A Possible Size-Determined Directional Selection in *Danaus plexippus* (Lepidoptera: Danaidae) with Evidence from Stable Isotope Analysis

by

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ABSTRACT

Cornelia Becker (M.A.)
Department of Ecology and Evolutionary Biology, August 2008
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Each fall, the North American population of monarch butterflies engage in one of the most extensive insect migrations of up to 4,800 km to their overwintering areas in central Mexico. Since monarchs make extensive use of soaring flight and may have to withstand adverse weather condition during the trip, their wing size may influence survival. Computational Fluid Dynamics analysis illustrated that larger wings produce more lift than smaller wings. The question whether there is directional selection for longer wings during the fall migration in eastern North American monarch butterflies was addressed. This was the first study which used the stable isotope technique to analyze monarch butterflies that had been sampled at several locations of their migration routes during the fall. Hydrogen stable isotope ratios in precipitation show a distinct geographic pattern across the North American continent, which is transferred to the monarch wing at the time and place of formation. This allows the assignment of butterflies to the latitude of their natal origin. The analyses indicate that butterflies with longer wings may have originated from farther north, which means that smaller monarchs may be selected against during the migration. Furthermore, monarchs collected at a location in Texas in 2007 had a larger average wing length and originated from higher latitudes than those sampled at the same location in the previous year. In addition, the general applicability of the stable isotope technique to assign monarchs to the site of their natal origin was addressed. Despite variation in

isotope values, the hydrogen stable isotope can be used to determine the latitude of origin.

ACKNOWLEDGMENTS

I would like to thank my committee, Chip Taylor, Joy Ward, and Rudolf Jander, for their time and guidance. My advisor Chip Taylor introduced me to the monarch butterfly, coordinated the monarch butterfly and milkweed sampling with our collaborators, took pictures of monarchs in Mexico for me, and discussed my thesis with me. Joy Ward gave me advice regarding the stable isotope technique and let me use her lab to prepare the wing and plant samples for stable isotope analysis. Rudolf Jander helped me to critically think about papers and was always very nice and encouraging.

I am thankful to Wonjin Jin for collaborating on the gliding simulations with me. He generated the grids and showed me how to use the FLUENT program.

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I am grateful to my parents, my sister, and my grandma for their support and love. My parents made it possible for me to come to KU in the first place. I would like to thank my friends in Germany for staying in touch with me.

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INTRODUCTION

The North American population of monarch butterflies east of the Rocky Mountains (*Danaus plexippus plexippus*) engages in one of the most extensive migrations of all insects. Each fall, they travel southward from their northern breeding grounds to overwintering sites in central Mexico (Calvert and Brower, 1986; Urquhart, 1987) (Figure 1).

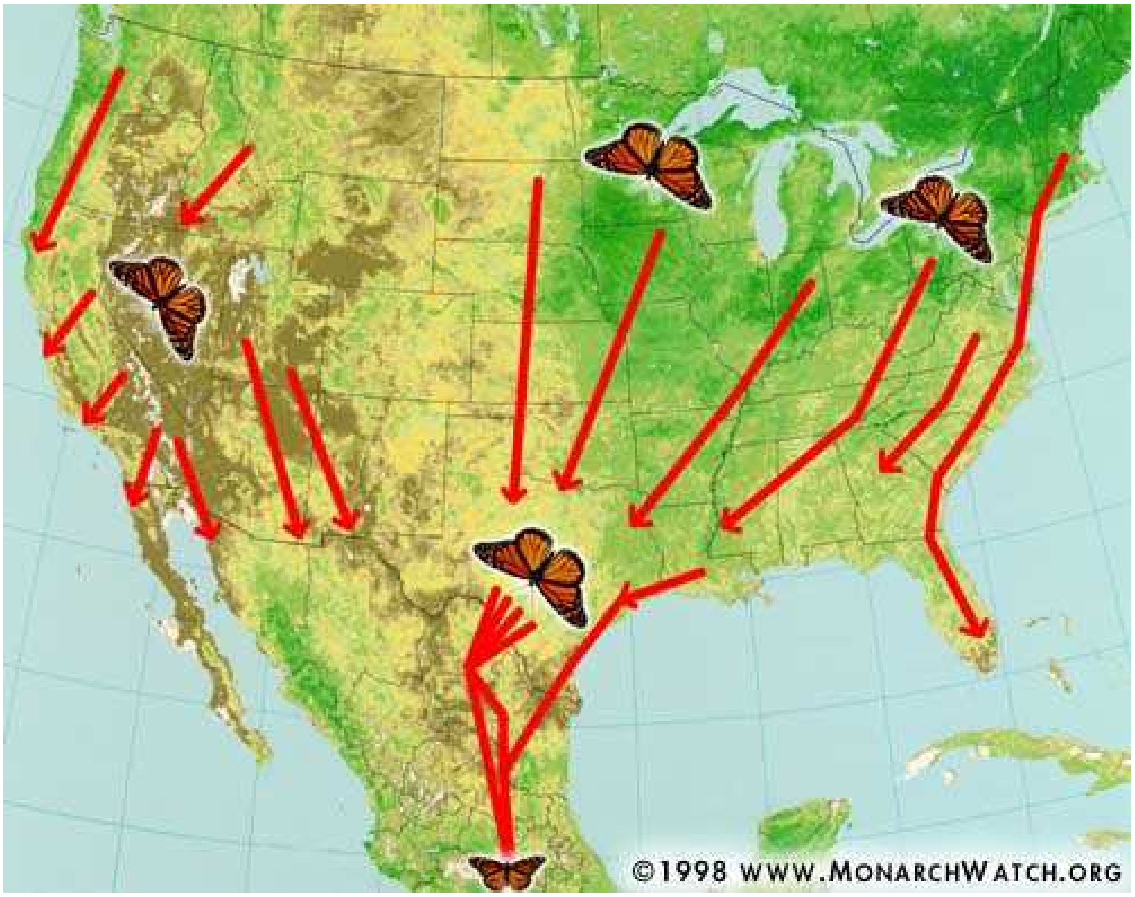

Figure 1. The Fall Migration of monarch butterflies in North America. This figure is an interpretation of the routes taken by monarchs during the fall migration. The pathways are based on tag recoveries and observations of migrating monarchs (Monarch Watch: http://www.monarchwatch.org/tagmig/fallmap.htm Accessed in 2006).

