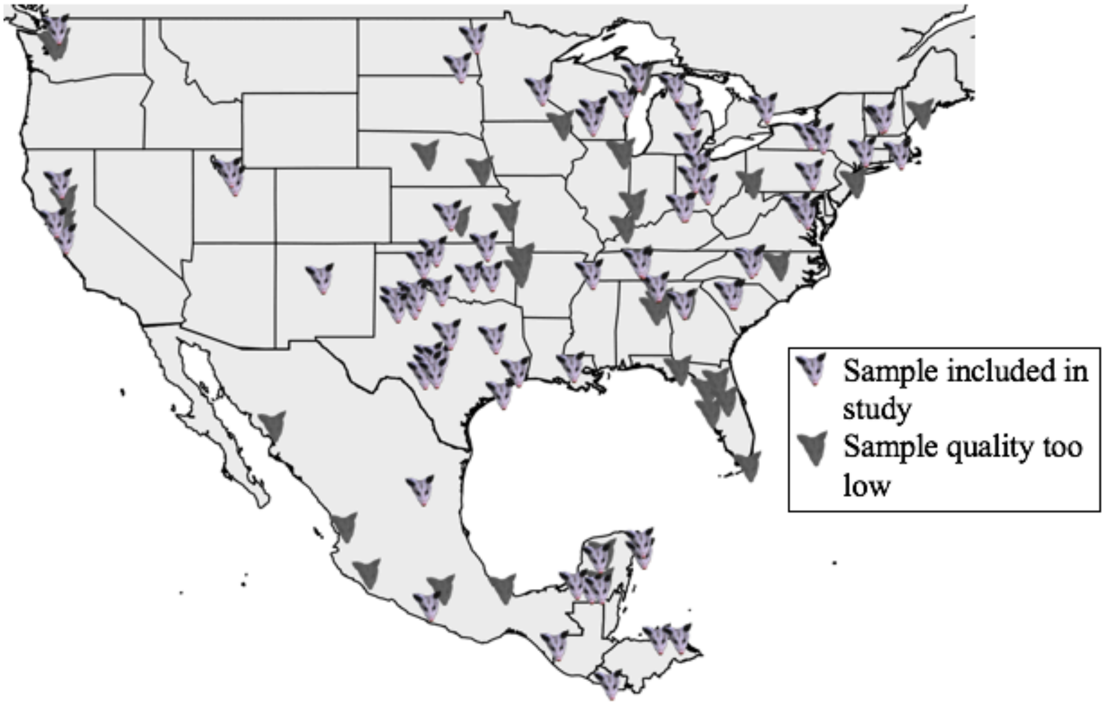

Figure A4.4 Virginia opossum (Didelphis virginiana) samples genotyped using ddRADseq. Gray markers (N = 22) indicate samples that yielded too few SNPs to be analyzed further.

