CHALLENGING THE PARADIGM OF MONARCH MIGRATIONS: BEHAVIORAL COMPLEXITY AND ISOTOPIC VARIATION OF THE EASTERN NORTH AMERICAN POPULATION OF *DANAUS PLEXIPPUS*

By

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To my wonderful family and friends for all their support
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<td>AMOVA</td>
<td>Analysis of molecular variance is a statistical test that evaluates the degree to which genetic variation is explained among and between groups.</td>
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<td>ANOVA</td>
<td>Analysis of variance is a statistical test that evaluates the degree to which variation in data is explained by different factors.</td>
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<td>IBD</td>
<td>Isolation-by-distance describes the distribution of genetic variation where increasing geographic distance is positively correlated with increasing genetic distance.</td>
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<td>PCR</td>
<td>The polymerase chain reaction is a laboratory technique for amplifying many copies of a target segment of DNA.</td>
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<td>VPDB</td>
<td>Vienna Pee-Dee Belemnite is the international standard reference against which carbon stable isotopes are measured.</td>
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<td>VSMOW</td>
<td>Vienna Standard Mean Ocean Water is the international standard reference against which hydrogen stable isotopes are measured.</td>
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CHALLENGING THE PARADIGM OF MONARCH MIGRATIONS: BEHAVIORAL COMPLEXITY AND ISOTOPIC VARIATION OF THE EASTERN NORTH AMERICAN POPULATION OF DANAUS PLEXIPPUS

By

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Movement is one of the key factors that influences evolution. My dissertation uses the unique migration behavior of the monarch butterfly (Danaus plexippus) to investigate the effects of migratory behavior on population structure. Although monarchs are not yet endangered as a species, their migration is facing a variety of threats. Migration may be key to the stability of the species, for example by preserving or redistributing genetic variation. Understanding how movement influences the distribution of genetic variation across an organism’s range is particularly important in conservation genetics. I used an approach that integrated genetic and stable isotope analyses to answer three research questions: 1) To what degree is the resident population in South Florida connected with the main migratory population? 2) Has the summer breeding range expanded northward? 3) Do monarchs from different origins mix in the Mexican overwintering colonies? I found that migrants from throughout the summer range enter the resident South Florida population, but that the degree of connectivity is different for males and females. Males immigrating to South Florida also appear to be in worse nutritional condition than either butterflies originating in the resident population or
migrants to Mexico, but are larger than resident males. My results indicate that monarchs are breeding well north of the published breeding limit, and that monarchs originating north of the putative breeding limit are successfully migrating to the overwintering colonies in Mexico. Finally, genetic variation is homogeneously distributed across the overwintering colonies in Mexico, but there are subtle differences between the overwintering colonies in origins of the butterflies at each colony. Thus, overwintering colonies are not equivalent. Overall, my dissertation shows that monarch movement produces patterns with greater fine-scale complexity than had previously been described. Because of the conservation implications this complexity, future work investigating the processes that produce these patterns is warranted.
Movement is a key behavior that affects evolution. This behavior can be classified into three basic categories: daily movement, dispersal, and migration (Rabb 1979). Because movement drives gene flow in animal populations, each type of movement has different implications for evolvability—the degree to which a species is able to produce the genetic variation that drives the capacity for change (Houle 1992; Houle et al. 1996; Kirschner & Gerhart 1998). Animal movement is particularly important for determining how genetic variation is distributed across a species’ range, since it is one factor governing whether the pool of potential mates comprises the entire species across the whole range, or just a small group clustered in a limited area. My dissertation centers on investigating how migratory movement in particular affects population structure.

**Importance of Movement**

Both daily and dispersal movement describe the behavior of individuals within or between populations, whereas migration describes the behavior of an entire local population. On a daily basis, individuals move to find food, water, and shelter, and to avoid predators. During reproductively active periods, they also search for mates, and reproduction sites. These daily movements determine a range comprising an organism's habitat (Hughes 1979). Both the characteristics of the landscape in which suitable patches of habitat are found and individual variation determine the amount and type of movement by organisms of the same species (Bowler & Benton 2005; Turlure et al. 2011). Daily movement behavior determines the number of potential mates that an animal encounters, and thus defines the demes—local interbreeding groups of
organisms of the same species (Gilmour & Heslop-Harrison 1954)—across a species’ range. In contrast, dispersal and migration behaviors determine gene flow between demes. The degree of movement in a population thus can affect the distribution of genetic variation in a population. As a result of movement, variation may be distributed relatively homogeneously through the population, or it may be partitioned among demes that are differentiated from each other.

The literature on animal movement is filled with various views on how (or whether) to differentiate between dispersal and migration, and the debate on how to define these behaviors has been ongoing for decades. Heape (1931) defined migration as requiring that animals return to the starting location, with one-way movements categorized as dispersal; Williams (1957) held that migration was movement between habitats, regardless of whether this move was temporary or permanent; Taylor and Taylor (1977) argued that dispersal and migration are fundamentally similar responses to adverse conditions; Fahrig and Merriam (1994) viewed dispersal as movement of animals between local populations in different habitat patches. I will be following the definitions used by Dingle (1972). Dingle defined dispersal as movement away from the emergence site, and thus among demes. Dispersal distance is often driven by population density, relative to the carrying capacity of the natal area. Thus, dispersal behavior influences evolvability by determining the amount of gene flow that occurs between demes in each generation. In contrast, Dingle (1972) defined seasonal migration as a life history adaptation to recurring shifts in climate and resource availability that involves the movement an entire population to a refuge site or sites. Migration behavior therefore may bring geographically separate demes into contact,
although the potential for gene flow between those demes depends on how reproductively active periods fit into the migration cycle. In my dissertation, I am focused on the effects of migration and its intersection with the reproductive period on population structure.

For insects in particular, Rabb (1979) describes four major types of sites between which movement occurs: feeding, mating, reproduction, and refuge. Mating sites include the sites of courtship and mating, whereas reproduction sites include the sites of oviposition, as well as larval, pupal, and adult emergence. Refuge sites are either daily or seasonal sites. The types of sites, their number, and spatial distribution vary by species, and sometimes by populations in different geographic areas within a species' range. Daily movement may occur between feeding, mating, oviposition, and daily refuge sites. Dispersal involves movement beyond the daily range. Though dispersal may similarly be between feeding, mating, oviposition, or refuge sites, dispersal should be among populations or demes of a species. Insect migration, on the other hand, generally involves movement to, or from, a refuge site, although in some cases, such as with Mormon cricket populations that migrate from feeding site to feeding site (Srygley & Lorch 2011), it can also involve movement between other types of sites.

**Relationship Between Movement and Population Structure**

Movement patterns between demes within a geographically distributed population affect genetic population structure. Random movement between demes leads to panmixia. In contrast, population structure develops as demes accumulate genetic differences with respect to one another when movement between them is either absent or consistently asymmetric. The null expectation for population structure is a straightforward isolation-by-distance (IBD) pattern (Wright 1943), where the degree of
genetic differentiation between demes is correlated with the geographic distance between them. Barriers to movement, however, may limit gene flow between some demes (reviewed by Manel et al. 2003). Vicariant barriers are geographic features that limit movement between demes; behavioral barriers, such as assortative mating or willingness to move between fragments of habitat patches, can limit movement between demes even in the absence of vicariant barriers. When barriers exist, population structure may show more complex patterns than IBD, such as locally isolated demes, source and sink areas, and metapopulations.

Depending on the degree of connectivity and ecological differences between demes, demes may accumulate differences in both the amount and character of genetic variation. Ecological differences, for example, may favor certain variants over others, so that allele frequencies may differ across demes. Smaller, more isolated demes may also harbor less genetic variation compared to other larger or more highly connected demes. In the most extreme case, when ecological differences are large and connectivity between demes is low, speciation may eventually occur (Darwin 1859). Islands are classic examples of locally isolated demes (MacArthur & Wilson 1967), but landscape features such as mountain ranges, rivers or fragmented habitats may also limit gene flow between demes on the same land mass (Johnson et al. 1992). Source and sink patterns occur when movement consistently occurs from one set of demes into another set, but rarely occurs in the opposite direction (Pulliam 1988). Another way to think about a collection of demes is as a metapopulation. Rather than describing how genetic variation is distributed across a stable collection of demes, a metapopulation consists of a collection of demes across which the population is relatively stable, but the individual
demes experience periodic local extinction and colonization events (reviewed by Hanski 1998). A variety of complex patterns in the distribution can thus develop. There is no clear association between specific complex patterns and particular types of movement, so the relationship between movement behavior and the distribution of genetic variation for a species must be determined empirically.

Both dispersal and migration are means by which gene flow occurs, but the two types of movement have distinct effects on population structure. For example, in the absence of vicariant barriers to dispersal, unidirectional movement away from the natal area leads to clear patterns of IBD that are primarily driven by the physiological ability of an organism to move between patches of habitat (Wright 1943). For seasonal migration, however, entire demes move between geographic areas, so IBD patterns created by dispersal are maintained only when behavior prevents or limits mixing between demes (Wright 1946). Examples of both intermingling (Combreau et al. 2011) and behavioral separation (Irwin et al. 2011) have been found in well-studied migratory bird taxa.

Conservation Genetics

New alleles are added to individual demes in only two ways: mutation or immigration. Mutation rates can be expected to be consistent across all demes, barring environmental influences on mutation rate that vary among demes. The number of new alleles introduced by immigration will be a function of the degree of connectivity between a deme and the rest of the population. Thus, well-connected demes will be more likely to add new variants than more isolated demes. The equilibrium fixation index ($\hat{F} = 1/[1 + 4Nm]$, where $N$ is population (deme) size, and $m$ is the migration rate) is a measure of differentiation, since it indicates the probability that different neutral
alleles will be fixed in two populations (demes). Since $\hat{F}$ approaches zero as the number of migrants approaches 1 migrant per generation ($Nm = 1$), this index illustrates that even a relatively low level of migration keeps demes “well-connected” (Hartl & Clark 2006).

Genetic variation can be removed by non-random mating, natural selection, or genetic drift. Non-random mating occurs when individuals of a population do not have an equal probability of mating with the entire pool of potential mates in the population. This may occur due to assortative mating (either positive—like with like—or negative—like with unlike), or to barriers within the population that prevent access to the complete pool of potential mates. Genetic variation is not necessarily removed by non-random mating, but it reduces the mixing of variants within the population. Both genetic drift and natural selection directly remove genetic variation. Natural selection removes variants by reducing the number of offspring for carriers of deleterious alleles, and increasing the number of offspring for carriers of advantageous alleles. Genetic drift, in contrast to selection, is a neutral process in which alleles are removed from the population by random chance. All three processes are acting at any given time, in any population or deme. Selection and drift tend to operate in opposition, with their relative strengths varying depending on the population size ($N$), the effective population size ($N_e$)—a measure of the size of the population that is successfully contributing genetic variants to future generations (Wright 1938)—and the strength of selection ($s$). Selection dominates when $4N_es >> 1$, whereas drift dominates when $4N_es << 1$ (Kimura 1983). For example, natural selection becomes less efficient as the size of the breeding pool decreases, so this process has a lower effect on isolated demes.
In isolated demes, both genetic drift and non-random mating are key processes by which genetic variants are removed. Because of the interplay between genetic drift, selection, and population size discussed earlier, in an isolated deme, genetic drift is equally likely to remove both detrimental and small-effect beneficial alleles. The net effect is that more isolated demes have a greater probability of less genetic variation, as well as lower quality alleles, than their well-connected counterparts.

Inbreeding does not reduce genetic variation (and thus evolvability) per se, but it does reduce the amount of heterozygosity by increasing the proportion of alleles that are identical by descent (Höglund 2009). As more homozygous individuals appear, inbreeding depression—decreased overall fitness in the deme due to inbreeding—can occur as rare deleterious alleles are expressed more frequently (Bruce 1910), and advantageous heterozygotes appear less frequently (East 1910). Thus, any negative effects of inbreeding accumulate over time as a smaller proportion of heterozygous individuals appear in each new generation.

Small, isolated demes are more susceptible to fixation of mildly deleterious alleles due to genetic drift and weak selection, as well as inbreeding depression (Lynch & Gabriel 1990). These mildly deleterious alleles increase the genetic load of the deme, and, given time, may go to fixation within the deme. Once a deleterious allele is fixed, there is no genetic variation on which selection can operate to give an advantage to a proportion of the deme. Thus, as mildly deleterious alleles fix within a deme, the principle of Muller’s ratchet applies to the fitness of the deme: the maximum fitness possible in that deme is reduced with respect to the population as a whole (Felsenstein 1974; Muller 1963). If a sufficient number of deleterious alleles accumulate within a
deme, these mutations may affect the size of the deme due to the action of natural selection. As the deme shrinks, the effects of inbreeding depression and genetic drift increase, which, in turn, increases the genetic load and the probability of fixing deleterious alleles (Lynch & Gabriel 1990). Lynch and colleagues (1995) termed this cycle “mutational meltdown”, and argued that once this cycle crossed a threshold of genetic load that is correlated with reproductive rate and carrying capacity of the deme, that extinction of the deme was essentially unavoidable. Therefore, if a population as a whole contains a large number of relatively isolated demes, then the population itself may go extinct as a result of processes acting independently on the individual demes.

**Tracking Movement Using Stable Isotopes**

Direct observation of movement is often both labor-intensive and expensive. Alternatively, one may attempt to indirectly observe movement by studies of genetic variation. However, genetic approaches have been criticized by Cockerham and Weir (1993). Although they supported the continued use of approaches that estimate population structure based on genetic differentiation—such as the analysis of $F_{ST}$—their concern was that such measures rely heavily on an underlying migration model. As with all models, there is no way to differentiate between violation of underlying assumptions and real signal. Stable isotope analysis provides an alternative for measuring movement that requires neither an underlying migration model, nor the need to directly observe movement.

Stable isotopes of carbon and hydrogen vary in a predictable pattern across North America (Hobson & Wassenaar 1997; Hobson et al. 1999). In contrast to radioactive isotopes, stable isotopes are variants of an element that do not degrade over time, but which vary in atomic weight. In the carbon and hydrogen isotope regimes
used to study animal movements, the lighter isotopes (H and $^{12}\text{C}$) are far more common than the heavy isotopes (Deuterium (D) has an abundance of 0.015% and $^{13}\text{C}$ has an abundance of 1.11%) (Sharp 2007). Because the absolute quantity of rare isotopes is so low, using a ratio rather than an absolute quantity increases the precision of the measurement (Sharp 2007).

The stable isotope profile of a sample is measured as the relative difference in the ratio of heavy to light isotopes in the sample compared to the ratios of these isotopes in an international standard, e.g., for hydrogen, $\delta D = \frac{(D_{\text{sample}}/H_{\text{sample}} - D_{\text{standard}}/H_{\text{standard}})}{(D_{\text{standard}}/H_{\text{standard}})} \times 1000$. Hydrogen isotopes are measured relative to ocean water (Vienna Standard Mean Ocean Water, VSMOW); carbon isotopes are measured relative to a mineral standard (Vienna PeeDee Belemnite, VPDB) (Coplen 1994). The isotope ratios found in these international standards are used as the zero point. Samples with ratios of heavy to light isotopes that are greater than the standard will have positive delta values (i.e., are enriched for the heavy isotope), while samples with ratios that are less than the standard will have negative delta values (i.e., are depleted for the heavy isotope).