Many butterflies do not survive the migration. The causes of mortality and the percentage of the migratory population that reaches the overwintering sites are unknown. However, the number of tagged butterflies recovered in Mexico suggests that the survival rate is at least 50% and could exceed 70% in some years (O. R. Taylor, pers. com.). The stress of the flight, winds and storms that blow the monarchs off course, predation, accidental deaths (vehicles), and lack of nectar all may contribute to this outcome. Since the migration occurs over a 4 to 6 week interval, referred to as a “migration window”, for each latitude, the timing of the flight for individuals could also influence survival. For instance, individuals in the early phase of the migration survive more often than those flying in later phases (Taylor and Gibo). The size of the individual monarch may also have a role in the probability of survival to reach Mexico, since larger wings allows longer gliding flight. I argue that there is selection for longer wings[1] and for reduced variation in wing length during the fall migration.

Hypotheses and Predictions

I hypothesized that eastern North American monarch butterflies are subject to directional selection during the fall migration which would result in longer wings and stabilizing selection, which would reduce wing variation. To test these hypotheses, I used a different approach from those of previous studies (Arango Velez, 1996; Davis and Altizer, 2003; Dockx, 2007). Instead of contrasting wing lengths of resident, i.e.,

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[1] Wing length is considered as a measure of wing size.

nonmigratory monarchs, against migratory monarchs, I compared wing lengths of monarchs sampled during the fall migration across latitudes and over the period of the migration. This assumes that monarchs are subject to selection pressures during the migration. Specifically, I tested the following predictions that go along with my hypotheses. I will include a review of previous studies which addressed these predictions.

(1) As monarchs move southward, there will be an increase in mean wing length of monarchs at lower latitude because there is a selection for butterflies with longer wings. There will be less variance in wing length in butterflies collected at southern latitudes. However, in a previous study, Borland et al. (2004) found that fall migratory monarchs collected in Texas had shorter wings than those collected in Minnesota and Wisconsin. They recorded forewing length from the point of attachment to the most distal tip. Likewise, Beall and Williams (1945) observed that monarchs collected during fall migrations of different years in Louisiana were shorter than those from farther north in Ontario. They had measured the distance from the proximal costal corner to the most distant point in the apex to the nearest millimeter. On the other hand, monarchs from Minnesota, collected during the migration, had shorter forewing lengths than those collected in Louisiana or Florida (Beall and Williams, 1945). It was not specified, however, during what time of the year the museum specimens from Florida had been collected.

One possible reason for the lack of agreement about the patterns of wing size at high and low latitudes is that the monarchs were sampled during different phases of the migration. This possibility was noted by Gibo and McCurdy (1993) when they found similar disagreements about the pattern of lipid accumulation in monarch butterflies. Accordingly, they controlled for phase of the migration and concluded that patterns in lipid acquisition were largely the result of seasonal changes in the age structure of the population. In Borland et al.'s study (2004), all monarchs from Minnesota and Wisconsin were collected between altitude angles of 62.9 and 52.4, i.e., early in or possibly before the fall migration, whereas monarchs from Texas had been sampled throughout the migration (AA between 70.8 and 39.9). Many studies document a decrease in wing length with date of capture, which might be a partial explanation for the lower mean wing length found in the Texas samples. Gibo and McCurdy (1993) observed that forewing length was similar for early- and middle-phase individuals, but declined in late-phase migrants in Ontario.[2] Borland et al. (2004) found that wing length tended to decrease with date of capture in Texas and in the north (Minnesota, Wisconsin).

Alternatively, if there is no selection but instead larger butterflies outfly smaller ones, I would expect the proportion of small monarchs to increase towards the end of the migration at any location because small butterflies are less likely to migrate as fast as large ones.

(2) Monarchs arriving in the overwintering sites in Mexico have a higher mean wing length than monarchs sampled during the migration. The butterflies with

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[2] Note that migration phases in the study by Gibo and McCurdy (1993) seemed to have been determined arbitrarily and thus differently from the way I determined migration phases. Nonetheless, a division allowed Gibo and McCurdy to detect differences among phases.

shorter wings are eliminated on their way to Mexico, which increases mean wing length.

(3) Monarchs with longer forewing length at any one location originate from farther north than small butterflies. This may indicate that large monarchs survive the migration, whereas small ones do not. Although previous studies established natal origin of monarch butterflies with the help of the stable isotope technique (Hobson et al., 1999; Wassenaar et al., 2000; Dockx et al., 2004), none of these studies examined the relationship of wing size to stable isotope ratio as indicator of place of origin.

In order to address the predictions, I compared the mean forewing lengths and wing variation of monarchs collected in seven different states of the U.S. during the fall migrations of 2001, 2003, 2006 and 2007. Next, I analyzed the hydrogen stable isotope ratio of several subsets of these samples to determine the latitude of their natal origin. Gliding simulations illustrated the potential role of wing size to flight efficiency. Determination of the stable isotope ratio of monarchs and milkweed of known origin explored the applicability of this technique to tracking migratory butterflies.

BACKGROUND

Monarchs

Migration of Eastern North American Monarch Butterflies

The migration of up to 4,800 km starts in mid-August in the northern breeding grounds with monarchs that are biologically and behaviorally different from the summer population (Monarch Watch: http//www.monarchwatch.org/tagmig/index.htm Accessed 2006). The migration advances at rates of close to 43 km per day from mid August to late October, when the first monarchs arrive at the overwintering area in Mexico (Urquhart, 1987; Calvert and Brower, 1986; O. R. Taylor, pers. com.). The Midwestern United States and surrounding areas are the breeding range for 95% of those monarchs that reach Mexico (Wassenaar and Hobson, 1998). Some of the eastern North American monarchs do not migrate to Mexico but instead spend the winter in Florida and Cuba (Dockx et al., 2004).