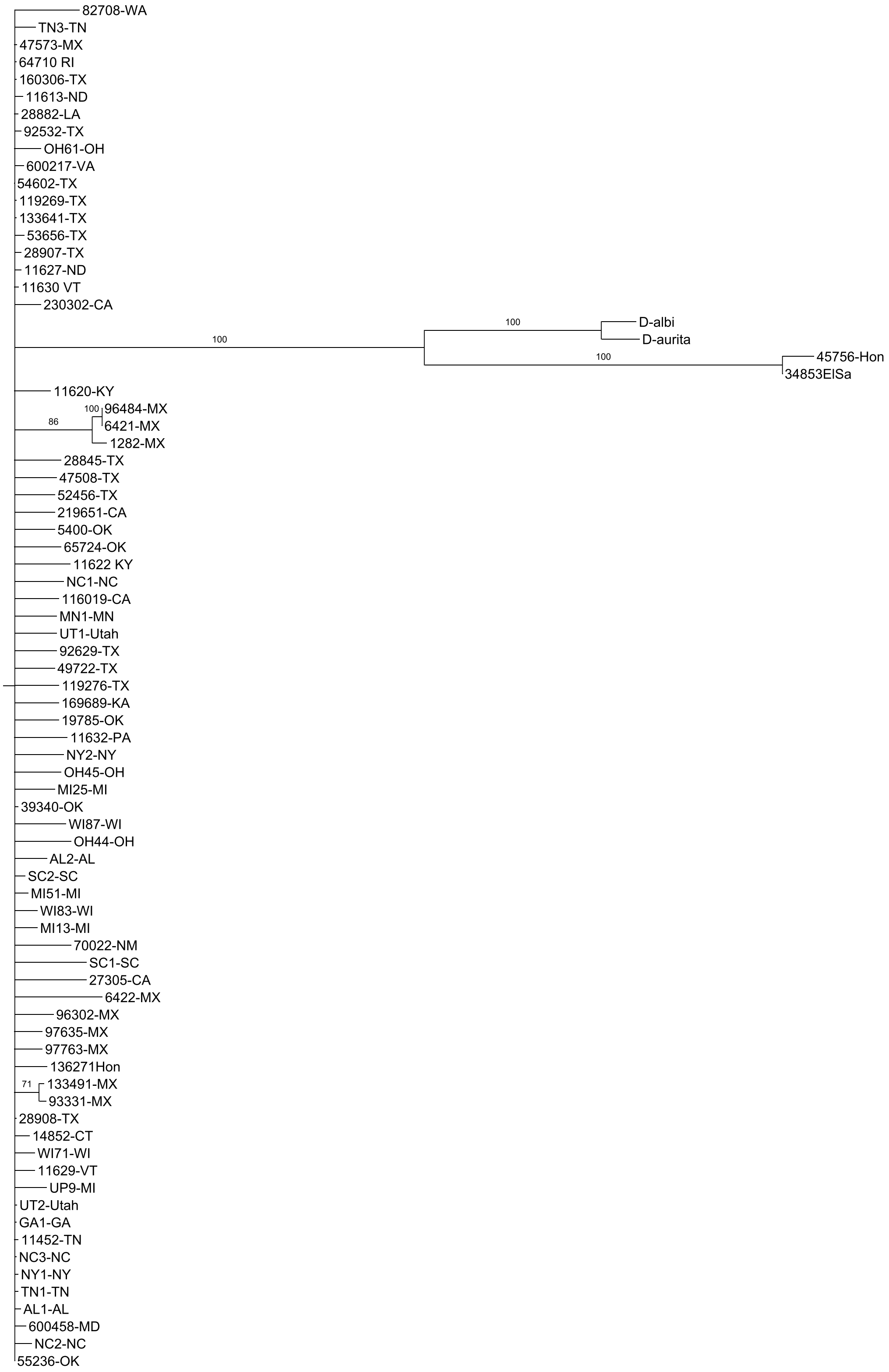

Figure A4.5 Maximum likelihood tree generated for 79 Didelphis samples (see Table A4.2) using 553 ddRADseq SNPs.