The geographic pattern of hydrogen ratios (Figure 1-1) varies based on precipitation (meteoric water), although temperature, altitude, latitude, position on the continent, and amount of rainfall all influence the ratio of deuterium to hydrogen (Sharp 2007). Because there is a seasonal effect on hydrogen stable isotope ratios, focusing on the ratios generated during the growing season is preferable when using hydrogen isotopes to study animal movements (Meehan et al. 2004).
There is also predictable variation in carbon isotopes based on geographic region, but the geographic patterns vary by type of plant (Sharp 2007). Plants fractionate, here meaning preferentially incorporate \(^{12}\)C, during photosynthesis. The degree of fractionation varies by the type of photosynthesis (C\(_3\), C\(_4\) or CAM) and by the amount of carbon dioxide in the surrounding air. Hobson and colleagues (1999) demonstrated that the carbon ratios of milkweed plants vary in a geographically predictable manner (Figure 1-1) across eastern North America.

Due to their associations with geography, stable isotopes of hydrogen and carbon have been used since the late 1990s to investigate migratory behavior (e.g., Hobson 1999; Hobson & Wassenaar 1997; Pinnegar & Polunin 2000; Rubenstein & Hobson 2004). By selecting tissues with little to no turnover, such as bones, wing membranes, and feathers, analysis of stable isotopes are useful intrinsic markers for tracking migration without the need to capture the same organism at both ends of the migration path. In contrast, mark-recapture methods, which have been extensively used to study migration, require capturing the same individual twice. Accordingly, mark-recapture can be limited by recovery of only a small fraction of the marked organisms (Lewis et al. 1997). Studies in a variety of taxa have combined genetic analyses with stable isotope analyses to address diverse issues, including the flight patterns of seabirds (Góméz-Díaz & González-Solis 2007), recent connectivity patterns in endangered fish (Cook et al. 2007), and trophic partitioning by different pods of killer whales (Foote et al. 2009).

**Study System: Monarch Butterflies**

Monarch butterflies (Danaus plexippus) present a unique system in which to study the effects of seasonal migration on population structure. The phenomenon of the
annual monarch butterfly migration in North America has attracted scientific interest since the mid-1800s. Despite this long history of scientific inquiry, however, there is still a great deal that is unknown about how these migration movements affect population structure in monarchs (Urquhart & Urquhart 1977; Wassenaar & Hobson 1998).

While the monarch genus *Danaus* most likely split from its sister *Tirumala* in the late Pliocene (~4.9 million years ago), *D. plexippus* is believed to have diverged from its closest relative, the queen butterfly (*D. gilippus*), as recently as 40,000 years ago (Lushai et al. 2003). Native populations of monarchs are found throughout the Americas and the Caribbean. Major introduced populations are found in Hawaii (Stimson & Kasuya 2000; Stimson & Meyers 1984), Australia (Hughes & Zalucki 1984; Zalucki & Suzuki 1987) and New Zealand (Pawson & Berndt 2004), with small, introduced populations also found scattered on a number of Pacific (Zalucki & Clarke 2004) and Atlantic islands (Hilburn 1989; Neves et al. 2001; Strecker & Wilkens 2000).

The monarch life cycle involves five larval instars, pupation, and an adult phase. While highly temperature-dependent, monarchs in the wild generally live six to eight weeks (Urquhart 1987). Monarchs eclosing in the fall months, however, emerge from pupation and enter reproductive diapause for migration to overwintering colony sites. The generally accepted paradigm is that monarchs in the eastern United States and southern Canada roughly follow the major flyway routes shown in Figure 1-2 (US Forest Service 2008) to overwinter at a small cluster of colonies in central Mexico (García-Serrano et al. 2004; Slayback et al. 2007; Urquhart & Urquhart 1977; Williams et al. 2007), while monarchs west of the Rocky Mountains migrate to a series of small colonies scattered along the California coast (Dingle et al. 2005; Leong 1990; Leong et
al. 2004). These migrants remain in diapause for the duration of the winter until exiting diapause in the early spring. Monarchs then mate within the overwintering colonies, and begin the northward migration before laying eggs in northern Mexico or southern Texas to complete the life cycle. Thus, the overwintering generation may live nearly ten times longer than its spring and summer counterparts: six to eight months, rather than six to eight weeks.

Monarch mating has often been described as a unique breeding system involving forced copulation by males, rather than female choice (Brower et al. 2007; Pliske 1975; Rothschild 1978). Males are ardent, and will attempt copulation with any butterfly—including other males—that they are able to take down (Hill et al. 1976; Oberhauser & Frey 1999). Other butterflies of the Danainae subfamily use elaborate courtship rituals that involve pheromones produced by alar glands on the hindwings of male butterflies and distributed by hairpencil structures that can be everted from the abdomen (Boppré 1993). These structures, however, are reduced in Danaus plexippus compared with other Danainae, which some researchers have used as evidence that males do not court females (Boppré 1993; Pliske 1975). Although mating behavior has been studied in the overwintering colonies of both Mexico and California, and in captive conditions, less is known about mating behavior throughout the summering range.

Both Frey (1999) and Solensky (2004), however, demonstrated that females exert a degree of choice in the mating process by manipulating the position of their abdomen to control male mating success. Females have been shown to vary the degree of these resistance behaviors based on male size (Van Hook 1993), wing
coloration (Davis et al. 2007), and previous mating (Frey 1999), suggesting that female choice does indeed exist in monarchs.

Monarchs tightly cluster into colonies that cover a mere 75 km$^2$ of high-altitude oyamel fir forest scattered across multiple peaks of the Transvolcanic Belt in central Mexico (Slayback et al. 2007). Most estimates indicate that over 10 million monarchs congregate at each colony in this small area (Brower et al. 1977; Calvert & Brower 1986; Slayback & Brower 2007). Overwintering survival is tightly coupled with the specific microclimate found at each colony site (Bojorquez-Tapia et al. 2003). Key characteristics for successful overwintering include temperature, tree density, degree of canopy cover, and composition of understory vegetation (Alonso-Mejia et al. 1992; Alonso-Mejia et al. 1993; Alonso-Mejia et al. 1998); these parameters vary at different colony sites, often due to differences in human activity (Ramirez et al. 2003). The forest characteristics are particularly important to protect overwintering monarchs from either freezing or desiccating (Bojorquez-Taipa et al. 2003). Because these characteristics vary by site, some sites are better suited to overwintering survival than others. Although the mechanisms driving monarchs to choose particular sites are unknown, colonies consistently form at major sites, even when the forest characteristics are no longer optimal due to human activities, such as logging (Garcia-Serrano et al. 2004; Williams et al. 2007). It is unclear whether monarchs select colonies at random with respect to their natal origins. If monarchs do choose colonies non-randomly with respect to geographic origin, then monarchs originating from areas associated with less desirable colony sites may be less likely to survive the overwintering season, potentially leading to non-random loss of particular summer demes. If monarchs assort into colonies
randomly with respect to origin, however, there is less chance of deterministic loss of
demes based on selection of suboptimal habitat, and an opportunity for gene flow
between different summer breeding areas or flyways. Random mating across demes
within the colonies has the same effect as migration with respect to increasing effective
population size and hence action of selection relative to drift, thereby shielding
monarchs from Muller’s ratchet or mutational meltdowns (Lynch et al. 1995) by
increasing the amount of purifying selection.

Since monarchs migrating northward in the spring are reproductively active, and
thus live only six-eight weeks, multiple generations are required to repopulate the entire
range into southern Canada. Monarchs are therefore mating throughout the northward
migration. As successive generations move northward, the migrating population fans
out across North America east of the Rockies, so butterflies following different
trajectories become increasingly geographically separated. Since four to five
generations may occur between overwintering generations, this geographic separation
could lead to an IBD pattern that is positively associated with increasing latitude.
Although there have been major efforts to identify and track monarch migration through
mass-mark-recapture and observational studies (Brindza et al. 2008; Garland & Davis
2002; Howard & Davis 2004; Urquhart & Urquhart 1978; Urquhart & Urquhart 1979a, b),
few studies have explicitly investigated how fine-scale movement behavior affects
population structure. One exception is the work of Eanes and Koehn (1978), who used
allozymes to calculate F-statistics for sites across the summering range. Contrary to
expectations, their data support panmixia even in the summer populations rather than
population structure (i.e., of IBD). However, as discussed in the discussion of
conservation genetics above, even occasional bouts of random mating across demes, such as are believed to be happening in Mexico, would be expected to obscure, or even eliminate, any genetic signals of population differentiation.

If population structure is established along the migratory routes during the spring and summer breeding season, it will only persist if there is some relationship between summer breeding sites, migratory routes, and the overwintering colonies. Monarchs generally follow the migratory flyways shown in Figure 1-2. The flyways indicate the general direction of monarch butterfly flight, rather than specific routes followed by migrating butterflies, but they do highlight two major barriers that tend to separate summer breeding populations: the Great Lakes and the Appalachian Mountains. Miller and colleagues (2011) demonstrated that the Appalachians form a porous barrier, as southward migrating monarchs can cross them. However, the degree to which monarchs breed across the Appalachians during the summer remains unknown. Thus, summer breeding sites seem to roughly correspond to migratory flyways. There is mixed evidence, however, regarding consistent associations between migratory routes and overwintering colony sites. Based on mass-mark-recapture data, Urquhart and Urquhart (1978) suggested that western colonies were populated from summering grounds in the Great Plains, whereas central and eastern colonies were populated from summering grounds roughly east of the Great Lakes. In contrast, studies using allozymes (Eanes 1979; Eanes & Koehn 1978) and stable isotope analyses (Hobson et al. 1999; Wassenaar & Hobson 1998) found no evidence for a clear relationship between breeding grounds and overwintering colony sites. This project therefore seeks to clarify the relationship between spring and summer breeding grounds and
overwintering colony sites by integrating stable isotope and genetic data for specimens collected across the full summer range and within colonies from throughout the overwintering range in Mexico.

In addition, non-migratory populations are scattered across the southern United States, but neither the long-term stability of these populations, nor their connectivity with the main migratory population, are known. Although poorly studied, there have been regular reports of continuously breeding, non-migratory monarch populations scattered throughout the southern United States (Brower 1961, 1985; Cohen 1983; Funk 1968; Urquhart 1960). By using a combination of observations, morphometric measures, mark-recapture efforts, and cardenolide fingerprinting (using chemical composition to analyze milkweed species), Knight (1998) characterized the southern Florida population and its relationship to the main migratory population. She found breeding monarchs throughout the year, with recapture data indicating a fairly stable number of monarchs at the two study sites, although insufficient data were collected to accurately estimate total population size. By using cardenolide fingerprinting to identify the milkweed species on which a captured monarch was a larva, Knight also found no evidence of a spring influx of monarchs returning to North America from overwintering sites in the Caribbean or Central America, but did find that a sizeable number of southward migrants enter south Florida in the fall. Dockx and colleagues (2004) also found that some of these southward migrants continued down the Florida peninsula to cross over to Cuba, but replicated Knight’s findings that monarchs did not return north once they had entered a resident population.
Research Questions and Approach

Even the most rapidly evolving genetic markers fail to detect most movement events occurring within a generation, but stable isotope analyses can detect such patterns. By using both genetic markers and stable isotopes, the movement patterns of monarch butterflies both within and across generations can be better evaluated than by using either approach alone. This integrated approach can be applied to study the relationship between migratory routes and overwintering colonies, as well as the relationship between the migratory population and the resident population in south Florida. A proof-of-concept study showed that stable isotope techniques that had been successfully used to track migratory birds were equally successful in tracking migratory monarch butterflies (Hobson et al. 1999). Stable isotope analyses of monarchs have been used to determine monarch origins (Wassenaar & Hobson 1998, Miller et al. 2011), but they have not been combined with genetic analyses to investigate pattern differences both within and across generations.

My dissertation builds on the broad patterns that have been identified by others over the past decades to how well the fine-scale patterns that monarch movement behavior has generated fit with the generally accepted paradigm of monarch migration. Chapter 2 focuses on the resident population in South Florida. This chapter addresses the key question of how and to what degree is this non-migratory population connected with the main migratory population in eastern North America. Chapter 3 examines movement patterns along the northeastern edge of the monarch summer range in Canada, and evaluates the degree to which range expansion may be occurring. Chapter 4 looks at the distribution patterns of genetic variation that occur at the Mexican overwintering colonies. Finally, Chapter 5 synthesizes my results to fill in some of the
gaps in our knowledge of fine-scale patterns of monarch movement behavior, and outlines future research directions suggested by my findings.

Figure 1-1. Heavy isotope depletion gradients for carbon and hydrogen in monarch wing tissue. Arrows point in the direction of increasing depletion of heavy isotopes ($^{13}$C and D). Orange = carbon, blue = hydrogen. Based on data from Wassenaar and Hobson (1998); hydrogen values modified according to Meehan and colleagues (2004).
Figure 1-2. Major migratory flyways of the fall monarch butterfly migration in eastern North America. Light orange arrows indicate the primary routes taken from summering locations throughout Canada and the United States to the overwintering colonies in central Mexico. Width of the arrows is roughly correlated with number of monarchs migrating along a flyway. Gold arrow indicates hypothesized flyway connecting migratory population with resident South Florida population. Monarchs migrate in a general south or southwest orientation throughout the eastern range, so migrating monarchs are also found in areas not directly covered by the arrows. Red diamonds indicate sampling locations. Due to size constraints of the map, the location of the overwintering colonies is shown northeast of its actual location in central Mexico. Based on the United States Forest Service (2008).
CHAPTER 2
SOUTH FLORIDA RESIDENT POPULATION

Alternative life history strategies are found in a wide variety of taxa, including
birds (Grönroos et al. 2012), butterflies (Velde & Van Dyck 2013), crickets (Judge et al.
2008), frogs (Leary et al. 2005), grasshoppers (Greenfield & Shelly 1985), horseshoe
crabs (Smith et al. 2013), lizards (Hews et al. 1994), and salmon (Paez et al. 2011).
While the details between strategies vary as widely as the taxa in which they are
observed, a key component in all cases is a trade-off, often between an optimal strategy
that requires “good” conditions, and alternate strategy that provides a fitness advantage
under “bad” conditions. The relevant conditions may be environmental, physiological or
a combination of both. Selection of a strategy therefore involves one or more
environmental or physiological thresholds that lead to an individual following a particular
strategy. For example, Velde and Van Dyck (2013) found clear trade-offs in the
alternative strategies employed by male speckled wood butterflies (Pararge aegeria) to
locate mates. Territorial males perch in a sunlight patch that is defended from other
males; non-territorial males fly throughout a large area of forest to search for mates.
The territorial strategy involves rapid launch and accelerate flight that is energetically
costly, whereas the non-territorial strategy relies on gliding flight that requires much less
energy. In addition, food resources in a given territory are limited. Velde and Van Dyck
demonstrated that only males that eclosed with sufficient lipid reserves would follow the
territorial strategy, and males with insufficient lipid reserves would not follow this
strategy, even when a suitable territory was available. Thus, to understand the
evolutionary implications of the alternative life history strategies in a particular species, it
is necessary to understand both the trade-offs and the triggers involved.
Monarch butterflies (Danaus plexippus) employ two contrasting life history strategies: migratory or resident. Individuals following the migratory strategy are found in the temperate zone. There are several generations through the spring and summer seasons. Although the average life span varies somewhat with temperature, butterflies in these generations usually have 6-8 week life spans. Monarchs that emerge in the late summer or fall, however, enter reproductive diapause immediately after emergence, then undertake the migration to the overwintering grounds. In the spring, these butterflies exit diapause and breed before beginning the journey north. Thus, the overwintering generation of monarchs have life spans nearly ten times as long as their spring and summer counterparts. In the eastern United States and southern Canada, these butterflies migrate during the fall along the major routes shown in Figure 2-1 to overwinter at a small cluster of colonies in central Mexico, while monarchs west of the Rocky Mountains migrate to a series of small colonies scattered along the California coast (Dingle et al. 2005; Leong 1990; Leong et al. 1995).