Since monarch butterflies are exothermal, they only travel at temperatures above 18°C and below 29.5°C (O.R. Taylor, pers. com.). On their way south, monarchs stop to feed on nectar, which they convert to lipids and store in their abdomen for the long flight and the overwintering period. The energy supply of the long-distance migration may be a problem in completing the trip. The average monarch accumulates 140 mg fat (Gibo and Pallett, 1979). From conservative calculations on maximum flying endurance, monarchs seem to have insufficient fuel reserves for the migration. They must frequently stop and feed at flowers to recharge depleted fat reserves, which they use as fuel (Gibo and Pallett, 1979). The butterflies arrive at their overwintering sites in the oyamel fir (*Abies religiosa*) forests (altitude: 2600 to 3600 m; Brower et al., 1977; Calvert and Brower, 1986) between late October and the first week of December. Millions of monarchs cluster in trees and remain there in a semi-dormant stage. In February, they start to mate and migrate back to the southern U.S. where they lay eggs on milkweed, the exclusive host plant of monarch larvae. The parental generation dies during the spring migration, but their offspring complete the roundtrip and migrate back to the breeding grounds of their ancestors (Malcolm et al., 1993).

Determination of Migration Phase

The altitude angle (AA), i.e., the angle of the sun above the horizon at noon, can be used as a means to standardize the migration phase of monarch butterflies observed in different locations. As monarchs move south during the fall migration, the peak of the migration, i.e., the abundance of butterflies traveling, occurs at different dates in different locations. The migration peak in the northern part of the breeding range occurs earlier in fall than the peak closer to Mexico. For instance, in Rochester, MN (latitude 44.02ºN), the migration peak according to AA was from September 4 to 9 in 2006, whereas the migration peaked between September 30 and October 6, 2006, in the more southern city Monticello, AR (latitude 33.38ºN).

Since the timing and pace of migration is probably based on celestial clues (Calvert, 2001; O. R. Taylor, pers. com.), a way to compare the time of migration peaks among sites is by considering the AA (Dively et al., 2004; O. R. Taylor, pers. com.). For the subsequent analyses, I determined all monarch butterflies collected on a day with a maximal AA between 51.0 and 53.0 at the collection locality fell into the middle phase of the migration because the peak of the migration seems to occur when the AA is 51 – 53 (Taylor et al.) Butterflies sampled on days with an AA between 53.0 and 57.0 were grouped into the early phase of the migration and the ones collected on days with an AA below 51.0 were designated to be late migrants. The AA at each collection site was determined using the U.S. Naval Observatory website.

Wing Size

The wing size of butterflies depends on environmental, genetic, and developmental factors. Fischer et al. (2003) found that lower temperatures induced *Bicyclus anynana* butterflies to lay significantly larger eggs. In general, egg size is correlated with other life-history traits such as body size and fecundity (Seko et al., 2006). Seko et al. (2006) found a positive phenotypic correlation between body size and egg size in the migrant skipper *Parnara guttata guttata* (Lepidoptera: Hesperiidae). Moreover, increased temperature during development leads to reduced size in the majority of exothermal organisms (Atkinson, 1994). Specifically, experiments on laboratory-reared migratory and resident monarchs demonstrated that lower temperature of at least 7ºC during larval development results in longer wings (Arango Velez, 1996). Reduced temperature is thought to be the proximate cause of larger body and egg size within insect species at higher latitudes (Chown and Gaston, 1999). However, the opposite relationship between size and latitude has been observed as well, and the decline in body size is ascribed to shorter seasonal development time (Chown and Gaston, 1999). Moreover, it has been proposed that larger individuals may be better able to resist starvation than smaller individuals, which may explain larger body sizes of some insects at higher latitudes, where unfavorable conditions tend to last longer (Chown and Gaston, 1999). Starving queen and monarch butterfly larvae for 40 hours led to significantly shorter wings in adults as compared to starving larvae for only 24 hours or not at all (Arango Velez, 1996). Similarly, a low nutrient content and biomass of the food plants during larval development later in the season may influence growth rate (Langvatn et al., 1996) and lead to shorter wings as well.

Besides these environmental factors, genetic sources are important in wing size regulation of butterflies. Body sizes in male and female *P. guttata guttata* had moderate and high heritability, respectively (Seko et al., 2006). In addition, male monarch butterflies seem to be generally larger than female ones (Beall and Williams, 1945; Borland et al., 2004; Herman, 1988; Monarch Watch: http://www.monarchwatch.org/class/studproj/mass.htm Accessed in 2006 and 2008; Oberhauser and Frey, 1999). Furthermore, previous studies found that migratory monarchs had longer wings than residents (Herman, 1998; Arango Velez, 1996; Davis and Altizer, 2003; Dockx, 2007). These observations were confirmed by my own measurements.

Moreover, interaction among body parts in development is part of the mechanism of wing size regulation. Wings compete for limiting resources with other traits within late-stage larvae (Nijhout and Emlen, 1998). Forewing size in *Precis coenia* (Lepidoptera: Nymphalidae) was significantly larger if imaginal discs, from which other traits like hindwings develop, had been removed.

Selection

Natural selection is nonrandom changes in the frequency of heritable traits in successive generations due to differential survival and reproduction of phenotypes which vary in average fitness. If one extreme phenotype is the fittest, selection is directional, and the mean of the population is shifted toward this extreme phenotype, for instance large wing size. In stabilizing selection, an intermediate phenotype is the fittest, and the variance of the trait is reduced (Futuyma, 1998; Dockx, 2007).

Previous studies have noted a correlation between migration or dispersal ability and wing size and shape in various groups of organisms. Fernández and Lank (2007) found that Western Sandpipers (*Calidris mauri*), which can travel at least 18,000 km in a round trip, have longer and more pointed wings during the nonbreeding season, which is consistent with selection on flight efficiency for longer migration distances. Individuals with longer wings migrated further (O'Hara et al., 2006). Furthermore, Hoffmann et al. (2007) suggested that *Drosophila* with long wings disperse further. Moreover, several presumed migratory butterflies have longer wings than non-migratory ones (Angelo and Slansky Jr., 1984). Arango Velez (1996) showed that migratory monarchs had a higher mean wing length than residents. She concluded that stabilizing selection has eliminated the production of significant variation in migratory monarchs. Likewise, Davis and Altizer (2003) compared wing size among monarchs from eastern North America (migrate the farthest distance), western North America (migrate a shorter distance), and South Florida (nonmigratory) and showed that resident South Florida monarchs had the shortest forewings and eastern North American butterflies the longest. This result corroborates earlier findings which compared eastern North American monarchs to those in western North America and Australia (Tuskes and Brower, 1978; James, 1984). Dockx (2007) found that Cuban migrant monarchs had significantly longer wings than resident monarchs, which supports the hypothesis of directional selection. According to Dockx (2007), migrants and residents in Miami, Florida, (Knight, 1998) and the Americas (Beall and Williams, 1945) showed the same trend. Calvert and Lawton (1993) speculated that larger monarchs may be more likely to survive the rigors of migratory flight.