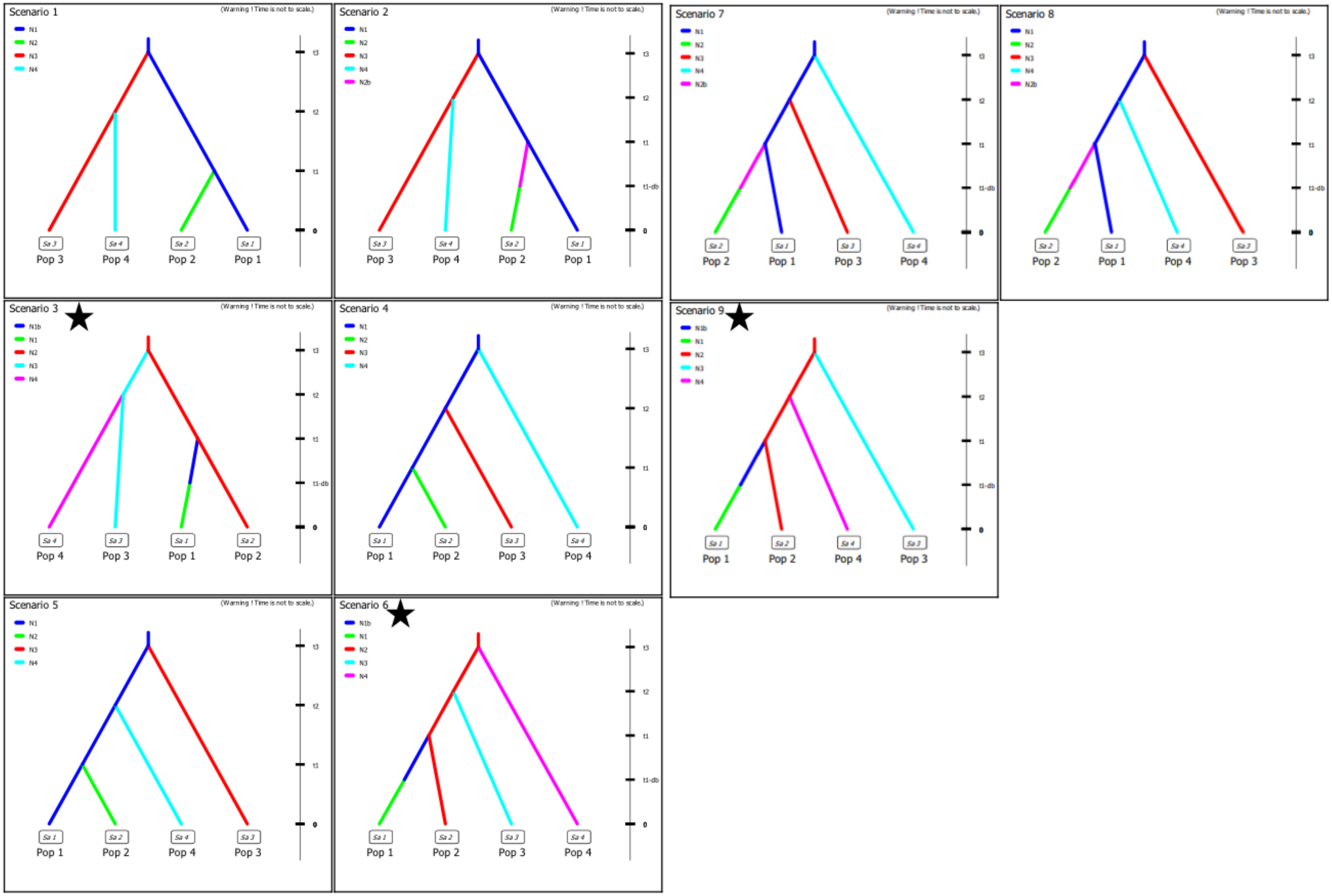

Figure A4.6 Nine migration scenarios with and without one temperate population being derived from another (indicated with color change along a branch). The best-performing scenarios in DIYABC are indicated with a black star.