Individuals following a resident strategy, however, neither enter diapause nor migrate, and are reproductively active throughout the year. These individuals also have short life spans throughout the year, similar the spring and summer animals from the migratory populations. Monarchs following the resident strategy are found in isolated colonies scattered across the southern United States (Dockx 2007; Knight & Brower 2009; Pyle 1999; Urquhart & Urquhart 1977). Decreasing photoperiod and lower temperature are environmental cues that trigger monarch diapause (Goehring & Oberhauser 2002), but the changes in photoperiod and temperature at sub-tropical and tropical latitudes are insufficient to trigger diapause. Thus, monarchs eclosing in a
resident population in sub-tropical or tropical latitudes are unlikely to enter diapause, and are therefore be unlikely to switch to a migratory strategy. However, it is possible that some monarchs eclosing into the main migratory population could enter resident populations and switch to a life history strategy that does not involve reproductive diapause.

I hypothesized that natal location would be a key determinant of life history strategy: butterflies eclosing in a resident population would follow a resident strategy, while butterflies eclosing elsewhere would follow a migratory strategy. However, it is possible that migrant animals of both sexes could join resident populations (immigration), or that northbound migratory females might mate with males from a resident population, and continue their migration (gene flow). Even for a well-studied resident population in South Florida (e.g., Brower 1961; Urquhart and Urquhart 1979; Knight 1998; Dockx 2002), the degree of connectivity with the migratory population is unknown. External factors such as displacement via storms could bring migrating monarchs into conditions that would cause them to drop out of diapause and start mating with resident animals or with one another. However, if internal factors such as nutritional condition are involved in determining migratory destination, then the trigger to switch to an alternate destination most likely occurs prior to or during migration toward the overwintering colonies. Therefore, I further hypothesized that this trigger would be condition-dependent.

Methods

I used stable isotope profiles to infer immigration from the main migratory population into the resident population in South Florida. Stable isotope ratios vary predictably with location, so these ratios can serve as intrinsic markers for tracing
animal movement behavior (Hobson 1999). The carbon and hydrogen isotope ratios found in monarch wings are determined by the origin of the host plants on which animals fed as caterpillars. The keratin proteins that make up the wing are stable following metamorphosis, so measuring the stable isotope ratios of wing tissue provides an estimate of the natal location of the butterfly (Wassenaar & Hobson 1998).

**Specimen Collection**

Specimens were collected from two sites in South Florida (18 specimens). For wing size comparison, specimens were collected from five overwintering colonies in Mexico (181 specimens). Live specimens were captured in South Florida, immediately placed in glassine envelopes, and stored at -20°C for at least 12 hours prior to measurement and dissection. In Mexico, specimens were collected from the pool of dead butterflies littering the forest floor at overwintering colony sites, and sorted into glassine envelopes at the end of the day. Details regarding collection sites and specimens are shown in Table 2-1. Sex was determined by presence or absence of the androconium on the dorsal side of the hindwing before separating wings from bodies, and wings were returned to the glassine envelopes for later stable isotope analysis. Bodies were preserved for later DNA extraction in 2.0 mL tubes filled with 95% ethanol. Both wings and bodies were stored at 4°C after specimens were brought to the lab.

**Natal Origin Analysis**

One hindwing from each South Florida specimen was washed in a 2:1 chloroform:methanol solution to remove surface impurities, lipids, and residual hemolymph, then air dried. Small sections of hindwing (0.350 ± 0.02 mg for hydrogen and 0.825 ± 0.05 mg for carbon) were cut and placed into capsules (3x5mm silver capsules for hydrogen and 5x9mm tin capsules for carbon) for stable isotope analysis.
To reduce small fluctuations in stable isotope profiles due to varying degrees of wing pigmentation, sections were consistently excised from the black portion of the wing margin (see Appendix). Carbon and nitrogen isotope profiles were measured at the Light Stable Isotope Mass Spec Lab at the University of Florida, and hydrogen isotope profiles were measured at the Colorado Plateau Stable Isotope Laboratory at Northern Arizona University. Approximate natal origins were assigned based on the combined isotope profiles roughly following the approach described by Wassenaar and Hobson (1998), except that the Online Isotopes in Precipitation Calculator (OIPC) plug-in for Google Earth (Bowen & Revenaugh 2003; Bowen et al. 2005; Bowen & Wilkinson 2002) was used to evaluate δD values for precipitation during the growing season, following the recommendations of Meehan and colleagues (2004).

**Wing Size Analysis**

Forewing size was measured as a proxy for body size. Width was measured from the white spot adjacent to the body to the point between the two white spots at the tip of the forewing (Figure 2-2). Although there were slight variations in wing patterns, these two markers were consistently used to ensure that homologous sections of the wing were measured. Measurements were recorded to the nearest millimeter. Differences in wing size by sex and origin were analyzed in R (R Development Core Team 2008). Because sample sizes were small (3 residents and 8 immigrants), I tested the difference in size between resident and immigrant males by permuting the variable “origin” five hundred times and observing the distribution of F-statistics from the one-way ANOVA of each permuted dataset. The observed value was found less than 5% of the time (Figure 2-3).
Larval Condition Analysis

The ratio of $^{15}$N relative to $^{14}$N indicates whether or not an animal is nutritionally stressed, and this ratio has been shown to hold across a wide range of taxa including insects (McCue & Pollock 2008). Unlike hydrogen and carbon, whose profile varies with geography, nitrogen varies according to the feeding status of the individual. Animals enduring starvation are enriched for $^{15}$N relative to $^{14}$N, such that the higher the relative amount of $^{15}$N, the worse the animal’s condition (Gannes et al. 1998). Feeding experiments have not been conducted on monarch butterflies; however, experiments in another Lepidopteran, the Japanese oak silkworm (Antheraea yamamai), are consistent with enrichment of $^{15}$N as a signal of response to larval starvation (Mizota & Yamanaka 2011). Tibbets and colleagues (2008) also found a similar pattern of enrichment pattern between larvae and adults in insects that undergo complete metamorphosis. Because the keratin proteins that comprise wing tissue are fixed after metamorphosis, I assessed larval condition using the ratio of nitrogen isotopes in adult wings as a proxy for larval condition. Data were analyzed using R (R Development Core Team 2008). As for wing size, sample sizes were small and thus I tested the difference in $\delta^{15}$N between resident and immigrant males by permuting the variable “origin” five hundred times and observing the distribution of $F$-statistics from the one-way ANOVA of each permuted dataset. The observed value was found approximately 10% of the time (Figure 2-4).

Results

Carbon and hydrogen stable isotope profiles for specimens collected in South Florida are presented in Table 2-2. These profiles indicate that eight specimens had a natal origin outside of Florida (Figure 2-5). Although both male and female specimens had natal origins in Florida, there was a significant excess of males originating outside
of Florida (Barnard’s exact test, two-sided: \( T = -2.063, N = 18, P = 0.047 \), see Table 2-3). Based on natal origin, migrant monarchs that entered the South Florida population originated from throughout the North American breeding territory, with each of the major migratory flyway routes roughly equally represented. No differences between the two South Florida collection sites were found, and the two sites were treated as a single population.

Migrant male monarchs that entered the South Florida population were larger than resident males (Figure 2-6; \( P = 0.022 \), see Methods for details). However, the size of migrant males collected in South Florida was not significantly different from migrant males collected in Mexico (ANOVA: \( F_{1,96} = 1.28, P = 0.26 \)). Migrant females collected in Mexico were also significantly larger than resident females from South Florida (ANOVA: \( F_{1,96} = 44.2, P < 0.0001 \)). There was no sexual dimorphism for body size in resident monarchs (Figure 2-6).

Based on the \( \delta^{15}N \) ratio, the immigrant males tended to be in worse condition than the resident males (Figure 2-7), though the difference was not statistically significant (\( P = 0.106 \), see Methods for details). In addition, immigrant males collected in Florida were in worse condition on average than males collected from Mexico (Figure 2-8; ANOVA, \( F_{1,76} = 6.76, P = 0.011 \)).

Discussion

My results suggest that migratory monarchs regularly enter the small, non-migratory South Florida population. It is easy to understand how monarchs following the Atlantic flyway could end up in South Florida simply by continuing to follow the Atlantic coast, rather than heading west across Georgia or the Florida peninsula to continue along the Gulf coast. However, my stable isotope data suggest that monarchs in South
Florida originated from locations throughout the summer range of the migratory population. All three proposed major migratory routes contribute butterflies to the South Florida population (Figure 2-9). Monarchs may arrive in South Florida by random processes such as displacement by weather, which serves as a null hypothesis against which to test other explanations, or they may pursue an alternate migration strategy that leads to their presence in South Florida.

The generally accepted paradigm is that monarchs emerging in the fall immediately enter reproductive diapause and remain in this state until the early spring (Urquhart 1960). A combination of temperature, day length, and light angle cues are believed to trigger both entry into and exit from diapause (Malcolm et al. 1987; Goehring & Oberhauser 2002; Reppert 2006). None of the known resident populations, including South Florida, has the appropriate conditions to trigger diapause. In fact, these same key environmental cues should trigger any diapausing animals entering a resident population to become reproductively active. For example, Knight and Brower (2009) demonstrated that breeding occurs in the resident population in South Florida throughout the year. Consistent with their results, when I collected in late February and early March 2011, I observed monarch adults in copula, larvae at various instars, and eggs during my collection period in the early spring. Thus, environmental conditions during this time in South Florida were conducive to reproductive activity, rather than diapause.

However, many of the animals I collected originated outside South Florida, indeed well north of the Gulf of Mexico. At this time, I do not know whether or not they were in diapause when they were collected. As monarchs that are reproductively active
only live for 6-8 weeks (Herman & Tatar 2001), the immigrants I collected could not have become reproductively active any earlier than late December. However, by the end of October, most monarchs have disappeared from all but the gulf coast states (Journey North 2013), so the immigrants I found—whose isotopic profiles suggest they originated outside of the Gulf Coast states—likely emerged no later than October. The conditions in South Florida should have triggered animals to exit diapause. Yet, the animals cannot have exited diapause prior to December, or they would not have been alive when I was collecting in late February and early March. This leaves at least a two month period unaccounted for—the monarchs could not be younger than October, yet could not be older than December, if diapause were not maintained. Although detailed responses to triggers for exiting diapause have not been studied in monarchs, Zhu and colleagues (2009) demonstrated that monarchs in a diapause manipulation experiment would exit diapause within approximately two weeks when exposed to a combination of chemical and environmental cues. Dingle (1974) also showed that milkweed bugs (Oncopeltus fasciatus) had fully exited diapause within a few weeks of being exposed to the environmental conditions triggering the return to reproductive competence.

Therefore, either the immigrant monarchs I collected in South Florida stayed in diapause for several months after arriving in South Florida—which seems unlikely, given that other studies suggest that the switch should occur within a few weeks at the most—or they spent at least a portion of the winter at an unknown location which had environmental conditions compatible with the maintenance of diapause prior to returning to South Florida.
Evaluating the diapause status of immigrants will involve further study. We can directly assess the reproductive condition of the animals at collection by dissecting the preserved gonadal tissue of the specimens I collected (Goehring & Oberhauser 2002). Regardless of the outcome, however, a key question that cannot be resolved from the set of animals I collected is how long do immigrants remain in diapause after encountering environmental conditions that trigger the termination of diapause, such as conditions in South Florida. A controlled experiment using captive monarchs held under different temperature and light regimes is needed to provide data to answer this question. If the results of such study suggest that monarchs entering South Florida are unlikely to remain in diapause long enough to explain the time delay I found, then they must have spent the winter elsewhere, and left this overwintering site well in advance of the bulk of the migratory population in Mexico. Thus, investigation of where these immigrants spend the winter is warranted.

Alternatively, males may employ condition-dependent alternative migration strategies. Since environmental conditions in South Florida do not favor diapause, monarchs entering this population are likely to exit diapause and begin reproducing without the need to overwinter for as long as their Mexican counterparts. Thus, this alternative migration strategy may provide a reproductive advantage for males whose condition makes it unlikely they would survive the entire winter in Mexico. Physiological triggers for condition-dependent responses have been shown to vary by sex for some Lepidoptera (Daniels 1995). Though I did not observe any female immigrants, barring sampling effort issues, it remains possible that females also employ an alternative
migration strategy, but with a threshold that was not triggered during the 2011-2012 winter season.

Other possible explanations for recovering large numbers of migrants in South Florida include biases in my sampling protocol, or releases of farmed butterflies. However, the proportions of males and females were similar at both collection sites, and migrant butterflies were distributed across both collection sites, so my sampling protocol does not include obvious biases that would explain my findings. Another possibility is that the animals I captured were farm-reared, and then released. However, the distribution of monarch farms (International Butterfly Breeders Association 2013) does not match the distribution of natal locations that I observed for migrants collected in South Florida. I thus plan to focus future efforts on testing the null hypothesis that immigrant monarchs arrive in South Florida due to random processes, the hypothesis that immigrant monarchs are entering South Florida from an unknown overwintering destination, and the hypothesis that immigrants are following an alternative migration strategy.

Wing size analyses showed there was no difference in South Florida migrant wing size relative to migratory males overwintering in Mexico. On the contrary, my results indicate that migratory males are bigger than their resident counterparts. Because larger butterflies are stronger fliers (Dudley & Srygley 1994), this size advantage may translate into a reproductive advantage if these larger immigrants are better able to take down resident females. Therefore, migratory males might increase their reproductive success by switching to a resident life history strategy, migrating to a
location where they would have the opportunity to mate immediately, rather than needing to survive until spring for mating opportunities at the overwintering colonies.

There are two components of condition, not mutually exclusive, that might serve as triggers for selecting either a migratory or a resident life history strategy. Larval condition could determine the strategy that an adult male would pursue, for example, as determined by the quality of milkweed upon which a larva feeds (Ladner & Altizer 2005; Atterholt & Solensky 2010). I used $\delta^{15}N$ profiles as a proxy for larval condition. Interestingly, the immigrant males tended to be in poor condition relative to the resident males, though the difference was not statistically significant. Of course, the most appropriate comparison to test for an alternative life history strategy would be between animals arriving in Florida and Mexico in the fall of the same year, rather than comparing animals collected after surviving the winter; I do not have such data. When I compare my early spring collected Florida immigrant animals from 2011 to the early spring Mexican animals collected in 2012, i.e. same timeframe but in the following year, the Florida immigrants are more highly enriched for $\delta^{15}N$ than the Mexican animals. Given the difference in collection years, these data must be interpreted with caution. For example, it is possible that all animals in 2012, including those arriving in Florida, were in better condition on average than all animals in 2011. However, my results are consistent with an alternative life history based on condition.