Arango Velez (1996) demonstrated that reduced wing-length variation was characteristic of four migratory lepidopteran taxa when compared to their resident populations, among them eastern North American monarch butterflies. In contrast, Dockx's (2007) comparison of wing size and shape between migrant and resident monarch butterflies in Cuba revealed no significant difference in variance and thus did not support the action of stabilizing selection. Likewise, Dockx (2007) reported that Van Hook's work (1996) on overwintering monarchs did not support that stabilizing selection acts on wing length.

Stable Isotopes

Monarch butterflies were the subject of the first comprehensive application of the use of hydrogen stable isotope measurements in the study of migratory animals (Hobson, 2008). Stable isotopes in precipitation and plants show patterns across the North American continent, and the monarch butterfly wing chitin reflects the isotopic composition of the place where wings are formed. This makes it possible to track back a monarch to its place of natal origin. First, an isotopic base map of monarchs throughout their breeding range was established during the summer of 1996 (Hobson et al., 1999). This map of isotopic hydrogen and carbon values (Figure 2) was used to assign monarchs sampled in the overwintering sites in Mexico to their natal sites (Wassenaar and Hobson, 1998). It was determined that monarch wintering colonies were panmictic, i.e., made up of a mix of butterflies from all over the breeding range, and composed of individuals originating mainly from the Midwestern United States. The same base map was used to validate that eastern North American monarch butterflies travel to Cuba during the migration period and possibly hybridize with resident populations (Dockx et al., 2004).

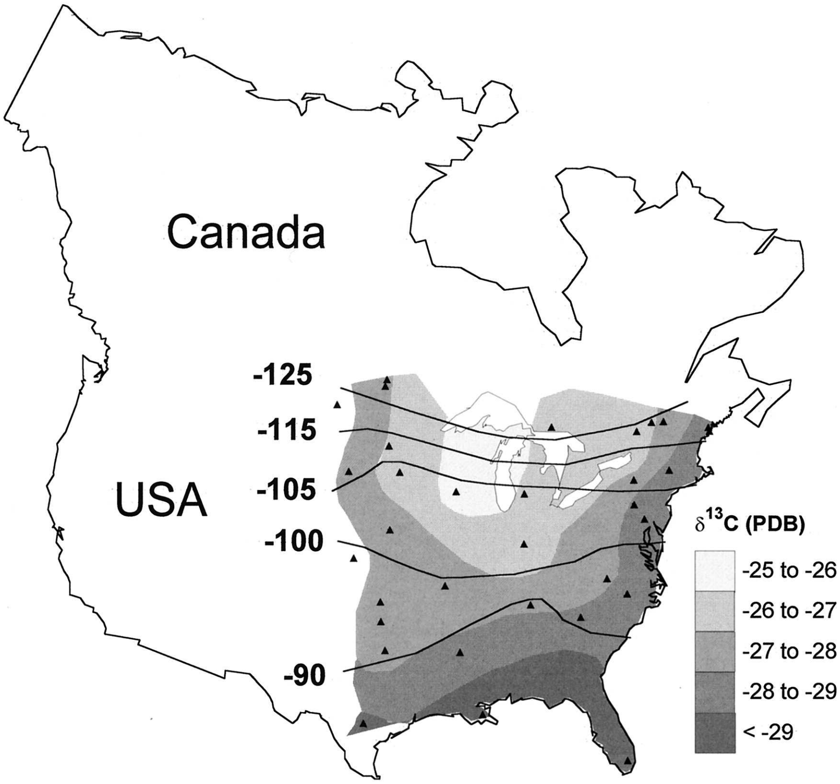

Figure 2. Geographic patterns of δD and δ^{13}C in monarch wings from natal sites across the breeding range of eastern North America. Solid triangles depict field-rearing sites, where monarchs originated that were isotopically analyzed in order to create this map (Wassenaar and Hobson, 1998; synthesized from Hobson et al., 1999).

Stable Isotope Technique

Isotope analysis provides a means to infer natal origins of animals and has many advantages over other tracking methods. It does not rely on the recapture of animals and allows analyses of many individuals because each bears the hydrogen isotope signature of water sources where the analyzed tissue was formed. Moreover, the animals' carbon isotope signature can be traced back to the plants on which they or their prey feed, the stable isotope signature of which is influenced by the local environmental conditions under which the plants grow.

Stable isotopes are naturally occurring stable forms of elements with different nuclear masses (e.g.; deuterium/hydrogen [D/H], carbon [$^{13}C/^{12}C$]). Their ratios, expressed in relation to a standard in δ notation, vary naturally, and I made use of the geographic stable isotope patterns of hydrogen and carbon.

The stable isotope ratios in precipitation and the atmosphere are passed on to milkweed, the exclusive host plant of monarch butterfly larvae. Larvae that feed on the leaves in turn incorporate the isotopic signature into their tissue and thus the butterfly wing tissue. The isotopic ratios are generally passed on with a certain δ offset due to fractionation, which occurs when a chemical reaction or a process results in a changing of the stable isotope ratios of the source or reactant because of the slight chemical differences arising from the subtle differences in mass (Wassenaar, 2008). An increase in the frequency of the light stable isotope is called depletion, whereas an increase in the frequency of the heavy stable isotope is called enrichment. Discrimination is biologically mediated isotope fractionation (Dawson et al., 2002), which is predictable and constant in time and space (Wunder and Norris, 2008).

The $\delta^{13}C$ value in milkweed is influenced by factors such as temperature, humidity, and salinity, and shows a general pattern of enrichment along a southwest to northeast gradient (Hobson et al., 1999). Plant processes, such as metabolic pathways or the response of stomata to water stress, are responsible for the carbon isotope signature.