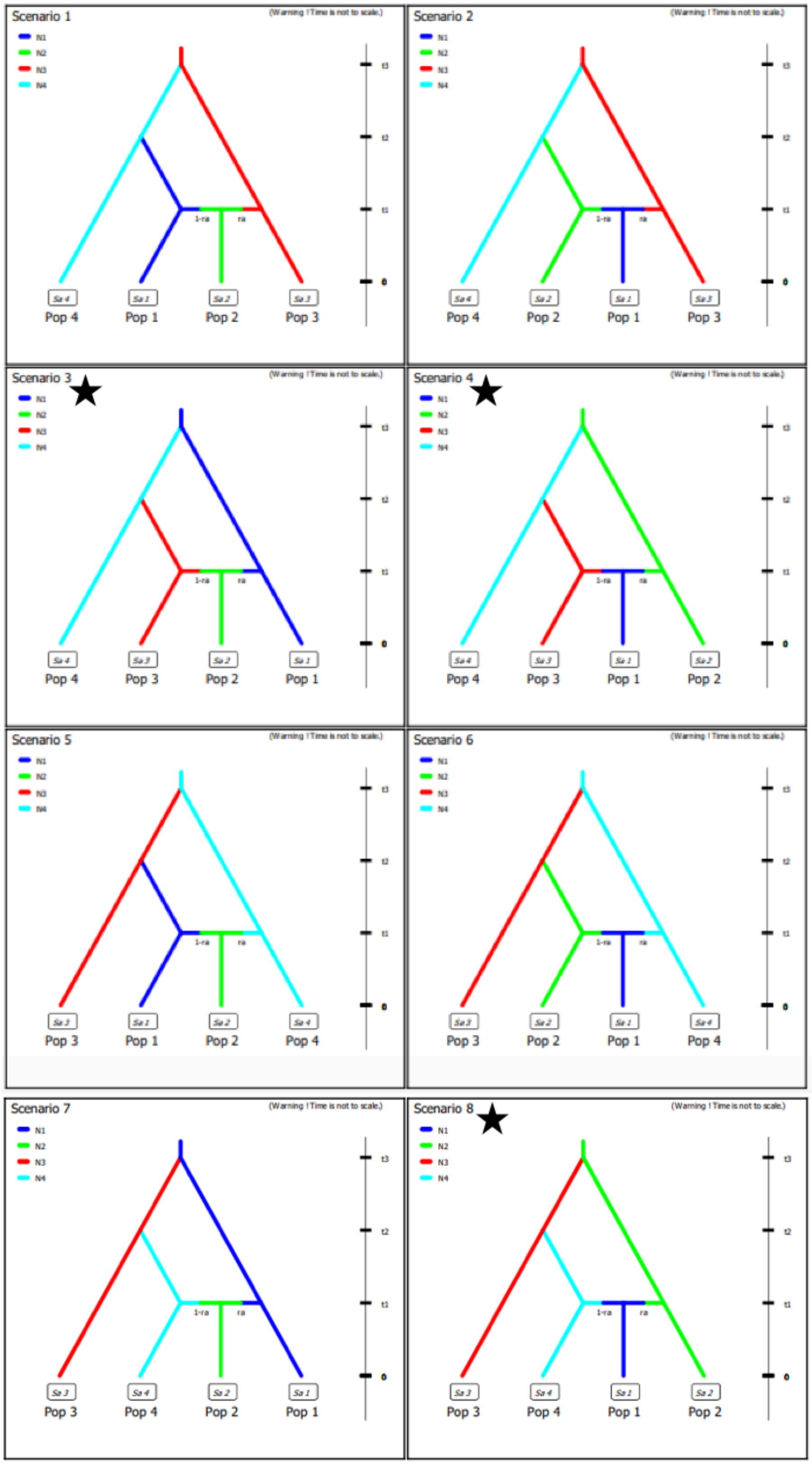

Figure A4.7 Eight admixture scenarios tested in DIYABC. The best-performing scenarios are indicated with a black star.