The life history strategy could also be determined by the condition of the adult, and could actually change in the midst of migration if lipid stores fall below a certain threshold. The fitness of migratory monarchs overwintering in Mexico depends not only on traversing thousands of kilometers from their summer range to the overwintering
colonies, but also on accumulating sufficient lipid stores as adults along this journey to survive the reproductively inactive winter period (Alonso-Mejia et al. 1997; Brower et al. 2006). Lipids could further be important via male investment in spermatophores, which is positively correlated with male mating success (Oberhauser 1988, 1989). Accordingly, males that are in poor condition may experience greater mating success if they pursue an alternative strategy by migrating a shorter distance, and/or to a location where they will be able to mate sooner. The size advantage I observed might be particularly important if males were in poor condition with respect to lipid stores. Future investigation of lipid stores in migrants entering South Florida, as opposed to overwintering in Mexico, is planned to directly assess adult condition of these butterflies.

Much of what is known about monarch migration patterns comes from large-scale, multi-decadal mark-recapture (Monarch Watch (2009)) or observational (Journey North (Howard & Davis 2004)) studies. These studies demonstrated that there are corridors of high migration, or “flyways”, from the northern summer breeding areas to the southern overwintering colonies. My results, combined with stable isotope data demonstrating trans-Appalachian flight (Miller et al. 2011), and genetic data which failed to detect population structure within North America (Lyons et al. 2012), indicate that migration routes may be more complex than these broad studies indicate. It is also possible that changing climate or other unmeasured variables have altered migration patterns within the last decade. Additional studies using techniques such as stable isotope analyses and more variable and/or additional genetic markers may add further detail to the overall pattern of the major routes.
Taborsky and Brockmann (2010) outlined three criteria that condition-dependent alternative strategies must meet to be evolutionarily stable: (1) environmental variation must be discrete, (2) environmental cues are reliable, (3) alternatives maximize fitness in different environments. Because the environmental cues in South Florida do not favor continuation of diapause over the winter season, the migratory and resident alternative strategies fit the first two criteria, and my results indicate that these strategies may also meet the third criterion. Further investigation of my hypotheses on condition-dependent alternate migration strategies is planned to evaluate how well these alternatives meet this third criterion, but the results of this study lead to an important overall conclusion: monarch migration behavior is more complex than implied by the major migratory routes. In particular, males and females do not seem to pursue the same strategies. The monarch migration is a unique phenomenon, and understanding sex-specific behaviors and the role of alternative life history strategies in the migration is critical to understanding the evolutionary and conservation implications of these movements.
Figure 2-1. Major migratory flyways of the fall monarch butterfly migration in eastern North America. Light orange arrows indicate the primary routes taken from summering locations throughout Canada and the United States to the overwintering colonies in central Mexico. Width of the arrows is roughly correlated with number of monarchs migrating along a flyway. Gold arrow indicates hypothesized flyway connecting migratory population with resident South Florida population. Monarch migration in a general south or southwest orientation throughout the eastern range, so migrating monarchs are also found in areas not directly covered by the arrows. Red diamonds indicate sampling locations. Due to size constraints of the map, the location of the overwintering colonies is shown northeast of its actual location in central Mexico. Based on the United States Forest Service (2008).
Figure 2.2. Landmarks used to measure wing width. Width was measured in millimeters with a ruler aligned just below the white spot adjacent to the body, and the two white spots closest to the tip. The white line indicates the axis along which measurement was made. Photo taken by author.

Figure 2.3. Distribution of $F$-statistics generated under the null hypothesis for permutation analysis of wing size. Vertical line indicates the $F$-statistic value observed without permuting the data.
Figure 2-4. Distribution of $F$-statistics generated under the null hypothesis for permutation analysis of nitrogen isotope profiles. Vertical line indicates the $F$-statistic value observed without permuting the data.
Figure 2-5. Approximate natal origins of specimens collected in South Florida, shown super-imposed on the major migratory flyways. Specimens collected at the two sites in South Florida were assigned natal areas based on their carbon and hydrogen stable isotope ratios. Eight of the 18 specimens (44%), all males, showed an origin outside of Florida. Dark red ellipses indicate the approximate origin of each of these eight specimens.
Figure 2-6. Wing size differences between monarchs collected in South Florida. Males shown in blue, females in red. Size and distribution of dots indicate individuals. Box limits show first and third quartiles, whiskers indicate $1.5 \times$ inter-quartile range, and horizontal bar indicates median.
Figure 2-7. Non-resident males collected in Florida tend to be in worse condition than resident males, as inferred from enrichment in $^{15}$N relative to $^{14}$N. Box limits show first and third quartiles, whiskers indicate $1.5 \times$ inter-quartile range, and horizontal bar indicates median.
Figure 2-8. Non-resident males collected in Florida in late February 2011 are in significantly worse condition than males collected at the Mexican overwintering colonies in early March 2012 as inferred from enrichment in $^{15}\text{N}$ relative to $^{14}\text{N}$. Box limits show first and third quartiles, whiskers indicate $1.5 \times$ inter-quartile range, and horizontal bar indicates median.
Figure 2-9. Revised migratory flyway map illustrating the connectivity between each of the major migratory routes and the South Florida population. Light orange arrows indicate the primary routes taken from summering locations throughout Canada and the United States to the overwintering colonies in central Mexico. Width of the arrows is roughly correlated with number of monarchs migrating along a flyway. Migrating monarchs are also found in areas not directly covered by the arrows. Red diamonds indicate sampling locations. Due to size constraints of the map, the location of the overwintering colonies is shown northeast of its actual location in central Mexico.
Table 2-1. Collection site and specimen details

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<th></th>
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<td>3</td>
</tr>
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<td>Cerro Pelón</td>
<td>N 19 22.152 W 100 15.618</td>
<td>21</td>
<td>18</td>
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<tr>
<td>Migratory</td>
<td>El Rosario</td>
<td>N 19 35.478 W 100 15.882</td>
<td>23</td>
<td>21</td>
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<tr>
<td>Migratory</td>
<td>Gota de Agua</td>
<td>N 19 22.296 W 100 16.242</td>
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<td>14</td>
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<td>Migratory</td>
<td>Piedra Herrada</td>
<td>N 19 06.450 W 99 50.802</td>
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<td>21</td>
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<tr>
<td>Migratory</td>
<td>Sierra Chincua</td>
<td>N 19 40.068 W 100 16.602</td>
<td>11</td>
<td>17</td>
</tr>
</tbody>
</table>

a Total 18 specimens (11 male, 7 female)
b Total 196 specimens (97 male, 99 female)
c Collected February 27 – March 2, 2011
d Collected March 4, 21 and 22, 2011
e Collected March 7-9, 2012

Table 2-2. Stable isotope profile data

<table>
<thead>
<tr>
<th>Collection site</th>
<th>Specimen</th>
<th>Sex</th>
<th>Hydrogen (δD) a</th>
<th>Carbon (δ13C) b</th>
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<tr>
<td></td>
<td>MB8.1B</td>
<td>Female</td>
<td>-96.22</td>
<td>-30.07</td>
</tr>
<tr>
<td></td>
<td>MB8.1C</td>
<td>Male</td>
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<td>-29.27</td>
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<tr>
<td></td>
<td>MB8.1D</td>
<td>Male</td>
<td>-81.02</td>
<td>-27.71</td>
</tr>
<tr>
<td></td>
<td>MB8.1E</td>
<td>Male</td>
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</tr>
<tr>
<td></td>
<td>MB8.1F</td>
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</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>MB8.1H</td>
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<tr>
<td></td>
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</tr>
<tr>
<td></td>
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<td>Male</td>
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<td>-28.79</td>
</tr>
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<td>SFL4.1F</td>
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<td></td>
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<tr>
<td></td>
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<td>Female</td>
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<td>Female</td>
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<td>-29.30</td>
</tr>
<tr>
<td></td>
<td>SFL4.1J</td>
<td>Female</td>
<td>-81.38</td>
<td>-29.98</td>
</tr>
</tbody>
</table>

a Relative to Vienna Standard Mean Ocean Water (VSMOW)
b Relative to Vienna Pee Dee Belemnite (VPDB)
Table 2-3. Natal origin results

<table>
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<th>Natal origin</th>
<th>Male</th>
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</thead>
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<td>Florida</td>
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<td>Not Florida</td>
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</tbody>
</table>
CHAPTER 3
CANADIAN POPULATION

The monarch breeding range is closely associated with the distribution of
milkweeds, since these plants are the exclusive food source for monarch larvae
(Urquhart 1960). During the spring and summer, milkweed grows throughout much of
North America, thus monarchs breed throughout the continent. The northern edge of the
eastern portion of this breeding range was determined by Urquhart and Urquhart (1979)
as running from the northern shore of Lake Superior to just south of Nova Scotia (Figure
3-2). Multiple milkweed species are native throughout southern Canada (Desmet &
Brouillet 2013). The Journey North project (2013) has tracked observations of monarch
butterflies since 1997, and milkweed plants since 2001. Eggs, larvae, and milkweeds
have all been reported throughout New Brunswick and Nova Scotia north of the putative
monarch breeding limit (Table 3-1, Figure 3-2). Milkweeds have also been introduced
on Labrador (Desmet & Brouillet 2013), although Journey North volunteers have not
reported any sightings that far north. Therefore, it is an open question whether
monarchs are now breeding and successfully eclosing north of previously established
breeding limit.

To test the hypothesis that the monarch breeding limit has shifted northward, I
used stable isotope analysis to determine the natal origin of monarch butterflies
collected both during their southward migration through Point Pelee National Park in
Ontario, Canada, and from their overwintering colonies in Mexico. Stable isotope ratios
vary predictably with location, so these ratios can serve as intrinsic markers for tracing
animal movement behavior (Hobson 1999). Because the carbon and hydrogen isotope
ratios found in monarch wings are determined by the origin of the host plants on which
animals fed as caterpillars and are stable following metamorphosis, measuring these ratios provides an estimate of the natal location (Wassenaar & Hobson 1998). I also used wing width measurements to evaluate whether monarchs originating from different geographic areas along the northern edge of the breeding range showed any obvious phenotypic differences from the rest of the migratory population.

Methods

I used stable isotope profiles to infer larval origins. Stable isotope ratios vary predictably with location, so these ratios can serve as intrinsic markers for tracing animal movement behavior (Hobson 1999). The carbon and hydrogen isotope ratios found in monarch wings are determined by the origin of the host plants on which animals fed as caterpillars. The keratin proteins that make up the wing are stable following metamorphosis, so measuring the stable isotope ratios of wing tissue provides an estimate of the natal location of the butterfly (Wassenaar & Hobson 1998).

Specimen Collection

The geography of southwestern Ontario serves as a funnel to Point Pelee, where monarchs are able to use the chain of small islands south of the Pelee peninsula as stepping stones to minimize the distance they must fly across open water to reach the southern shores of the Great Lakes. Each year, monarchs consistently migrate through Point Pelee in late September (Parks Canada 2011), so this location is well-suited to sampling monarchs migrating south from the northeastern summer range. Monarchs were collected near the tip of Point Pelee National Park in Ontario, Canada, and from five colonies spread across the overwintering region in Mexico (Table 3-2). Live specimens were captured at Point Pelee, immediately placed in glassine envelopes, and stored at -20°C for at least 12 hours prior to sex determination, dissection, and
measurement. Although 196 butterflies were collected in Mexico, only the 16 whose stable isotope profiles indicated an origin north of the published breeding limit are included in this chapter (Chapter 4 discusses the analysis of the complete set of monarchs collected at the Mexican overwintering colonies). Due to permit restrictions in Mexico, specimens were collected from the pool of dead butterflies littering the forest floor at overwintering colony sites, and sorted into glassine envelopes at the end of the day. Sex was determined by the presence or absence of the androconium on the dorsal side of the hindwing before separating wings from bodies, and wings were returned to the glassine envelopes for later stable isotope analysis. Bodies were preserved for later DNA extraction in 2.0 mL tubes filled with 95% ethanol. Both wings and bodies were stored at 4°C after specimens were brought to the lab.

**Natal Origin Analysis**

One hindwing from each South Florida specimen was washed in a 2:1 chloroform:methanol solution to remove surface impurities, lipids, and residual hemolymph, then air dried. Small sections of hindwing (0.350 ± 0.02 mg for hydrogen and 0.825 ± 0.05 mg for carbon) were cut and placed into capsules (3x5mm silver capsules for hydrogen and 5x9mm tin capsules for carbon) for stable isotope analysis. To reduce small fluctuations in stable isotope profiles due to varying degrees of wing pigmentation (see Appendix), sections were consistently excised from the black portion of the wing margin. Carbon isotope profiles were measured at the Light Stable Isotope Mass Spec Lab at the University of Florida, and hydrogen isotope profiles were measured at the Central Appalachians Stable Isotope Facility at the University of Maryland Center for Environmental Science Appalachian Laboratory. Approximate natal origins were assigned based on the combined isotope profiles following the approach
described by Wassenaar and Hobson (1998) and Miller and colleagues (2011), except that the Online Isotopes in Precipitation Calculator (OIPC) plug-in for Google Earth (Bowen & Revenaugh 2003; Bowen et al. 2005; Bowen & Wilkinson 2002) was used to evaluate δD values for precipitation.

**Wing Size Analysis**

Forewing size was measured as a proxy for body size. Width was measured from the white spot adjacent to the body to the point between the two white spots at the tip of the forewing (Figure 3-1). Although there were slight variations in wing patterns, these two markers were consistently used to ensure that homologous sections of the wing were measured. Measurements were recorded to the nearest millimeter. Differences in wing size were analyzed in R (R Development Core Team 2008).

**Results**

Stable isotope profiles indicate that 3 monarchs collected in Point Pelee, as well as 17 monarchs collected in Mexico, had approximate natal origins north of the accepted breeding limit (Table 3-3). Natal origins are shown in Figure 3-2, along with observations collected by the Journey North project for comparison. Origin did not significantly differ for males and females.

The hydrogen stable isotope profile for the specimen (ER2.3AJ) with the northernmost origin (Figure 3-2) indicated an origin in northern Quebec. Although this origin is well above the published northern limit of the monarch range, citizen scientists have reported sightings at similar latitudes to the Journey North project (2013). All samples measured at CASIF were calibrated against USGS42, USGS43 standards, and a keratin quality control sample. Neither the readings of these standards nor the
readings of samples measured before and after ER2.3AJ showed skew in their readings that would indicate a systematic error.

Butterflies collected in Canada ($N = 15$) were significantly smaller than those collected in Mexico ($N = 17$; Figure 3-3; ANOVA, $F_{1,28} = 10.086, P = 0.004$). Specimens also showed sexual dimorphism, with females significantly larger than males (Figure 3-3; ANOVA, $F_{1,28} = 4.2186, P = 0.049$). The interaction of collection country and sex was not significant (ANOVA, $F_{1,28} = 1.5514, P = 0.223$).

**Discussion**

The Journey North project relies on volunteers to report sightings of monarch eggs, larvae, and adult butterflies. However, sightings of adult animals could represent dispersal from animals eclosing within the breeding limit. Observations of eggs and caterpillars are strongly suggestive, but not definitive proof, that animals are successfully breeding. My results are consistent with the hypothesis that the monarch breeding limit has indeed moved northward. Stable isotope profiles show that some animals collected from both Point Pelee and Mexico had successfully eclosed north of the published breeding limit, and these butterflies are arriving at the Mexican overwintering colonies. Thus, my data confirm the prediction from observational data that successful breeding is indeed happening north of the established limits.