The δD value in precipitation varies in response to a number of physical and meteorological parameters such as latitude, altitude, distance form the coast, amount of precipitation, and surface air temperature (Dansgaard, 1964; Gourcy et al., 2005). Isotopic fractionation associated with phase changes of water, such as condensation of atmospheric water vapor and evaporation, are the underlying cause for variation of δD (Gourcy et al., 2005). The air masses lose water through condensation as they move along surface temperature gradients (Gourcy et al., 2005). For instance, the hydrogen stable isotope value of precipitation decreases the farther away clouds move from the coast, since the heavier isotope is rained out closer to the coast, leaving a more negative isotope signature of precipitation in the interior of continents (continental effect). Furthermore, the δD value decreases as moist air masses travel from low to high latitudes (latitudinal effect) and from low to high altitudes (altitudinal effect) (Dansgaard, 1964; Meehan et al., 2004). On the other hand, evaporation leads to enrichment of the source water. In North America, these effects lead to a δD pattern that varies with latitude. Through milkweed the stable hydrogen isotope ratio of precipitation is fixed into the monarch butterfly tissue with an offset due to fractionation that occurs during plant carbohydrate synthesis (Meehan et al., 2004).

Since the butterfly wing tissue is metabolically inert, i.e., its composition does not change after it has been formed (Wassenaar, 2008), its stable isotope signature allowed me to trace it back to the location of the milkweed, i.e., the place of the monarch's origin. A spatially interpolated model is necessary to derive a continuous surface over which animals can be assigned to specific locations (Wunder and Norris, 2008). Hobson et al. (1999) empirically modeled geographic hydrogen and carbon isotope patterns of monarch butterflies for eastern North America. They solicited volunteers from throughout eastern North America to raise monarch butterflies from eggs on naturally occurring milkweed, determined the $\delta^{13}C$ and δD of the monarch wings, and interpolated the isotopic pattern to create the map, which was the basis for my analyses[3] (Figure 2).

However, during my analyses, I came across several problems with using the established isotope patterns. Due to interannual climatic variability at different locations and maybe some unknown variables, the base map did not completely align with the stable isotope pattern from the years of my sampling, 2006 and 2007. For instance, the presumed natal origin of several monarchs according to their δD value was south of the sampling sites. It is, however, unlikely that monarch butterflies travel north during the fall migration. Moreover, I found discrepancies between the place of origin that was assigned using δD and the one assigned using carbon stable isotope. One site may be very stable year-to-year over many years, while another might experience highly variable climate and drought. This potential variability seems to be more of a problem for assigning low trophic level organisms like insects to the place of their origin than for assigning species higher up in the food chain to the place of their natal origin. Despite the possible interannual variations, deuterium is the only stable isotope that is a spatially continuous predictor, which allowed me to infer natal origin of monarch butterflies relative to each other. There is no such

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[3] Hereafter referred to as 1996 base map.

assurance for $\delta^{13}C$, which is why I decided to solely concentrate on δD in my analyses (Len Wassenaar, pers. com.).

Aerodynamics

Since it appears that monarchs have insufficient fuel reserves for their trip to the overwintering sites in Mexico, selection should favor any flying techniques that reduce the energy expenditure during cross-country travel and minimize the effects of unfavorable weather (Gibo and Pallett, 1979). The most efficient flying strategy is soaring, which is using rising air to remain aloft while gliding (Gibo and Pallett, 1979; Alexander, 2002). Soaring flight results in substantial energy savings (Gibo and Pallett, 1979). Gliding is using wings to produce lift while descending through the air, but not actively producing any thrust (Alexander, 2002). Lift is a force perpendicular to a wing's motion through the air and usually has an upward component to offset a flyer's weight (Alexander, 2002) (Figure 3). If the force in the upward direction is greater than the flyer's weight, the flyer will rise, if it is equal, the flyer will remain at a constant altitude, and if the upward force is less than the weight, the flyer will descend.

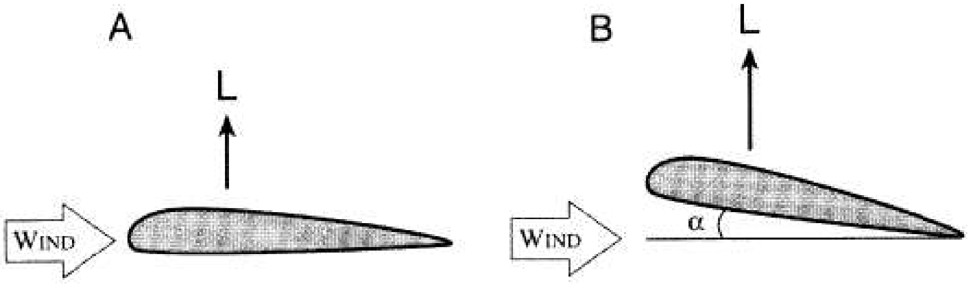

Figure 3. Angle of attack, α. Lift, L. A. No angle of attack, moderate lift. B. As the angle of attack increases, lift also increases (Alexander, 2002).

Monarch butterflies observed in southern Ontario make extensive use of soaring flight during their annual migration to Mexico (Gibo and Pallett, 1979). In a study by Gibo and Pallett (1979), more than 90% of the observed migratory monarchs were using soaring as their main method of flight. The substantial energy savings probably allows them to soar for 1060 hours with the same fat supply they would use for 11 hours of powered flight (Gibo and Pallett, 1979). The glide ratio of monarchs has been estimated as 4:1 (Gibo, 1981), which means that they glide four units horizontally for each unit of descent. Glider pilots reported that monarch butterflies were soaring in thermals at altitudes greater than 1200 m, which would enable them to glide almost 5 km before nearing the ground. Thermals are air masses rising because they are warmer and lighter than the surrounding air (Gibo, 1981).

Since lift is directly proportional to the surface area of a wing, larger wings produce more lift (Anderson, 1989), and maximizing lift allows butterflies to stay aloft longer. Herman (1988), who compared immigrant, local, and emigrant monarchs in Minnesota, suggested that larger wings might be more efficient for soaring and gliding. This may be one reason why emigrant monarchs, which leave the breeding grounds to migrate south, have longer wings.

MATERIALS AND METHODS

Sample Collections

I measured the forewing length and analyzed the stable isotope ratios of monarch butterflies that had been sampled during the annual fall migration and in Mexico. Collaborators and I collected a total of 743 monarch butterflies during the fall migration of 2006 in six different collection sites (Table 1, Figure 4). Harlen Aschen sent me 137 monarchs that he had caught during the fall migration of 2007 at the same Texas site as in the previous year. I photographed each butterfly with closed wings, lying on its right side, against a colored background (Figure 5). I took pictures of the left wings in cases where the right wings were damaged. The camera used was a Nikon Coolpix 995. The sex of each butterfly was noted.