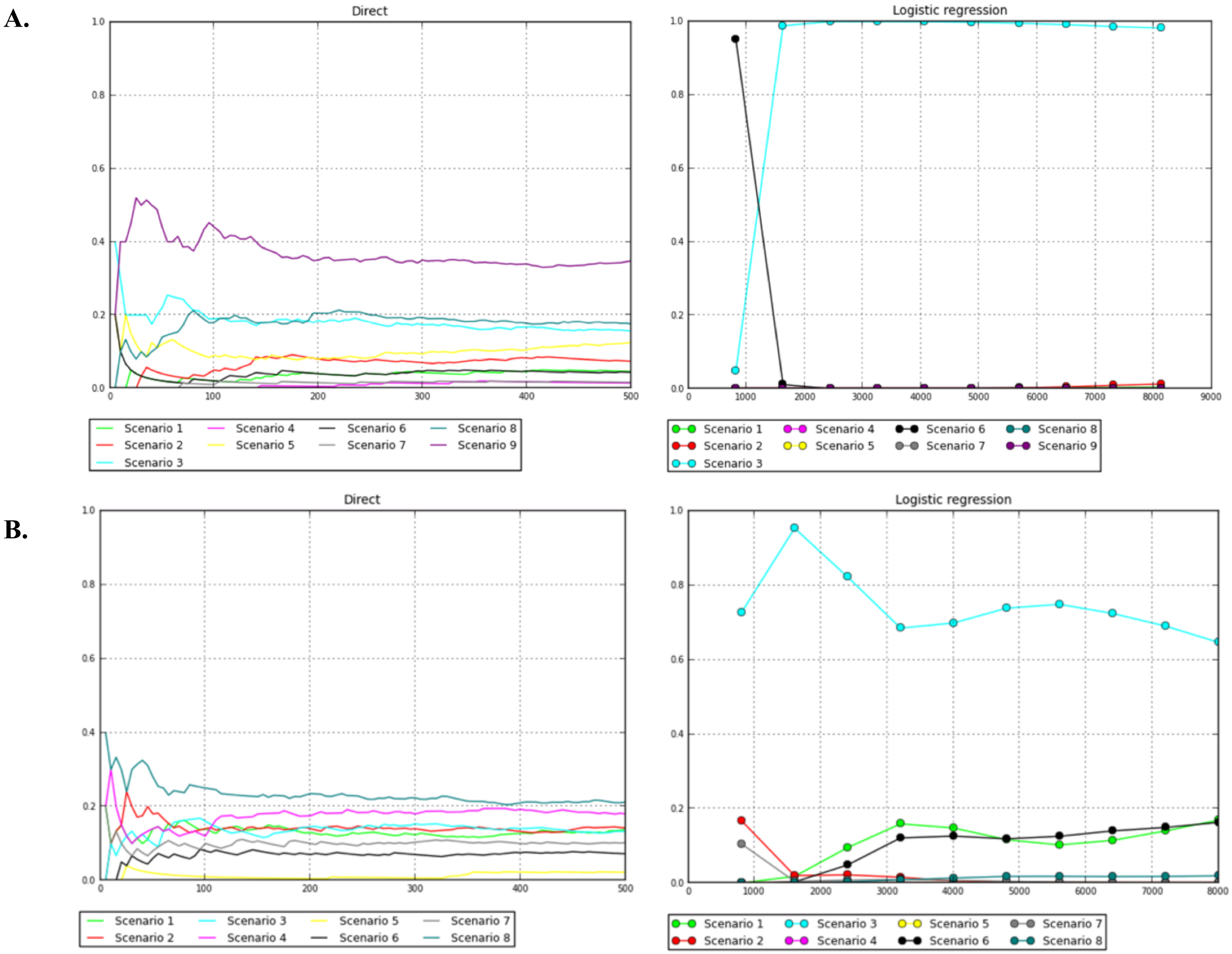

Figure A4.8 Direct and logistic regression plots for A) nine migration scenarios shown in Figure A4.6 and B) eight admixture scenarios shown in Figure A4.7.