Stable isotope data complement observational, citizen scientist-collected data, which are concentrated in populated areas. Since much of northeastern North America is sparsely populated, this limits the areas in which observations are likely to occur. Stable isotope profiles indicate larval origins, so my approach can detect butterflies that originated from both populated and unpopulated areas. Unsurprisingly, butterflies that were collected at Point Pelee originated from some of the same breeding areas.
previously reported by Journey North, but also from areas not included in the Journey North observations. Animals collected in Mexico, in particular, show origins from wilderness regions of Quebec where citizen scientist observations are unlikely to occur.

I found no evidence of phenotypic differences in monarchs originating from northern latitudes. Although size differences were detected between animals collected in Canada and animals collected in Mexico, specimens north of the breeding limit did not drive these differences. Using a captive population reared in controlled laboratory conditions, Wensler (1977) demonstrated that monarchs reared in colder temperatures—such as those found at northern latitudes—followed Bergmann’s rule (Van Voorhies 1996), and accordingly tend to be larger than monarchs reared in warmer temperatures. Moreover, I did not find a significant association between size and latitude (data not shown). Thus, my findings run counter to expectations.

Butterflies were collected on the wing in Point Pelee. In Mexico, however, monarchs were collected from natural mortality on the forest floor at the end of the overwintering season. Thus, it is possible that the different collection methods used influenced the results; it is also possible that the difference between my two studies can be explained by natural selection. Accordingly, I have developed two complementary hypotheses: 1) I was not able to capture the full size range of animals in Canada. Since larger butterflies tend to be more vigorous flyers (Dudley & Srygley 1994), they may have evaded capture or been roosting higher in the trees. If animals in Mexico died randomly with respect to size, then the Mexican samples represent a random sample of the size range of the population, while the Point Pelee ones may not. 2) Alternatively, if smaller animals are less likely to survive the migration, then the population in the
overwintering colonies is likely larger on average than animals beginning the journey. Again, I make the (untested) assumption that mortality in Mexico is random with respect to size. Since the sample sizes collected at Point Pelee were relatively small, future study will be necessary to evaluate these two hypotheses, including capturing animals in Point Pelee in a way that is less likely to result in size bias; and similarly collecting a live sample in Mexico in the same, unbiased way. Moreover, the apparent failure to follow Bergmann’s rule in field-caught animals is surprising, and bears further study.

All the monarchs collected for this study were from the 2011-2012 southward migration and overwintering season, so the effects of annual variations in breeding season weather on the degree of monarch breeding in the far north are unknown. Environmental conditions conducive to monarch breeding are generally found during a fairly brief period at these latitudes. Also, monarch larval success, including susceptibility to parasites, has been shown to vary with species of *Asclepias* on which larvae feed (e.g., Bartholomew & Yeargan 2001; Calvert 1999; Cohen & Brower 1982, de Roode *et al.* 2008), and the set of *Asclepias* species found at northern latitudes differs from that found at southern latitudes (Desmet & Brouillet 2013). The combined effects of a short breeding season, cooler temperatures, and northern *Asclepias* species on monarch fitness are unknown, but a possible hypothesis is that monarch breeding in the far north produces fewer viable offspring for the same reproductive investment.

In addition, wing size is a fairly limited proxy for phenotypic differences that may arise due to northerly breeding. At a minimum, phenotypic characteristics that are correlated with migration and overwintering success, such as lipid content, are important to investigate to determine the degree to which breeding at northern latitudes...
affects these traits, if at all. Solensky and Larkin (2003) also found that monarch larvae exhibit a degree of phenotypic plasticity in their coloration, with the degree of melanization inversely correlated with temperature. While darker larvae are better able to maintain body temperatures suitable for development, they hypothesized that this was accompanied by a tradeoff in greater susceptibility to predation. Given that monarch larvae at northern latitudes are likely to experience colder temperatures than their southern counterparts, the degree to which melanization increases in northern latitudes, as well as the effects of this melanization on predation, are additional phenotypic traits that should be investigated to evaluate the overall effect of northern breeding on monarch fitness.

My data indicating that animals from beyond the previously established breeding limit indeed successfully mature and undertake the journey south are important additions to the growing literature on shifting population ranges, not only of monarchs, but of many plant and animal species (reviewed in Parmesan & Yohe 2003) in the face of climate change. Ecological niche modeling has predicted monarch range shifts northward with changing climate (Batalden et al. 2007). The stable isotope data presented here confirm the model predictions, and underscore the utility of stable isotopes in studies of range expansion.
Figure 3-1. Landmarks used to measure wing width. Width was measured in millimeters with a ruler aligned just below the white spot adjacent to the body, and the two white spots closest to the tip. The white line indicates the axis along which measurement was made. Photo taken by author.
Figure 3-2. Map of approximate origins, based on $\delta^{13}$C and $\delta$D, of monarch butterflies collected at Point Pelee National Park (red and blue circles). Specimens collected at five overwintering colonies in Mexico that originated north of the published breeding limit are also shown (red and blue triangles). Longitude for the Mexico specimens is especially approximate, since these specimens could also be placed in the Canadian Great Plains based on their carbon stable isotope profiles, although still at latitudes north of the published breeding limit. The brown dashed line indicates the previously published limit of the monarch breeding range. Purple dots mark sites where monarch eggs or larvae were reported north of the breeding limit by the Journey North project, 2006-2013.
Figure 3-3. Size differences of specimens collected at Point Pelee and Mexico. Size of points indicate number of individuals in a given size class; points for Canadian specimens with origins north of the published breeding limit are circled in gold (all Mexican individuals shown here originated north of the established limit). Females shown in red; males in blue. Box limits show first and third quartiles, whiskers indicate $1.5 \times$ inter-quartile range, and horizontal bar indicates median.
3-1. Sightings, by year of observation, from the Journey North project suggesting northward expansion of the monarch breeding range.

<table>
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<tr>
<th>Year</th>
<th>Milkweed(^a)</th>
<th>Eggs(^b)</th>
<th>Larvae(^b)</th>
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\(^a\)Milkweed sightings have only been tracked since 2001.
\(^b\)Egg and larvae sightings have only been tracked since 1998.

3-2. Collection site and specimen details

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<tr>
<th>Country</th>
<th>Site name</th>
<th>Location (Lat/Long)</th>
<th># Specimens</th>
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<td>Point Pelee</td>
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<tr>
<td>Mexico(^b)</td>
<td>Cerro Pelón</td>
<td>N 19 22.152 W 100 15.618</td>
<td>1 Male 0 Female</td>
</tr>
<tr>
<td></td>
<td>El Rosario</td>
<td>N 19 35.478 W 100 15.882</td>
<td>4 Male 2 Female</td>
</tr>
<tr>
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<td>Gota de Agua</td>
<td>N 19 22.296 W 100 16.242</td>
<td>1 Male 2 Female</td>
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<tr>
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<td>Piedra Herrada</td>
<td>N 19 06.450 W 99 50.802</td>
<td>2 Male 1 Female</td>
</tr>
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<td>Sierra Chincua</td>
<td>N 19 40.068 W 100 16.602</td>
<td>4 Male 0 Female</td>
</tr>
</tbody>
</table>

\(^a\)Collected September 12-14, 2011
\(^b\)Collected March 7-9, 2012
3-3. Stable isotope profile data

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\(^a\)Relative to Vienna Standard Mean Ocean Water (VSMOW)

\(^b\)Relative to Vienna Pee Dee Belemnite (VPDB)

\(^c\)Canadian specimens with origins north of the published breeding limit

\(^d\)Only includes specimens collected in Mexico with origins north of the published breeding limit
CHAPTER 4
MEXICAN OVERWINTERING COLONIES

Since the ability to adapt to changing conditions is correlated with the amount of genetic variation in a species, a major concern in species conservation efforts is the preservation of adequate genetic variation both for the species as a whole, and in its distribution across the species’ range. Thus, a key aspect of the planning process is assessment of the level of genetic variation and its distribution. For species such as the monarch butterfly (*Danaus plexippus*) that are threatened, but not yet endangered, investigating the distribution patterns of genetic variation, and the processes that lead to them, provides critical data for the development of appropriate conservation policy.

Bowlin and colleagues (2010) argued that migratory species offer unique natural systems in which to study natural variation in the responses to environmental change. Monarch butterflies in eastern North America undergo a seasonal migration from their summer range throughout the eastern United States and southern Canada to a cluster of overwintering colonies in central Mexico (Urquhart & Urquhart 1977). This migration is so unique that the area containing the overwintering colonies was named a UNESCO World Heritage site in 2008 (UNESCO 2008). Although monarch butterflies are not listed as an endangered species, the phenomenon of the monarch migration has been a conservation concern for over three decades. The International Union for Conservation of Nature and Natural Resources (IUCN) added the monarch migration as a “threatened phenomenon” to its Red List in 1983 (Wells *et al.* 1983). Mexico first began officially protecting the overwintering colony areas in the early 1980s; the Monarch Joint Venture was formed in the United States as a partnership between 13 federal, state, non-governmental, and academic organizations in 2008; the Committee on the Status of
Species at Risk in Ontario, Canada listed monarchs as a “species of special concern” in 2009. (COSSARO 2009). The type of threats varies throughout the monarch range in eastern North America. Logging of the oyamel fir habitat has been a major concern for the overwintering colonies in Mexico (e.g., Ramirez et al. 2003; Calderón et al. 2004; Ramirez et al. 2007). In both the United States and Canada, possible negative effects of genetically modified crops on monarch reproduction and the loss of suitable breeding habitat have been focal areas of conservation research and policy (e.g., Zangerl et al. 2001; Macdonald & Yarrow 2003; Oberhauser & Rivers 2003; Anderson et al. 2005; Marvier et al. 2007; Prasifka et al. 2007). Because of this variation in the degree and type of threat across the eastern North American monarch range, the distribution of genetic variation, and the degree of connectivity between monarchs found in geographically distant areas, are key for monarch conservation.

Monarchs breed throughout North America during the spring and summer months, with 4-6 generations produced during this period. Butterflies that emerge in the late summer or fall, however, enter reproductive diapause immediately after emergence, then undertake the arduous migration to the overwintering grounds. In the eastern United States and southern Canada, these butterflies migrate during the fall along the major routes shown in Figure 4-1 to overwinter at a small cluster of colonies in central Mexico, while monarchs west of the Rocky Mountains migrate to a series of small colonies scattered along the California coast (Dingle et al. 2005; Leong 1990; Leong et al. 1995). These butterflies remain in diapause until the early spring, when they exit diapause and breed before leaving the overwintering colonies to begin the journey north. This overwintering generation of monarchs generally migrates far enough north to
lay eggs in suitable milkweed habitat in southern Texas, and each subsequent
generation continues the northward migration to fan out across the full range of North
America east of the Rockies. Because breeding occurs continuously during this
migration, a possibility exists for an isolation-by-distance (IBD) pattern to form along the
various migration routes. The overwintering colonies in Mexico, however, may provide a
means for monarchs from widely separated summering areas to interbreed, thus
breaking down IBD patterns that may form during the spring and summer.

After decades of intense effort, the precise location of the overwintering area in
Mexico was discovered (Brower et al. 1977; Urquhart & Urquhart 1977). Early efforts to
study monarch movement and its effects on population structure relied on observational
or mark-recapture approaches. These studies were able to determine the major
migratory flyways (Calvert & Brower 1986; Howard & Davis 2004; Howard & Davis
2009; Malcolm 1987; Schmidt-Koenig 1985). Despite these major findings, however,
both observational and mark-recapture approaches are of limited utility for investigating
fine-scale movements and population structure.

In contrast, both genetic sequences and stable isotope profiles are intrinsic
markers that do not rely on following the movements of individuals, nor on capturing the
same individuals at multiple sites. Thus, they are potentially more powerful, less prone
to bias, and useful for investigations at a more fine scale. Prior to the widespread
availability of gene sequencing, allozymes (naturally occurring variants of the same
enzyme) were used to infer genetic differences. Eanes and Koehn (1978) used
allozyme analysis to evaluate population structure in monarch butterflies, and found no
evidence for geographic patterns of population structure. Because allozymes are post-
translational markers, they may mask genetic differences that are not carried forward to the protein such as synonymous and intronic mutations. Such mutations can be detected, however, if genomic DNA is sequenced. Thus, DNA may provide higher resolution and greater power to detect subtle differences than allozymes. Brower and Jeansonne (2004) found a low degree of genetic variation in monarch butterflies using DNA sequences of gene fragments, and Lyons and colleagues (2012) used microsatellite markers to show that monarchs in North America form a single panmictic population.

While DNA markers are a means of directly assessing the distribution of genetic variation, movement patterns can only be inferred indirectly by analyzing the degree of differentiation between groups. In contrast, stable isotope analysis uses predictable geographic patterns in the distribution of different isotopes of key elements to measure animal movement between the location at which isotopes were incorporated into tissues, and the location at which specimens were collected. Because keratin in wing tissue is laid down at the time of metamorphosis, and does not change subsequently, isotopes of the carbon and hydrogen incorporated in these tissues can be used to assign natal origin to butterflies (Wassenaar & Hobson 1998). Thus, the ratio of light to heavy isotopes in an adult animal reflects the milkweed on which the larva fed. In monarch wings, hydrogen isotopes vary in conjunction with the isotopic signature of local meteoric water; carbon isotopes vary depending on the milkweed species on which a larva fed prior to pupation (Hobson et al. 1999). As shown in Figure 4-2, hydrogen isotope ratios in eastern North America exhibit a roughly southeast-northwest gradient, with increasing depletion of deuterium (D) to the northwest, whereas carbon
isotope ratios show increasing depletion of $^{13}$C moving from northeast to southwest. By determining stable isotope ratios for carbon and hydrogen, Wassenaar and Hobson (1998) found that there was no clear association between natal origin and overwintering colony site, and that a preponderance of monarchs originated from the Midwest or western Great Lakes.

In contrast to previous studies, I integrated genetic and stable isotope analyses to test the hypothesis that monarchs at the Mexican overwintering colonies are part of a single panmictic population. To evaluate possible phenotypic differences in monarchs found at different colonies, I used wing size as a proxy for overall phenotype. Genetic sequences reflect movements that persist over generations, wing size is a function of both genetics and of the condition of the larva, and stable isotopes reflect the conditions under which the larva fed. Thus, each line of evidence produces data that resolve patterns at different time scales, and integrating these three provides a more complete picture than any one alone.

**Methods**

I used stable isotope profiles to infer larval origins. Stable isotope ratios vary predictably with location, so these ratios can serve as intrinsic markers for tracing animal movement behavior (Hobson 1999). The carbon and hydrogen isotope ratios found in monarch wings are determined by the origin of the host plants on which animals fed as caterpillars. The keratin proteins that make up the wing are stable following metamorphosis, so measuring the stable isotope ratios of wing tissue provides an estimate of the natal location of the butterfly (Wassenaar & Hobson 1998).
Specimen Collection

Monarchs consistently congregate at a collection of sites scattered across the mountains of central Mexico northwest of Mexico City. Colonies to sample (Figure 4-3) were selected from throughout this geographic range based on accessibility, safety, and collection permit restrictions. In March 2012, 181 specimens were collected from five overwintering colonies in Mexico (Table 4-1). Due to permit restrictions, monarchs were collected from the pool of dead butterflies littering the forest floor at overwintering colony sites, and sorted into glassine envelopes at the end of the day. Sex was determined by presence or absence of the androconium on the dorsal side of the hindwing before separating wings from bodies, and wings were returned to the glassine envelopes for later stable isotope analysis. Bodies were preserved for later DNA extraction in 2.0 mL tubes filled with 95% ethanol. Both wings and bodies were stored at 4°C after specimens were brought to the lab.