For the purpose of testing the validity of the previous isotope analysis (Hobson et al., 1999), I received monarch butterflies with known place of origin from seven sample sites and milkweed leaf samples from 11 locations, sampled in the fall of 2007 (Table 2 and 3, Figure 6). All butterflies were put in individual glassine or paper envelopes and stored in a freezer until analyzed.

Furthermore, I analyzed the hydrogen stable isotope ratio of 33 tagged monarch butterflies which had been recovered in the Mexican overwintering site Cerro Pelon in March 2003. I obtained the location and date of tagging from the searchable tag recovery database of the Monarch Watch organization (http://www.monarchwatch.org/scgi-bin/search2.pl Accessed June 2008) (Table 4).

In addition, my advisor Orley R. Taylor took photos of 210 monarch butterflies at one monarch overwintering site in the area of Cerro Pelon, Mexico, on February 28, 2007. There were no living butterflies at this colony site; they had probably been killed by cold temperatures in January (O. R. Taylor, pers. com.; Monarch Watch: http://monarchwatch.org/blog/2008/03/21/deforestation-and-monarch-conservation Accessed June 14, 2008). The wing length of those monarchs probably reflect the size of monarchs reaching Mexico since Calvert and Lawton (1993) found that forewing length did not change in samples during immigration and stable phases of the overwintering period between November and mid-February.

Figure 4. Sampling sites of monarch butterflies collected in fall 2006 and fall 2007 and the overwintering site Cerro Pelon in Mexico.

Table 1. Sites, coordinates, dates, migration phase, number (N), sex (F = female, M = male) of individuals collected and collectors of monarchs for wing length measurements and stable isotope analyses. No stable isotope analyses of monarchs from Mexico.

Sample Site (Total N)	**Latitude**	**Longitude**	**Date of Collection**		**Migration Phase**	**N**	**N***	**F**	**M**	**Collectors**
Ames vicinity, Iowa (110)	42.01	-93.37	13-14 Sep	2006	middle	18	18	8	10	Royce Bitzer
			15-Sep - 1-Oct	2006	late	92	91	40	51	
Lancaster, Holtwood, Mountville, Pennsylvania (791)	40.00 and 40.02	-76.18 and -76.26	23-July - 2-Sep	2001	before early phase	31	23	8	23	Gayle Steffey
			4-14-Sep	2001	early	284	284	75	209	
			16-17-Sep	2001	middle	86	86	35	51	
			21-Sep - 10-Oct	2001	late	390**	330	128	260	
Lancaster, Holtwood, Pennsylvania (1047)	40.00 and 40.02	-76.18	19-Aug - 4-Sep	2003	before early phase	106	101	43	63	Gayle Steffey
			6-15-Sep	2003	early	199	197	64	135	
			16-20-Sep	2003	middle	120	120	32	88	
			21-27-Sep	2003	late	622	620	213	409	
Lawrence vicinity, Kansas (282)	38.57	-95.15	11-Sep	2006	early	30	30	16	14	Orley Taylor, Cornelia Becker
			14-Sep - 20-Sep	2006	middle	91	91	45	46	
			23-Sep - 9-Oct	2006	late	161	160	79	81	
Oklahoma City vicinity, Oklahoma (112)	35.28	-97.30	23-Sep	2006	early	40	39	16	23	David Walker
			30-Sep	2006	middle	38	38	17	21	
			7-Oct	2006	late	34	34	16	18	
Monticello vicinity, Arkansas	33.37	-91.47	12-Oct	2006	late	40	40	21	19	Jim Edson
Folly Beach and Seabrook Island, South Carolina (150)	32.49	-79.55	8-Oct	2006	middle	30	30	7	23	Billy McCord
			23-Oct - 5-Nov	2006	late	120	119	43	76	
Port Lavaca vicinity, TX (40)	28.36	-96.37	17-Nov - 28-Nov	2006	late	40	40	12	28	Harlen Aschen
Port Lavaca vicinity, TX (137)	28.36	-96.37	24-Oct - 26-Nov	2007	late	137	137	37	100	H. Aschen
Cerro Pelon, Mexico (210)	19.03	-100.1	photographed 28-Feb 2007	2007	n/a	210	188	n/a	n/a	Orley Taylor

* N used for FWL measurements

** includes two monarchs with unidentified sex

Table 2. Milkweed samples with coordinates and elevation of sampling site. Summer temperature (T) and precipitation (P) measured from June to August for the collection state.

Site	**Latitude**	**Longitude**	**Date of Collection (2007)**	**Collectors**	**δD Value (‰)**	**Summer T 2007 (°C)**	**Summer P 2007 (L/m^2)**	**Elevation (m)**	**Distance to Coast (km)**
Wicklow, Ontario	43.6	-77.58	end of Sep	Don Davis*	-149.2	n/a	n/a	108	435
Wicklow, Ontario	43.6	-77.58	end of Sep	Don Davis*	-143.48	n/a	n/a	108	435
Shell Rock, IA	42.71	-92.58	Fall	Anna Nicholas	-152.74	22.6	425.0	925	1410
Shell Rock, IA	42.71	-92.58	Fall	A. Nicholas	-133.93	22.6	425.0	925	1410
Penitentiary Glen Park, Ohio	41.37	-81.20	2-Oct	Jerry Wiedmann	-132.28	22.3	319.4	202	599
Frewsburg, New York	42.05	-79.16	Fall	Barbara Case	-126.67	19.4	263.5	396	495
Frewsburg, New York	42.05	-79.16	Fall	Barbara Case	-122.84	19.4	263.5	396	495
Painesville, Ohio	41.72	-81.25	28-Sep	J. Wiedmann	-140.09	22.3	319.4	215	618
Western Douglas County, KS	38.54	-95.29	22-Oct	Orley Taylor	-122.49	25.0	300.8	263	1040
Coblentz Marsh, Douglas Co., KS	38.54	-95.3	31-Sep	O. Taylor	-117.08	25.0	300.8	271	1040
Richmond, KS	38.4	-95.26	Fall	Todd Mildfelt	-134.7	25.0	300.8	312	977
Richmond, KS	38.4	-95.26	Fall	T. Mildfelt	-137.02	25.0	300.8	312	977
Union, West Virginia	37.59	-80.54	1-Oct	Ba Rae	-108.01	21.8	322.5	628	465
Greensboro, North Carolina	36.07	-79.79	Fall	Charles Cameron	-126.09	24.9	253.8	266	270
Greensboro, NC	36.07	-79.79	Fall	C. Cameron	-143.62	24.9	253.8	266	270
James Island, SC**	32.44	-79.56	Fall	Billy McCord	-90.23	26.5	306.4	3	0
James Island, SC**	32.44	-79.56	Fall	B. McCord	-106.14	26.5	306.4	3	0