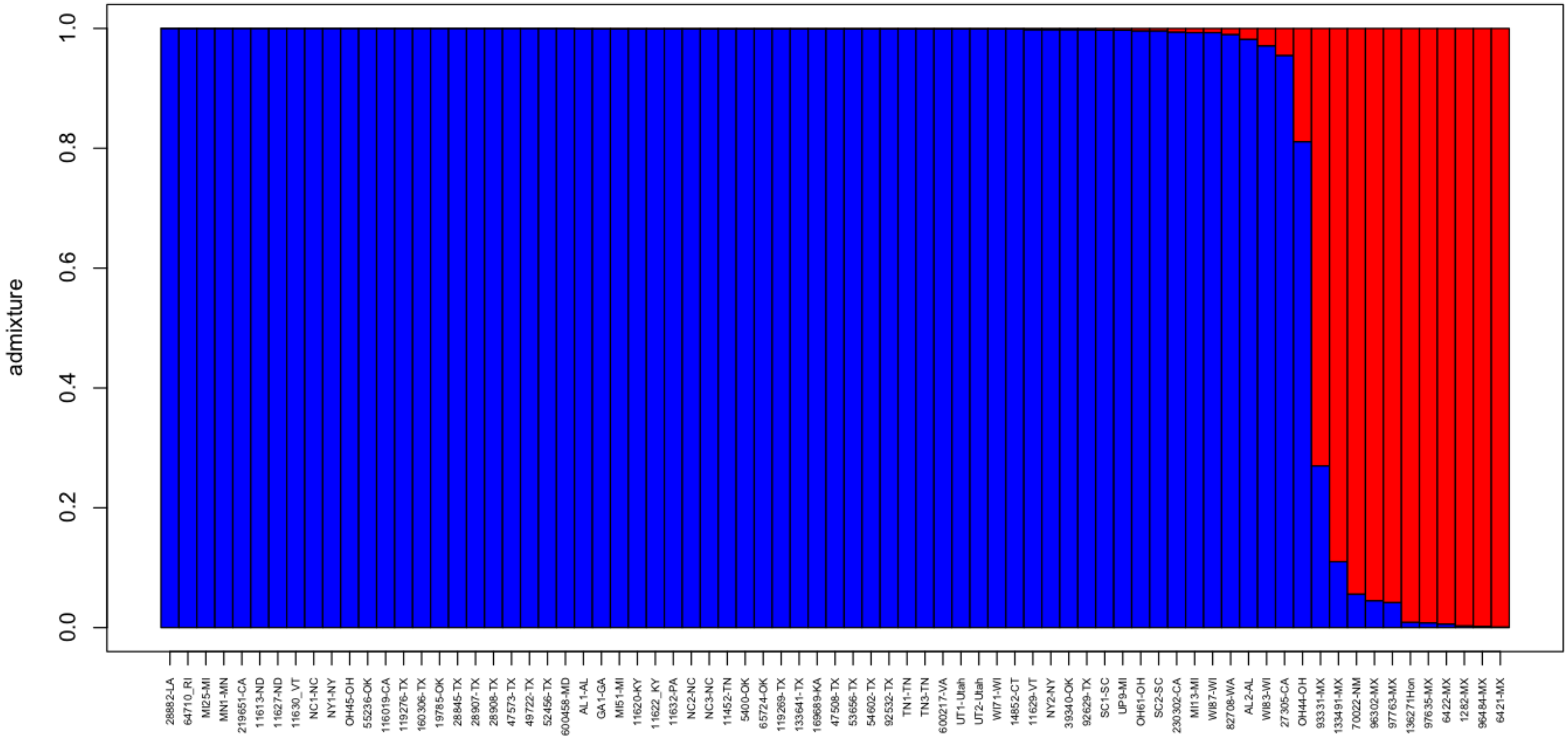

Figure A4.9 Individual cluster assignment bar plots for 75 opossums sampled, with two clusters identified in STRUCTURE. The Y axis indicates the cumulative assignment coefficient (Q) value for each of the clusters per individual. Colors indicate the genetic cluster to which the individual has been assigned: blue is cluster 1 and red is cluster 2.

Conclusion

Although fossil and morphometric work has shown that marsupials are less developmentally constrained than initially presumed (reviewed by Sánchez-Villagra, 2013), the claim that marsupials are less adaptively competitive than placental mammals remains a pervasive concept in mammalogy (e.g. Vaughan et al., 2015). This perspective on marsupials has largely ignored the geographic ranges of roughly a dozen marsupial species now found in North America as a result of the Great American Interchange (reviewed by Voss and Jansa, 2009). Within the broader context of marsupials, the historical northward movement of *D. virginiana* from Central America and its ongoing expansion into Canada and the Midwest are unprecedented success stories of an extant marsupial in the context of mammalian faunas overwhelmingly composed of placental mammals (Amador and Giannini, 2016).

For over two centuries, opossums have been expanding northward into both the Midwest and Northeast (reviewed by Gardner and Sunquist, 2003). With an evolutionary history rooted in the Neotropics, opossums face extreme winter climates at the northern edge of their range and yet have spread further north than thermoregulation models predicted (Gardner and Sunquist, 2003; Kanda, 2005). In this dissertation, I leveraged the success story of the opossum to examine the influences that climate, landscape, and demographic history have on a species distribution. I analyzed stable isotope ratios, genetic markers, and occurrence data to better understand how the opossum expanded its range.

First, I sampled captive and wild opossums to evaluate the evidence that opossums annually or biannually molt using stable isotope analysis. Establishing their molt pattern allowed me to search for a putative adaptation to extreme winter conditions and determine the approximate time captured in opossum guard hair. Second, I evaluated the stable isotopic composition of opossums across their range to measure how their niche varies across space and identify potential climate variables driving these patterns. Third, I used carbon isotope ratios in northern opossums to test the hypothesis that opossums survive at the northern edge of their range by foraging on processed anthropogenic food from trash. Finally, I used genetic markers to examine the relatedness among opossums and evaluate the evidence for one or multiple expansions into temperate North America.