DNA Analysis

DNA was extracted from head and thorax tissue using the UltraClean Tissue & Cells DNA Isolation Kit (MO-BIO Laboratories, Inc., Carlsbad, CA) according to the manufacturer’s protocol for difficult tissue extractions. As summarized in Table 4-2, only a subset of the total number of specimens collected was used for analysis. Unused material was archived for analysis in future studies. To meet collection permit requirements, only 25 µL aliquots of DNA extracts from Mexico were brought back to the University of Florida for PCR amplification and sequencing; the remaining specimen material was archived (DNA @ -20°C, bodies @ 4°C) in the Laboratorio de Genética de la Conservación at UNAM Campus Morelia.
Amplification of a hyper-variable fragment of the nuclear Tektin gene was performed following the methods described by Whinnett and colleagues (2005) for the TektinA/Tektin3 primer pair. Each 25 µL reaction volume contained 2 µL (roughly 5-20 ng) genomic DNA extract, 12.5 µL 2X PCR Master Mix (Promega, Madison, WI), 10 µM each primer and ddH₂O to bring the final volume to 25 µL. After optimizing the original PCR conditions, amplifications were performed with an initial denaturing of 2 minutes at 94°C, followed by 35 cycles of 60 s at 94°C, 60 s at 47.9°C, 90 s at 72°C, and a final extension of 10 minutes at 72°C. PCR products were prepared for sequencing using either a QIAquick PCR Purification Kit (QIAGEN, Inc., Valencia, CA) or polyethylene glycol (PEG) and ethanol precipitation (Paithankar & Prasad 1991). All purified PCR products were sequenced at the DNA Analysis Facility on Science Hill at Yale University using the same primers as those used for PCR.

Sequences were imported into Geneious (Drummond et al. 2012), forward and reverse reads were matched, and ends were trimmed to give a final product of 626 bp. Trimmed contigs were aligned using the MUSCLE option (Edgar 2004) of Geneious with the default settings. Alignments were imported into Network (Fluxus Technology Ltd., Clare, Suffolk, UK) to generate median-joining genetic network diagrams (Bandelt et al. 1999). Final diagrams illustrating the breakdown of genotypes by sex, and by overwintering colony were prepared in Adobe Illustrator (Adobe Systems Inc., San Jose, CA). Alignments were also used to generate basic neighbor joining tree with 10,000 bootstrap replicates using built-in Geneious tree builder with the default settings.

Haplotypes were reconstructed using the PHASE 2.1 (Stephens et al. 2001, Stephens & Scheets 2005) option of DnaSP v5 (Librado & Rozas 2009) with the default
parameters, except with 1,000 iterations instead of 100. As recommended in the PHASE 2.1 documentation, reconstruction was run five times with different starting seed values, with one run of 10,000 iterations. Results were compared for consistency (mean likelihood ranged from -932.527 to -932.014), and the results from the run with the highest likelihood were used for subsequent analyses. All subsequent analyses were performed in Arlequin 3.5 (Excoffier et al. 2007). To evaluate the degree to which genetic variation was unevenly distributed across the overwintering colony sites, measures of molecular diversity ($\pi$ (Tajima 1983), $\theta_\pi$ (Tajima 1983), $\theta_S$ (Watterson 1975)) were determined from these haplotypes for both the migratory population as a whole (all five colonies as a single population), and for each colony separately. The degree of differentiation between the colonies was determined by calculating the pairwise genetic distances between the populations ($F_{ST}$; Wright 1943), running an AMOVA with 1,000 permutations under the assumption that each colony represents a distinct population, and performing Raymond and Rousset’s exact test of population differentiation (1995) with 100,000 Markov steps, which tests against a null expectation of panmixia.

**Natal Origin Analysis**

One hindwing from each South Florida specimen was washed in a 2:1 chloroform:methanol solution to remove surface impurities, lipids, and residual hemolymph, then air dried. Small sections of hindwing (0.350 ± 0.02 mg for hydrogen and 0.825 ± 0.05 mg for carbon) were excised from the wing and placed into capsules (3x5mm silver capsules for hydrogen and 5x9mm tin capsules for carbon) for stable isotope analysis. To reduce small fluctuations in stable isotope profiles due to varying degrees of wing pigmentation, sections were consistently excised from the black and
white portion of the wing margin (see Appendix). Carbon isotope profiles were measured at the Light Stable Isotope Mass Spec Lab at the University of Florida, and hydrogen isotope profiles were measured at the Central Appalachians Stable Isotope Facility at the University of Maryland Center for Environmental Science Appalachian Laboratory.

Approximate natal origins were assigned based on the combined isotope profiles following the approach described by Wassenaar and Hobson (1998) and Miller and colleagues (2011), except that the Online Isotopes in Precipitation Calculator (OIPC) plug-in for Google Earth (Bowen & Revenaugh 2003; Bowen et al. 2005; Bowen & Wilkinson 2002) was used to evaluate δD values for precipitation. Origins were assigned to one of the three major migratory flyways (United States Forest Service 2008). In some cases, origins could not be assigned to a single flyway because of the complex distribution of carbon and hydrogen isotopes in North America.

**Wing Size Analysis**

Forewing size was measured as a proxy for body size. Width was measured from the white spot adjacent to the body to the point between the two white spots at the tip of the forewing (Figure 4-4). Although there are slight variations in wing patterns, these two markers were consistently present to ensure that analogous sections of the wing were measured. Measurements were recorded to the nearest millimeter. Differences in wing size by sex and origin were analyzed in R (R Development Core Team 2008). Because sample sizes were small (13 females/colony, 11-16 males/colony), I tested the difference in size between overwintering colonies by permuting the variable "origin" five hundred times and observing the distribution of F-statistics from the one-way ANOVA of each permuted dataset.
Results

A 626 bp hypervariable region of the autosomal locus Tektin was analyzed to evaluate the possibility for genetic structure among the overwintering sites. There were 39 segregating sites (~6%), 21 of which were parsimony informative. All but one site had two alleles; the other had three. Of 126 butterflies analyzed, 92 (73%) were heterozygous for at least one site.

Molecular diversity statistics are summarized in Table 4-3. Genotype networks show that haplotypes are homogenously distributed across the five sampled colonies (Figure 4-5). Moreover, haplotypes are distributed approximately equally between males and females (Figure 4-6). Pairwise genetic distances were small, and permutation tests of these distances, as implemented in Arlequin, showed that none were statistically significant (Table 4-4; 1,023 permutations, 0.13 < P < 0.96). The distribution of genetic variation among the colonies was also not significant (Table 4-5; AMOVA, P = 0.817 ± 0.012). Finally, Raymond and Rousset’s exact test of differentiation was unable to reject panmixia on either a global (P = 0.79) or pairwise basis (ranging from 0.21 < P < 0.92).

Phylogenetic analysis also supports a single homogenous population distributed across the overwintering colonies. Two Queen butterfly (D. gilippus) specimens collected in South Florida were also sequenced for the same region of Tektin to serve as an outgroup. Comparison between monarchs and this sister taxon shows 100% bootstrap support for separation between these taxa, but there was no support for differentiation within monarchs (Figure 4-7).

I identified natal origin by using carbon and hydrogen stable isotopes to test the null hypothesis that animals congregate in particular overwintering sites without respect to their origin. I found no significant relationship between origin (via δD and δ13C) and
overwintering colonies with respect to north-south ordering (linear regression, $R^2 < 0.022$, $F < 0.53$, $P > 0.47$). Although there is no clear association between specific flyways or summering areas and overwintering colony sites, sites do significantly differ with respect to origin (Figure 4-8; ANOVA, main effect of site, $F_4 = 3.35$, $P = 0.012$). Sexes do not differ by origin (Figure 4-8; ANOVA, main effect of sex, $F_4 = 1.00$, $P = 0.319$), and site x sex is also not significant ($P = 0.068$). The latter interaction seems to be driven primarily by exceptional females found in the Sierra Chincua colony (females at this site tend to be from the most southern locations).

Wing size varied significantly between the overwintering colony sites (Figure 4-9; ANOVA, $F_4 = 4.18$, $P = 0.003$). There were no significant differences in the size of male vs. female monarchs ($F_1 = 0.44$, $P = 0.507$), nor was there a significant site x sex interaction ($F_4 = 0.670$, $P = 0.670$). No significant differences were found between females from different colonies ($F_4 = 1.40$, $P = 0.24$). Males at the Sierra Chincua colony were significantly smaller than males at other colonies ($F_4 = 3.42$, $P = 0.012$), but no significant differences were found between males from different colonies when Sierra Chincua was excluded from the analysis ($F_3 = 0.806$, $P = 0.494$). In addition, I found no significant relationship between origin (via $\delta D$), size, and colony (Figure 4-10, linear regression for each colony, $R^2 < 0.064$, $F < 1.72$, $P > 0.20$).

**Discussion**

Taken together, my genetic and stable isotope results support the hypothesis that monarchs at the Mexican overwintering colonies are part of a single panmictic population. The overall amount of genetic variation at the *Tektin* locus was low. Across all measures of diversity ($\pi$ (Tajima 1983), $\theta_\pi$ (Tajima 1983), $\theta_S$ (Watterson 1975)), El Rosario consistently shows the highest, and Cerro Pelón the lowest, amount of genetic
variation. $\pi$ is a measure of the mean number of pairwise differences. Both $\theta_\pi$ and $\theta_S$ are estimates of the population parameter $\theta$, which describes the amount of variation that can be expected under the neutral theory (Kimura & Ohta 1971). This parameter is defined as $\theta = 4N_e\mu$, where $N_e$ is the effective population size, and $\mu$ is the mutation rate. $\theta_\pi$ estimates $\theta$ based on $\pi$ (Tajima 1983); $\theta_S$ is based on $S$—the number of segregating (variable) sites (Watterson 1975). Since $\theta_\pi$ is based on $\pi$, agreement between these two statistics is to be expected. Comparison between the colonies, however, shows that these differences in the distribution of genetic variation are not significant, since both the AMOVA—which compares the amount of genetic variation among and within colonies—and Raymond and Rousset’s test—which uses a Markov chain approach to test for panmixia—found no support for differentiation between the colonies. In addition, the Tektin genotype network indicates that this genetic variation is distributed fairly uniformly across the five sampled overwintering colonies, and fairly equally between the sexes. The degree of concordance between these different approaches to evaluating the distribution of genetic variation indicates that my finding of a single panmictic population is robust under different sets of assumptions. This lack of genetic population structure is consistent with previous work that has shown no genetic differentiation within the North American migratory monarch population (Eanes & Koehn 1978; Lyons et al. 2012).

Stable isotope results complement the genetic data by providing evidence that monarchs sort into overwintering colonies randomly with respect to geographic origin. No relationship between geographic origin and colony was detected, but distribution of origins across the colonies was not uniform. One requirement of panmixia is that
animals originating from different geographic origins must have the opportunity to mate with one another. Since monarchs begin breeding before leaving the overwintering colonies in the spring, the mix of geographic origins at the colonies fulfills this requirement, and is consistent with the hypothesis that any geographic structure that may develop during the spring and summer would be broken down at the overwintering colonies. Thus, while the genetic results support the hypothesis that the migratory population of monarch butterflies in eastern North America is genetically homogenous, it is the stable isotope data that strengthen the inference this homogeneity is due, at least in part, to panmixia.

Although my findings are consistent with the hypothesis that monarchs at the Mexican overwintering colonies are part of a single panmictic population, my data also detected subtle differences between the overwintering colonies. These results suggest that, while monarchs from throughout the summering range mix, the colony sites are not equivalent. The ecological conditions are the colony sites I sampled vary widely in tree density, understory composition, exposure to weather, and colony size. Since all of these factors are important for monarch survival over the winter (Alonso-Mejia et al. 1992; Alonso-Mejia et al. 1998; James 1984), this ecological variation could be correlated with some of the differences I detected. For example, El Rosario is the largest colony, whereas Cerro Pelón is one of the smallest. The colony site at El Rosario is distributed along an altitudinal gradient with consistent cover of old-growth oyamel firs, and butterflies in this colony tend to form massive clusters high in these trees. In contrast, the butterflies in Cerro Pelón are found in smaller numbers and lower densities. Large stands of old-growth oyamel fir are also lacking at Cerro Pelón, and the
forest habitat is interspersed with open meadows. Thus, ecological conditions at the El Rosario site are better suited to overwintering survival than Cerro Pelón, even though monarchs consistently return to both sites each year (L. Brower, personal communication). El Rosario displayed the largest variance in origins; Cerro Pelón displayed the largest variance in wing size. Thus, a greater variety of origins were found at the overwintering colony with some of the best ecological conditions for overwintering survival, whereas some of the smallest animals were found at the colony with some of the worst ecological conditions. These differences in both origin and size indicate that different colonies attract different types of monarchs, but the precise relationship between origin, size, and quality of ecological conditions at the overwintering colonies needs additional study.

Ecological differences in the colony sites may correlate with the patterns I observed, but detailed study of these potential correlations is needed. In particular, multi-year observations are necessary to demonstrate whether or not between-colony variation with respect to origin is a recurrent pattern, and the degree to which this pattern is consistent across years.

My results lead to several questions that warrant future investigation. One key question is the persistence of the patterns I found across years. These specific patterns may consistently appear from year to year; differences between colonies may appear each year, but the particular pattern may differ; or colonies may show differences in some years, but not in others. Moreover, I collected towards the end of the overwintering period. Thus, the profiles of animals arriving at the site with respect to
genetics, natal origin, and size could be different from the animals found towards the spring, particularly if mortality is nonrandom with respect to any of the traits I studied.

Finally, I collected dead specimens, so the patterns for live butterflies may differ. For example, the majority of monarchs I collected originated in the areas that feed the Atlantic or Great Lakes flyways, but previous studies found a greater proportion of butterflies from the Midwest (Howard & Davis 2009; Wassenaar & Hobson 1998). Differences could be due to different methods used, random variation between years, or actual changes in monarch behavior patterns. However, although Howard and Davis used monarch sighting data collected via the Journey North project, Wassenaar and Hobson used a similar approach to ours (stable isotopes from dead animals), suggesting that different approaches are unlikely to explain my differing results. Determining if and how patterns vary across years is the first step in investigating the mechanism(s) that produce these patterns.