* sent by Rachel A. Powless

** milkweed species *Asclepias curassavica*

Table 3. Monarch butterfly samples with coordinates and elevation of place of natal origin. Milkweed species refer to the plants on which the larvae were raised. Temperature (T) and precipitation (P) were measured from June to August for the collection state.

Site	**Latitude**	**Longitude**	**Date of Collection (2007)**	**Collectors**	**δD Value (‰)**	**Summer T 2007 (°C)**	**Summer P 2007 (L/m^2)**	**Elevation (m)**	**Distance to Coast (km)**
Union Springs, New York	42.84	-76.69	3-Sep	Gary Stell	-119.72	19.4	263.5	125	320
Cohasset, Massachusetts	42.24	-70.80	mailed 22-September	Velma Begley	-108.83	20.1	219.3	6	0
Cohasset, Massachusetts	42.24	-70.80	mailed 22-September	V. Begley	-103.77	20.1	219.3	6	0
Spring Mills, PA	40.54	-77.34	end of Aug	Rose Franklin	-126.68	20.5	341.4	337	267
Spring Mills, PA	40.54	-77.34	end of Aug	R. Franklin	-117.79	20.5	341.4	337	267
Lawrence, KS	38.97	-95.24	14-Oct	Orley Taylor	-101.24	25.0	300.8	263	1040
Lawrence, KS	38.97	-95.24	14-Oct	O. Taylor	-106.52	25.0	300.8	263	1040
Union, West Virginia	37.59	-80.54	17- Oct	Ba Rae	-98.18	21.8	322.5	628	465
Greensboro, North Carolina	36.07	-79.79	Fall	Charles Cameron	-81.95	24.9	253.8	266	270
James Island, SC*	32.44	-79.56	18-Sep	Billy McCord	-90.69	26.5	306.4	3	0
James Island, SC**	32.44	-79.56	25-Sep	B. McCord	-99.42	26.5	306.4	3	0
James Island, SC***	32.44	-79.56	6-Sep	B. McCord	-104.21	26.5	306.4	3	0
James Island, SC	32.44	-79.56	6-Sep	B. McCord	-109.71	26.5	306.4	3	0
James Island, SC***	32.44	-79.56	28-Sep	B. McCord	-96.32	26.5	306.4	3	0
James Island, SC****	32.44	-79.56	29-Sep	B. McCord	-101.46	26.5	306.4	3	0
James Island, SC****	32.44	-79.56	29-Sep	B. McCord	-72.56	26.5	306.4	3	0
James Island, SC****	32.44	-79.56	30-Sep	B. McCord	-71	26.5	306.4	3	0

Milkweed species: * emerged indoors on *Seutera augustifolia*
** emerged indoors on *Asclepias tuberose*
*** *Asclepia curassavica* exlarvae
**** *Seutera augustifolia*

Table 4. Tagging and δD information of monarch butterflies recovered in Mexico in March 2003. Report City was Cerro Pelon, Mexico, in all cases. * indicates replicates.

δD Value (‰)	Tag Code	Tag State	Tag City	Tag Date	Report Date
-142.51	AGB 064 JM	MN	Cannon Falls	8/17/2001	3/5/2003
-139.32	AJR 308 LM*	KS	Wamego	9/19/2001	3/5/2003
-136.63	AJR 308 LM	KS	Wamego	9/19/2001	3/5/2003
-135.69	BDI 063 JM	IA	Grand Mound	9/30/2002	3/5/2003
-134.88	AHL 135 LM	n/a	n/a	n/a	3/5/2003
-132.88	AGY 088 JM	TX	Del Rio	10/12/2001	3/5/2003
-130.93	AIC 183 JM	n/a	n/a	n/a	n/a
-130.66	ACF 469 LM	MI	Grand Rapids	9/12/2001	3/5/2003
-125.94	AHW 806 LM	SD	Baltic	9/2/2001	3/5/2003
-125.85	AND 788 LM	n/a	n/a	n/a	n/a
-124.61	ACY 086 JM	MN	Rochester	8/16/2001	3/5/2003
-123.96	ACS 643 LM	IA	Dysart	9/2/2001	3/5/2003
-120.88	ACS 643 LM*	IA	Dysart	9/2/2001	3/5/2003
-123.62	ABY 529 LM	n/a	n/a	n/a	n/a
-121.69	ADS 268 LM	MN	Campbell	8/19/2001	3/5/2003
-120.69	ADF 318 LM	NE	Hastings	8/23/2001	3/5/2003
-119.56	AJP 528 FE	TX	Dripping Springs	n/a	3/5/2003
-118.6	AHR 319 LM	IA	Carroll	9/5/2001	3/5/2003
-116.85	AIP 578 LM	NE	Hebron	9/18/2001	3/5/2003
-116.59	AFK 776 LM	TX	Del Rio	10/13/2001	3/5/2003
-114.91	BIX 404 JM	ON	Grand Bend	10/9/2002	3/5/2003
-114.61	ACD 460 LM	n/a	n/a	n/a	n/a
-113.34	YL 495 LM	MN	Rochester	9/5/2001	3/5/2003
-112.9	AHM 717 JM	n/a	n/a	n/a	3/5/2003
-111.64	ACC 872 LM	KY	Henderson	9/15/2001	3/5/2003
-111.3	AFT 779 JM	OK	Oklah. City	n/a	3/5/2003
-110.44	ACK 810 LM	IA	Jesup	9/2/2001	3/5/2003
-104.88	ACK 810 LM*	IA	Jesup	9/2/2001	3/5/2003
-108.16	AJZ 175 JM	KS	Lawrence	9/15/2001	3/5/2003
-107.92	ADJ 678 LM	MN	Maplewood	9/10/2001	3/5/2003
-98.21	BER 165 JM	IL	Lomax	9/11/2002	3/5/2003
-94.47	AEI 063 JM	KS	Lawrence	9/15/2001	3/5/2003
-91.9	BKL 065 JM	Mexico	Monterrey	10/5/2002	3/5/2003
-90.64	AJR 453 JM	KS	Wamego	9/20/2001	3/5/2003
-83.9	AGF 822 JM	IA	Tripoli	9/12/2001	3/5/2003
-81.81	ADA 083 JM	WI	Green Bay	9/7/2002	3/5/2003

Figure 5. Wing length measurement (white line) of a monarch butterfly collected in Kansas in 2006 with the program ImageJ.