The Virginia opossum has spread north into temperate North America over the centuries, raising questions regarding how opossums are adapting to survive harsh winters. In my first chapter, stable isotope values were used to search for evidence of seasonal molting, one winter adaptation exhibited in mammals (Beltran et al., 2018). The stable isotope values of both captive opossums changed over time, mirroring their documented shifts in diet. In addition, opossums trapped in different seasons did not have significantly different stable isotope values. Evidence for a shift in isotope values in captive opossums and the absence of any difference between winter and summer opossum isotope values suggest that the opossum's seasonal change in pelage composition is minimal, supporting growth and continuous shedding rather than seasonal molting. Virginia opossums do not significantly alter their metabolic rate seasonally, although they are the only didelphid opossum that can retain substantial amounts of body fat (Hsu et al., 1988; Gardner and Sunquist, 2003). Seasonally shedding their hair may be too metabolically expensive for this short-lived species. The results suggest that analysis of stable isotope values from guard hair will not be compromised by sampling opossums collected during various seasons.

Although generalists are becoming increasingly abundant and widespread, little is known about their response to ecological variation they encounter across their range (Prugh et al., 2009). In my second chapter, I used nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) stable isotope values from Virginia opossums collected across their range to quantitatively examine the hypothesis that a generalist's isotopic niche is larger in regions with higher habitat heterogeneity. I found that habitat heterogeneity (calculated using landcover types and diversity index equations) was positively correlated with isotopic niche size. When I compared the range expansion fronts in the Midwestern USA and Northeastern North America, I found the Midwest had a much larger isotopic niche size. These results highlight the ecological role local habitat and habitat heterogeneity may play in facilitating range expansions, especially in generalists. I also used WorldClim variables to evaluate how opossum stable isotope values are impacted by their local environment. $\delta^{15}N$ values were positively correlated with aridity variables, while $\delta^{13}C$ values were positively correlated with climate conditions conducive for C_4 plant abundance. Collectively, my data suggest generalist isotope values reflect their local habitat's isotopic composition. My results reveal that a generalist's stable isotope signature reflects its local environment, demonstrating their flexible diet is captured with stable isotopes and supporting the "generalist habitat heterogeneity hypothesis."

The opossum's ability to consume anthropogenic refuse due to its generalist diet has been referenced to explain their unexpected persistence at the northern edge of their range (e.g. Kanda, 2005; Walsh and Tucker, 2018). In my third chapter, I evaluated this hypothesis using $\delta^{13}C$ values from two geographic expansion fronts. I observed non-significant relationships between latitude or winter harshness and opossum $\delta^{13}C$ values, but variance in $\delta^{13}C$ values increased in Midwestern opossums after 1970. $\delta^{13}C$ values compared across space and time indicate that the opossum's expansion in northern areas is not due to anthropogenic refuse, but bolster evidence for the influence of agricultural development on the opossum's range expansion.

In my fourth chapter, I genotyped opossums across their range to better understand how they spread north out of Mesoamerica and to determine their current genetic structure. Clustering algorithm results and approximate Bayesian computation support the hypothesis that a single ancestral lineage migrated into temperate North America. Two co-occurring clusters in temperate North America suggest a recent divergence event or incomplete lineage sorting due to rapid expansion. The strongly supported genetic structure between Yucatan, Mesoamerican, and temperate North American clusters are congruent with changes in optimal habitat since the last glacial maximum.

Collectively, my chapters underline that opossums spread quickly, even reaching areas (e.g. North Dakota) not predicted habitable for 2050 (Walsh et al., 2017) despite lacking traditional mammalian adaptations to harsh winters such as hibernation and seasonal molting. My results suggest that rather than primarily consuming human trash, opossums are consuming abundant plants and animals in their habitat, which often includes agricultural landscapes. Genetic (Walsh and Tucker, 2018) and isotopic results indicate agriculture plays a beneficial role in supporting expanding opossum populations and influences their foraging habits. My research indicates that opossums should not be linked to trash but considered as generalists that succeed in the face of changing landscapes. The genetic structure identified between Yucatan Peninsula opossums, Mesoamerican opossums, and temperate opossums warrants further evaluation with more samples analyzed from Mexico. Because the opossum's generalist behavior appears to allow them to spread into a wide variety of habitats, it is surprising that only a single ancestral lineage successfully spread into temperate North America. Additional genetic and physiological analysis of opossums from the Yucatan, Mesoamerica, and temperate North America would help me better understand the putative barriers that prevented additional expansion fronts.

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