Awareness of the fine-scale differences between overwintering colonies can better inform conservation efforts focused on the overwintering colony sites. In addition to the concerns raised over the years by various conservation organizations, the 2013 survey of the Mexican overwintering colonies by the World Wildlife Foundation-Telcel Alliance, and Mexico’s National Commission of Protected Areas (CONAP) showed that the number of monarchs congregating at these colonies was the lowest it has been in over two decades (World Wildlife Foundation 2013). Microclimate conditions at colony sites are key for monarch survival over the winter (Alonso-Mejia 1996; Anderson & Brower 1996; Brower et al. 2009; Brower et al. 2008), but environmental conditions at different colony sites vary widely (Brower et al. 2002). My results show that the
monarchs found at these sites vary as well with respect to genetic variation, origin and size, and that colony sites contain distinct combinations of animals, such that loss of any one of these sites would decrease the diversity of the species. The source of mortality is important in setting conservation priorities. For example, if mortality is associated with particularly degraded sites, then restoring these sites may improve overwintering survival. If, however, mortality is due more to site-extrinsic factors such as availability of suitable nectar sources and roosting sites along the migratory routes, then policies focused on the migration itself may be more important. Understanding the patterns and processes that lead to differences at the overwintering colony sites will provide a more complete picture of the potential impacts of different threats on the monarch migration.
Figure 4-1. Major migratory flyways of the fall monarch butterfly migration in eastern North America. Light orange arrows indicate the primary routes taken from summering locations throughout Canada and the United States to the overwintering colonies in central Mexico. Width of the arrows is roughly correlated with number of monarchs migrating along a flyway. Monarchs migrate in a general south or southwest orientation throughout the eastern range, so migrating monarchs are also found in areas not directly covered by the arrows. Due to size constraints of the map, the red diamond indicating sampling at the overwintering colonies is shown northeast of the actual location in central Mexico. Based on the United States Forest Service (2008).
Figure 4-2. Heavy isotope depletion gradients for carbon and hydrogen in monarch wing tissue. Arrows point in the direction of increasing depletion of heavy isotopes ($^{13}$C and D). Orange = carbon, blue = hydrogen. Based on data from Wassenaar and Hobson (1998); hydrogen values modified according to Meehan and colleagues (2004).
Figure 4-3. Map of overwintering colony sites. Cerro Pelón and Gota de Agua are on opposite sites of the same mountain massif; all other sites are on separate massifs. Generated using Google Earth.

Figure 4-4. Landmarks used to measure wing width. Width was measured in millimeters with a ruler aligned just below the white spot adjacent to the body, and the two white spots closest to the tip. The white line indicates the axis along which measurement was made. Photo taken by author.
Figure 4-5. Network illustrating the distribution of genotypes among the overwintering colonies in Mexico. Colonies are listed from north to south in legend. Node size is proportional to the number of samples sharing a genotype, while each segment is proportional to the number of samples found at each overwintering colony. Lines connecting nodes represent a base pair difference. Small white circles represent unsampled intermediates.
Figure 4-6. Network illustrating the distribution of genotypes by sex. Node size is proportional to the number of samples sharing a genotype, while each segment is proportional to the number of samples of each sex. Lines connecting nodes represent a base pair difference.
Figure 4-7. Neighbor-joining tree illustrating the clear separation of monarchs (*D. plexippus*, orange nodes) from their sister species, the queen butterfly (*D. gilippus*, blue nodes), but no strong differentiation within monarchs. Branch labels are bootstrap support based on 10,000 replicates. Although only the branch separating *D. plexippus* from *D. gilippus* is significant, other bootstrap values are shown to illustrate degree of support of branching within *D. plexippus*. Colored circles at the tips indicate overwintering colony of specimen. Overwintering colonies are listed from north to south in the legend.
Figure 4-8. Origin differences at each overwintering colony site, as indicated by hydrogen isotope ratios (δD). Vertical axis reversed, so that more southerly origins (less negative δD values) are shown closer to the x-axis. Colonies listed along x-axis from south to north. Box limits show first and third quartiles, whiskers indicate $1.5 \times$ inter-quartile range, and horizontal bar indicates median. Females shown in red; males shown in blue.
Figure 4-9. Distribution of wing sizes at Mexican overwintering colonies. Colonies listed along x-axis from south to north. Box limits show first and third quartiles, whiskers indicate $1.5 \times$ inter-quartile range, and horizontal bar indicates median. Females shown in red; males shown in blue.
Figure 4-10. Relationship between δD and wing size at the different overwintering colony sites. More northerly origins are indicated by more negative δD values. Colonies are listed from north to south in the legend. Colored lines show the linear regression for each colony, the black line is the overall regression, and the grey shadows show the confidence intervals for each regression. Circles indicate females; triangles indicate males.
### Table 4-1. Overwintering colonies at which specimens were collected.

<table>
<thead>
<tr>
<th>Colony</th>
<th>GPS</th>
<th>Elevation</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sierra Chincua</td>
<td>N 19 40.068</td>
<td>3,300m</td>
<td>3/9/12</td>
</tr>
<tr>
<td></td>
<td>W 100 16.602</td>
<td></td>
<td></td>
</tr>
<tr>
<td>El Rosario</td>
<td>N 19 35.478</td>
<td>3,200m</td>
<td>3/7/12</td>
</tr>
<tr>
<td></td>
<td>W 100 15.882</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerro Pelón</td>
<td>N 19 22.152</td>
<td>2,650m</td>
<td>3/8/12</td>
</tr>
<tr>
<td></td>
<td>W 100 15.618</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gota de Agua</td>
<td>N 19 22.296</td>
<td>2,900m</td>
<td>3/9/12</td>
</tr>
<tr>
<td></td>
<td>W 100 16.242</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Piedra Herrada</td>
<td>N 19 06.450</td>
<td>3,150m</td>
<td>3/8/12</td>
</tr>
<tr>
<td></td>
<td>W 99 50.802</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Colonies are listed north to south*

### Table 4-2. Summary of specimens collected and analyzed.

<table>
<thead>
<tr>
<th>Colony</th>
<th># Specimens Collected</th>
<th># Specimens Analyzed</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>Sierra Chincua</td>
<td>34</td>
<td>34</td>
<td>16</td>
</tr>
<tr>
<td>El Rosario</td>
<td>38</td>
<td>34</td>
<td>15</td>
</tr>
<tr>
<td>Cerro Pelón</td>
<td>27</td>
<td>24</td>
<td>14</td>
</tr>
<tr>
<td>Gota de Agua</td>
<td>23</td>
<td>21</td>
<td>11</td>
</tr>
<tr>
<td>Piedra Herrada</td>
<td>32</td>
<td>34</td>
<td>15</td>
</tr>
</tbody>
</table>

*Colonies are listed north to south*

### Table 4-3. Summary of molecular diversity statistics

<table>
<thead>
<tr>
<th>Colony</th>
<th>n</th>
<th>S</th>
<th>$\pi$ $^{b,c}$</th>
<th>$\theta_r$ $^{c,d}$</th>
<th>$\theta_S$ $^{c,d}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sierra Chincua</td>
<td>21</td>
<td>13</td>
<td>0.0035 ± 0.00034</td>
<td>2.20 ± 0.382</td>
<td>3.02 ± 0.326</td>
</tr>
<tr>
<td>El Rosario</td>
<td>27</td>
<td>23</td>
<td>0.0038 ± 0.00032</td>
<td>2.37 ± 0.303</td>
<td>5.05 ± 0.354</td>
</tr>
<tr>
<td>Cerro Pelón</td>
<td>27</td>
<td>12</td>
<td>0.0031 ± 0.00027</td>
<td>1.96 ± 0.361</td>
<td>2.63 ± 0.294</td>
</tr>
<tr>
<td>Gota de Agua</td>
<td>27</td>
<td>16</td>
<td>0.0035 ± 0.00029</td>
<td>2.17 ± 0.339</td>
<td>3.51 ± 0.317</td>
</tr>
<tr>
<td>Piedra Herrada</td>
<td>24</td>
<td>13</td>
<td>0.0037 ± 0.00033</td>
<td>2.32 ± 0.397</td>
<td>2.93 ± 0.312</td>
</tr>
<tr>
<td>OVERALL</td>
<td>126</td>
<td>39</td>
<td>0.0035 ± 0.00014</td>
<td>2.01 ± 0.216</td>
<td>6.39 ± 0.264</td>
</tr>
</tbody>
</table>

*Colonies are listed north to south*

<table>
<thead>
<tr>
<th>b</th>
<th>Number of individuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>c</td>
<td>Values are mean ± standard error</td>
</tr>
<tr>
<td>d</td>
<td>Per sequence</td>
</tr>
</tbody>
</table>

### Table 4-4. Pairwise $F_{ST}$ values for Mexican overwintering colonies

<table>
<thead>
<tr>
<th>Colony</th>
<th>Sierra Chincua</th>
<th>El Rosario</th>
<th>Cerro Pelón</th>
<th>Gota de Agua</th>
</tr>
</thead>
<tbody>
<tr>
<td>El Rosario</td>
<td>0.0177</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerro Pelón</td>
<td>0.0013</td>
<td>0.0027</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gota de Agua</td>
<td>0.0133</td>
<td>-0.0082</td>
<td>-0.0011</td>
<td></td>
</tr>
<tr>
<td>Piedra Herrada</td>
<td>-0.0045</td>
<td>0.0022</td>
<td>-0.0089</td>
<td>-0.00274</td>
</tr>
</tbody>
</table>

*Colonies are listed north to south*
Table 4-5. AMOVA under the assumption that each overwintering colony is a separate population

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Variance component</th>
<th>% of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among colonies</td>
<td>4</td>
<td>4.482</td>
<td>0.00041</td>
<td>0.04</td>
</tr>
<tr>
<td>Within colonies</td>
<td>247</td>
<td>271.728</td>
<td>1.10011</td>
<td>99.96</td>
</tr>
<tr>
<td>TOTAL</td>
<td>251</td>
<td>276.210</td>
<td>1.10052</td>
<td></td>
</tr>
</tbody>
</table>

Fixation Index ($F_{ST}$) = 0.00037
1,023 permutations, $P = 0.817 \pm 0.0119$
CHAPTER 5
SYNTHESIS

My findings show that monarch butterflies have complex movement behaviors during and in addition to their mass migration. Therefore, the generally accepted paradigm that monarchs are distributed throughout North America during the spring and summer, but migrate to overwintering colonies masks fine-scale complexity that has implications for monarch conservation and evolution. The monarch population lacks detectable genetic structure. Therefore, it can be considered a single panmictic population. However, my studies from three distinct geographic areas—a putatively resident population in Miami, animals in Canada embarking upon the journey south, and animals overwintering in Mexico—all provided evidence of monarch movement behavior that is more complex than previously thought. The resident population in South Florida may serve as an alternative overwintering destination; viable adult butterflies are originating north of the published breeding limit; and the different Mexican overwintering colonies display subtle, but significant, differences in the geographic origins of their inhabitants. All of these previously undocumented patterns raise interesting questions about monarch butterfly behavior and physiology that warrant further investigation. I discuss possible follow-up studies for each in turn.

**Resident Populations as Alternate Migratory Destinations**

By collecting monarchs throughout the year in South Florida, additional data on the degree of immigration and the reproductive status of immigrant butterflies can also be collected. Since juvenile hormone is a key signal in the maturation of monarch gonadal tissue, and its levels correlate with diapause status, determining the titer of juvenile hormone in the hemolymph of freshly caught specimens provides a second
means of assessing diapause status (Lessman *et al.* 1989). In addition to providing data about diapause status, the nutritional condition of monarchs entering the South Florida population throughout the year could also be determined by measuring their lipid content (Gibo & McCurdy 1993), which would indicate whether immigrants show evidence of following a condition-dependent alternate migration strategy.

If males immigrating to South Florida are following a condition-dependent alternate migration strategy, then identifying the condition thresholds leading to migration to South Florida is critical to understanding the mechanism underlying this behavior. Another key question is whether these thresholds vary across the range, with time, or with genetic variants. In addition, since I found that immigrant males were larger than resident butterflies, another unanswered question is whether this gives these males a reproductive advantage in South Florida that they would not experience in the Mexican overwintering colonies.

I found no evidence for females using South Florida as an alternate migration destination. It is unclear whether females do not employ an alternate migration strategy, whether females have different condition thresholds that were not met during the 2010-2011 overwintering season, whether they use a different alternate destination, or whether as a result of my small sample size, I simply failed to capture female immigrants. Across widely divergent taxa, alternative tactics appear more often in males than in females, most likely because of the greater energetic investment of females due to anisogamy (Taborsky & Brockmann 2010), so it is possible that monarch females do not employ an alternate migration strategy. Collecting additional specimens in South
Florida, particularly throughout the overwintering period, would indicate whether sample size was the reason I did not observe any immigrant females.

In addition to more intensive sampling of the South Florida population, careful study of additional putatively resident populations in the United States would be desirable to see whether or not males following alternative migration strategies are found at multiple sites, as well as whether females are only found at some sites. Other possibly resident populations have been reported in southern Texas and Arizona (Funk 1968; Journey North 2013). The locations and persistence of these populations may vary, and may shift with climate change or development pressure. The frequency with which immigrants from the main migratory population enter these additional resident populations is also unknown. Evaluating the degree to which monarchs employ multiple alternate destinations is key to understanding the use of alternate migration strategies.

**Phenotypic Variation in the Mexican Overwintering Colonies**

Monarch phenotypes vary across both their summer breeding range and the overwintering colonies in Mexico. I found size differences at the overwintering colonies, which raises the question of whether there is additional phenotypic variation between the colonies. For example, the shades of orange wing pigments fall along a gradient from yellow-orange (lighter color) to red-orange (darker color), and these shades have been found to co-vary with size—larger monarchs have redder hues (Davis 2009). Both adult wing and larval coloration vary with temperature, although these relationships appear to be different for males and females, and for monarchs originating from different parts of North America (Davis et al. 2004; Davis et al. 2005). Insect size is also known to co-vary with temperature (Dingle 1972; Wensler 1977), so it may be that the
relationship between color and size is controlled by temperature, though this hypothesis needs investigation.

Importantly, females have shown a significant, consistently strong preference for males with less saturated orange shades (Davis et al. 2007), which should also have been smaller males, given the size-color relationship described previously (Davis 2009). Perhaps surprisingly, Davis and colleagues found that females tended to prefer larger males, although this preference was not significant across all experiments. Thus, investigating whether wing colors differ between overwintering colonies would provide insight into whether mating advantages vary across the colony sites for butterflies with different wing colors. Only one forewing for each specimen I collected was used for stable isotope measurements, so the digital image analysis used by Davis and colleagues could be applied to these specimens to evaluate wing color. My goal in such an analysis would be to evaluate whether butterflies at different overwintering colonies vary in their wing colors, whether color varies by natal origin as determined by hydrogen and carbon stable isotope profiles, if and how color co-varies with size, and whether color varies by larval condition, as indicated by nitrogen stable isotope profiles. Data from such a color analysis could also inform design of a controlled experiment to evaluate the interaction of temperature and larval nutritional condition on adult wing color and body size.

**Differentiation of the Mexican Overwintering Colonies**

Wassenaar and Hobson (1998) found more animals from the western Great Lakes and Midwest regions than the Atlantic region. Although my sampling regime was similar, my study found a preponderance of butterflies from the eastern portion of their range. This discrepancy may be due to normal year-to-year variation, the period in the
overwintering season in which the collections were made (their collections were in February, mine were in March), or shifts in the distribution of the monarch population amongst the regions of their summer range.

Suitable breeding habitat varies widely throughout the summer range, so understanding how monarchs from different areas within the range arrange themselves within the colonies provides information on the importance of particular parts of the summer range for the monarch population as a whole. I found that the monarchs from different summer ranges sorted into the colonies in different proportions. Since I analyzed dead monarchs, I have no data to indicate whether these proportions hold for monarchs that survive the overwintering season, or whether butterflies that originate from certain summering areas are more likely to perish during the winter, perhaps in a site-specific manner due to microclimate differences among overwintering sites, and different sensitivity to condition based on natal origin. In addition, mortality at the overwintering colonies has often been correlated with the severity of winter storms (Anderson & Brower 1996; Brower et al. 2004; Calvert et al. 1983). Gathering data that show how survival patterns differ at the various colonies could help the authorities of the monarch reserves in Mexico set colony-specific policies for tourism, logging, and agriculture.

My study at the Mexican overwintering colonies used dead monarchs collected from the forest floor near the end of the overwintering season. A logical follow-up study would be to determine whether or not the patterns I observed are reproducible from one year to the next using the same sampling scheme. In addition, it would be useful to understand the association between patterns found in live butterflies and patterns found
in dead ones. An additional question of interest would be if and how differentiation between colonies by natal origin changes throughout the overwintering season.

In addition, although colonies appear to be differentiated from one another with respect to natal origin, there is no obvious pattern in how they are differentiated. I began the study with the null hypothesis that the overwintering colonies would not show differentiation with respect to natal origin. The obvious alternative hypothesis was that there would be a clear pattern—perhaps from north to south in the range reflecting north to south natal origin; or perhaps clustering between colonies by origin, but without clear patterning with respect to the colonies themselves. Instead, butterflies from areas covered by different flyways were present in different proportions at the different colony sites, rather than there being any direct associations between flyways and specific sites.

Clear separation by natal origin would have been particularly interesting to observe, as such a pattern would have lent credence to the hypothesis that monarchs mate non-randomly with respect to natal origin. Of course, that animals are in close proximity does not mean there is mating going on. It remains unclear whether animals mate randomly with respect to geography; my finding of panmixia, along with the findings of previous studies (Lyons et al. 2012; Wassenaar & Hobson 1998), suggests that they do. However, Van Hook (1993) found that non-random, dissortative mating based on size was occurring near the end of the overwintering season. Unfortunately, she did not identify the natal origins of specimens in her study. Capturing animals in copula and analyzing their origins would be the definitive test of hypotheses regarding random mating with respect to geography.
The overwintering colonies may be the basis for panmixia. However, it is also possible that there is high connectivity across the summer range, a possibility that has not been rigorously explored. Applying my approach that integrates genetic and stable isotope analyses to butterflies collected at various sites during the summer breeding season would provide information on how areas within the summer breeding range are connected. Investigating butterflies in the summer range, particularly if studies were timed to sample each successive generation moving northward out of Mexico, could provide detailed data on the connectivity between areas, and would complement the detailed data available for the southward migration (e.g., this study, Brindza et al. 2008; Monarch Watch 2009; Wassenaar & Hobson 1998). In addition, the stable isotope/natal origins approach could be particularly useful at the margins of the summer range, such as along the eastern edge of the Rocky Mountains, the northwestern edge of the Canadian Great Plains, and of course the northeastern Canadian range into the Maritimes. Given the evidence I found of range expansion along the latter northeast margin of the breeding range, it is possible that the summer range has extended through the Rocky Mountains to connect the eastern and western monarch populations.

**Future Directions**

Since I concentrated on regions in eastern North America, similar investigation of adjacent habitats, such as western North America, the Caribbean, Bermuda, and Central America, would indicate the effects of monarch movements in these areas. Substantial vicariant barriers (e.g., the Rocky Mountains and the Sonoran desert) exist that might impede gene flow between monarch populations in eastern and western North America. In addition, the generally accepted paradigm is that migrants from the west coast do not overwinter in the Mexican colonies (Urquhart & Urquhart 1977).
However, no evidence of genetic variation has been found between these regions (Lyons et al. 2012). Using stable isotopes to determine the natal origins of butterflies found at the overwintering colonies on the California coast may provide some insights into the pathways that connect these populations.

A map of carbon stable isotopes for milkweeds throughout the range of Danaus plexippus is an essential tool for continued use of stable isotopes to rigorously study monarch movements throughout their range. The map developed by Hobson, Wassenaar and Taylor (1999) is focused on the eastern breeding range, and was generated from stable isotope data collected for specimens from 33 sites distributed across the range. However, few sites were located either in the southeastern United States or along the margins of the breeding range, and no sites outside the eastern breeding range were sampled. In addition, it would be useful to include data about variation in carbon isotopes for different milkweed species within the same geographic area. Expansion of this map would be a prerequisite to applying my approach to investigate some of the key open questions suggested by my findings.

Additional investigations looking at the fine-scale processes at work throughout the monarch population will expand on the foundation laid by decades of investigation focused on the monarch migration. My findings show that the paradigm of a single migratory population in eastern North America needs to be revisited. The mass migration of the eastern population of D. plexippus overlays fine-scale patterns that indicate complexity in both migratory and breeding behavior. Rather than a monolithic entity, this panmictic monarch butterfly population exhibits subtle differences in space and time that have evolutionary and conservation implications for D. plexippus.
APPENDIX
ISOTOPE FRACTIONATION IN DIFFERENT WING COLORS OF MONARCH BUTTERFLIES (DANAUS PLEXIPPUS)

Introduction

Stable isotopes have been used to as intrinsic markers to study wildlife migration. Because stable isotopes are incorporated into various tissues at different times, certain tissues carry isotopic signatures that reflect the geographic location in which the tissue was created. Bird feathers (Kelly et al. 2008), fish otoliths (Miller et al. 2010) and butterfly wings (Hobson et al. 1999) are all tissues whose isotopic composition does not change after initial deposition. Thus, these tissues can be used to track movements between locations that exhibit different isotopic signatures (e.g., Bowen et al. 2005; Brattström et al. 2008; Clegg et al. 2003; Miller et al. 2011; Szymanski et al. 2007). A key assumption underlying this technique is that samples from a particular type of tissue within a single individual are relatively homogenous, so that the isotopic composition measured in a small sample is representative of the tissue as a whole.

The unique seasonal migration of monarch butterflies in eastern North America has been investigated using analysis of stable carbon and hydrogen isotopes in wing tissue. Because monarch wings are composed chiefly of keratin proteins that are laid down during metamorphosis from larva to adult butterfly, wing tissue reflects the isotopic composition of the larval area (Hobson et al. 1999). In monarch wings, hydrogen isotopes vary in conjunction with the isotopic signature of local meteoric water; carbon isotopes vary depending on the milkweed species on which a larva fed prior to pupation (Hobson et al., 1999). Hydrogen isotope ratios in eastern North America exhibit a roughly southeast-northwest gradient, with increasing depletion of D
to the northwest, whereas carbon isotope ratios show increasing depletion of $^{13}$C moving from northeast to southwest (Figure A-1).

In contrast to the predictable geographic distribution of carbon and hydrogen isotopes, nitrogen isotopes in monarch wings are correlated with the nutritional condition of the larva. Animals enduring starvation are enriched for $^{15}$N relative to $^{14}$N, such that the higher the relative amount of $^{15}$N, the worse the animal’s condition (Gannes et al. 1998). Feeding experiments have not been conducted on monarch butterflies; however, experiments in another Lepidopteran, the Japanese oak silkworm (*Antheraea yamamai*), are consistent with enrichment of $^{15}$N as a signal of response to larval starvation (Mizota & Yamanaka 2011). Across animal taxa, nitrogen isotope enrichment consistently occurs between trophic levels, with higher trophic levels associated with greater enrichment in $^{15}$N (Gannes et al. 1998). Tibbets and colleagues (2008) also found a similar pattern of enrichment pattern between larvae and adults in insects that undergo complete metamorphosis. Thus, higher $\delta^{15}$N ratios are consistently found when nitrogenous compounds are recycled.

A major assumption of previous studies using stable isotopes in monarch tissue (Hobson et al., 1999; Miller et al., 2011; Wassenaar & Hobson, 1998) is that homogenizing wing tissue is adequate to ensure consistent stable isotope compositions for all butterflies from a given area. Monarchs, however, exhibit some degree of natural variation in the intensity of their wing colors, from light to dark orange, and in the ratio of orange to black (Davis 2009; Davis et al., 2007). Fractionation of stable isotopes during synthesis and deposition of pigments involved in these wing colors could therefore
affect stable isotope compositions of wings, but the degree to which this fractionation occurs has not been determined.

The goal of this study was to test several hypotheses regarding isotope fractionation in orange and black monarch butterfly wing tissue. I hypothesized that both carbon and nitrogen would fractionate differently in different colors of monarch butterfly wing tissue. I also hypothesized that these fractionation differences would lead to different inferences about location based on stable carbon isotope ratios. Because stable nitrogen isotope ratios have not shown significant differences in wing tissue between adults raised in similar conditions in previous studies (Tibbets et al. 2008), I further hypothesized that fractionation differences in nitrogen in orange and black segments would be too small to affect inferences about nutritional condition.

Methods

Specimen Selection

To reduce the effects of possible confounding factors such as variability due to milkweed species or local variations in the composition of meteoric water, all specimens used for this analysis were collected from monarch butterflies raised in captivity at the Cockrell Butterfly Center at the Houston Museum of Natural Science. All butterflies were raised in common cages with the same species of milkweed and the same source of local Houston water. In addition, all 13 specimens were from the same generation, so they were exposed to the same meteoric water. Finally, all wing sections were excised from homologous portions of the right hindwing of each specimen to minimize the effects of differences in pigment deposition across the wing (e.g., wing sections adjacent to the body tend to be lighter).
Stable Isotope Analysis

All wing tissues were first washed in a 2:1 chloroform:methanol solution to remove surface contaminants and lipids. Specimens were air-dried; next, orange and black segments (~1.0 mg) were excised for analysis. Segments were loaded into tin capsules, and carbon and nitrogen isotope compositions were measured at the University of Florida Light Stable Isotope Mass Spec Lab. Isotope ratio measurements were corrected by the University of Florida Light Stable Isotope Mass Spec Lab using a calibrated lab standard measured before and after each wing color. $\delta^{15}$N values are given relative to air, with a precision of 0.06‰; $\delta^{13}$C values are given relative to VPDB, with a precision of 0.08‰.

Isotope ratio data were grouped by wing color before analyzing in R (R Development Core Team 2008). Measurements within each group were tested for normal distribution using the Shapiro-Wilk test (Shapiro & Wilk 1965). Variances were compared using F-tests, and Student’s two-sample t-tests were used to compare mean isotope ratios for each wing color.

Results

Corrected stable isotope ratio measurements for the 13 specimens included in this study are shown in Table A-1. Measurements within each color were normally distributed (Shapiro-Wilk, $0.931 \leq W \leq 0.963$, $0.356 \leq P \leq 0.792$), and variances for each color were equal for both carbon ($F_{12} = 1.067$, $P = 0.912$) and nitrogen ($F_{12} = 1.029$, $P = 0.961$). Black wing segments were significantly more depleted in $^{13}$C than orange segments (Figure A-2, $t_{24} = -3.039$, $P = 0.006$), but no significant differences in $\delta^{15}$N were found between wing colors (Figure A-3, $t_{24} = 0.718$, $P = 0.479$).
Discussion

My results are consistent with the hypothesis that carbon fractionates differently in different colors of monarch butterfly wing tissue. However, I found no support for fractionation differences with nitrogen. Although the difference in carbon stable isotope ratios in orange and black wing segments was statistically significant, this difference is generally too small to significantly affect the identification of natal location. Although these differences mean that orange segments could nominally indicate more southerly origins than black segments, the mapping of carbon stable isotope profiles is at a coarse scale (Figure A-1, bands are in 1‰ increments), and the fractionation differences (mean ± standard error = -0.47 ± 0.06‰) fall well below this scale. Thus, homogenized wing tissue is acceptable for identification of larval area, but using wing tissue homogenate may increase the amount of noise in the data. Since the intensity of orange pigments vary in monarch wings (Davis et al., 2007), any fractionation differences due to pigment density would further increase the amount of noise. Black pigments, however, fluctuate little, so using black wing tissue would introduce as little noise as possible into stable isotope measurements.

The fractionation differences in the two colors present in monarch wings raise questions about the kinetics involved in the physiological processes that create and deposit pigments in wing tissue. The wing is composed entirely of keratin proteins, but the pigments embedded in these keratins vary with color. Investigation of the molecular pathways involved in pigment deposition could illuminate the mechanisms underlying the differential fractionation observed in this study. In addition, specimens from wild populations should be analyzed to see if these results hold for wild populations. Finally, hydrogen isotopes should be analyzed in different wing colors to determine if hydrogen
fractionation also occurs in these tissues. If both hydrogen and carbon fractionate differently in wing tissue, then small errors in geographic assignment to larval area may be compounded and lead to misleading inferences. The results of this study indicate this is unlikely to be a severe problem, but repeating the approach to analyze hydrogen in each wing color would provide the data necessary to evaluate the validity of this conclusion.
Figure A-1. Heavy isotope depletion gradients for carbon and hydrogen in monarch wing tissue. Arrows point in the direction of increasing depletion of heavy isotopes ($^{13}$C and D). Orange = carbon, blue = hydrogen. Red star indicates the sampling location at the Cockrell Butterfly Center in Houston, TX. Based on data from Wassenaar and Hobson (1998); hydrogen values modified according to Meehan and colleagues (2004).
Figure A-2. Mean isotope fractionation in different colors of monarch butterfly wings is significantly different for carbon. Values are relative to VPDB. Box limits show first and third quartiles, whiskers indicate $1.5 \times$ inter-quartile range, and horizontal bar indicates median.
Figure A-3. Mean isotope fractionation in different colors of monarch butterfly wings is not significantly different for nitrogen. Values are relative to air. Box limits show first and third quartiles, whiskers indicate $1.5 \times$ inter-quartile range, and horizontal bar indicates median.

Table A-1. Stable isotope data for different colored wing sections.

<table>
<thead>
<tr>
<th>Specimen ID</th>
<th>$\delta^{15}$N (‰)</th>
<th>$\delta^{13}$C (‰)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Black</td>
<td>Orange</td>
</tr>
<tr>
<td>CC.1A</td>
<td>4.13</td>
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<tr>
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<td>1.29</td>
</tr>
<tr>
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<tr>
<td>CC.1D</td>
<td>2.46</td>
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</tr>
<tr>
<td>CC.1E</td>
<td>4.11</td>
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<td>CC.1F</td>
<td>3.86</td>
<td>3.47</td>
</tr>
<tr>
<td>CC.1G</td>
<td>4.53</td>
<td>4.28</td>
</tr>
<tr>
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</tr>
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</tr>
<tr>
<td>CC.1M</td>
<td>3.41</td>
<td>3.02</td>
</tr>
</tbody>
</table>

*aDifference between measurements for black and orange wing sections.
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BIOGRAPHICAL SKETCH

Carol Chaffee is broadly interested in how movement affects the distribution of genetic variation, and how this distribution affects evolution. Migration, dispersal, even daily movement around a local territory can all influence how genetic variation is distributed across an organism’s range. For her dissertation, she investigated how the unique migration behavior of the monarch butterfly (Danaus plexippus) affects the distribution of genetic variation in eastern North America. Carol followed a non-traditional path toward her doctorate. She graduated from the University of California, Berkeley, in 1989 with a Bachelor of Arts in social science, then went on to earn a Master of Arts in communications management at the University of Southern California. Following this, she pursued a career in software engineering with a focus on database design and information management. In 2002, Carol decided to switch career paths, and returned to San José State University to earn a Bachelor of Science in molecular biology with a minor in chemistry. She joined the Doctor of Philosophy program in zoology at the University of Florida in the fall of 2007. Carol plans to pursue a career focused on undergraduate biology education.