Figure 6. Sampling sites of milkweed and monarchs of known origin collected in 2007.

Wing Length Measurements

I measured the forewing length (FWL) of all photographed monarch butterflies using ImageJ 1.37v software (National Institutes of Health, downloaded at http://rsb.info.nih.gov/ij/). This software for scientific image processing and analysis allows one to draw a line onto a picture and gives the length of the line. I calibrated the line with a ruler that I included in the pictures when I photographed the monarchs. This procedure allowed me to measure the wing lengths to the nearest 0.1 mm. The forewing was measured from the white spot at the wing base on the underside of the wing to its apex (Figure 5). Where the rim of the apex had white spots, I took the length to the black rim. In some butterflies, the apex was torn away, so wing length could not be obtained.

In addition to these samples, I considered the wing size of monarch butterflies which were sampled in Pennsylvania in 2001 and 2003[4] before and during the fall migration (Table 1). The wing length in these data was measured to the nearest 1 mm. Gayle Steffey shared this information with me.

Stable Isotope Analyses

Several preparatory steps were necessary in order to ready wing and milkweed samples for stable isotope analysis. I chose different sample sizes for each subset of analysis. For the determination of δD and $\delta^{13}C$ of wings of monarchs collected during the fall 2006 migration, I chose 10 monarchs from three states, Iowa, Oklahoma, and

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[4] Hereafter referred to as Pennsylvania 2001 (PA2001) and Pennsylvania 2003 (PA2003) monarch butterflies or collectively as Pennsylvania (PA) butterflies.

South Carolina[5], sampled in the middle migration phase. Furthermore, I selected 10 butterflies from Arkansas as well as 10 from Texas[6] collected during the late phase of the migration and 50 monarchs collected in Kansas[7] throughout the migration. Since I intended to determine whether the size of monarchs depends on their natal origin, I picked large, middle-sized, and small butterflies in each sample by eye.

Second, I compared the natal origin of monarch butterflies sampled in Oklahoma and Kansas between collection sites as well as between the first and second half of the migration in 2006. In order to compare the relative migration periods between the two localities, I divided the samples according to AA. Samples collected on days with AAs above 52 were considered the first migration half, those below 52 the second migration half. I did not select monarchs collected in Kansas on days with an AA greater than 54.3, which was the maximal AA in the Oklahoma samples. Moreover, I did not consider monarchs sampled on days with an AA lower than 48.9, which was the minimal AA in butterflies from Oklahoma. This helped to maintain comparability between migration periods considered. Using the statistics program Minitab, I randomly selected 20 monarch butterflies from Oklahoma and Kansas from the first migration half respectively and 20 monarchs from the second migration half respectively, totaling 80 samples. For those wing samples, I was interested in the δD value only. Next, I compared the δD value of monarchs collected

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[5] Hereafter referred to as Iowa (IA), Oklahoma (OK), and South Carolina (SC) monarch butterflies.

[6] Hereafter referred to as Arkansas (AR) and Texas (TX) monarch butterflies.

[7] Any monarch collected in Kansas hereafter referred to as Kansas (KS) monarch butterflies.

in Texas in 2006 to those collected in 2007[8]. For this, I randomly selected 20 monarch butterflies from each year.

In addition, I analyzed the δD value of all tagged monarchs recovered in Mexico in 2002 as well as the milkweed and monarchs from 2007 of known origin. All butterflies that were selected multiple times for stable isotope analysis were only analyzed once. A few replicates, however, tested the repeatability of the analysis.

Before the analysis, I cleaned surface lipids from the wings using a Soxhlet apparatus (Soxhlet, 1879) because the isotopic composition of the lipids can be different from the wing tissue (Wassenaar, 2008). For this procedure, pieces of forewing and hindwing tissue from each selected sample were punched out using a paper punch. This technique can be used instead of grinding the whole wing and obtaining a homogenous powder because there is no intra-sample hydrogen isotopic variation in monarch butterfly wings (Len Wassenaar, pers. com.). The wing tissue of each monarch was placed into a labeled thimble made of thick filter paper, which was sealed with an Impulse Sealer, and put into the main chamber of the Soxhlet apparatus. The samples were rinsed with a 2:1 chloroform:methanol solution for 24 hours before being air-dried (Hobson et al., 1999; Dockx et al., 2004). The milkweed leaves were oven-dried at 70ºC for 24 hours and ground with liquid nitrogen.

For determining carbon stable isotope values, I weighed 40 to 50 μg of wing tissue with a Mettler UM3 scale and sealed it into 3x5 mm tin capsules. The analysis was conducted with a continuous-flow, gas-source mass spectrometer (Finnigan MAT

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[8] Hereafter referred to as Texas 2007 (TX2007) monarch butterflies.

253 Isotope Ratio Mass Spectrometer) coupled to an elemental analyzer (Costech 4010 model) in the W.M. Keck Paleoenvironmental & Environmental Stable Isotope Laboratory at the University of Kansas. For the analysis of the stable isotope ratio of deuterium in wings and milkweed, 400 to 600 μg of each sample was weighed with a Mettler UM3 and a Mettler Toledo scale, sealed into a 3x5 mm silver capsule, and sent to the Washington State University Laboratory for Biotechnology and Bioanalysis. There, the samples were equilibriated with a keratin standard developed by Wassenaar and Hobson (2003). Usually, the exchangeable hydrogen in the wing tissue exchanges readily and uncontrollably with ambient water hydrogen; however, through equilibration, any alterations to the δD signature of the wings were accounted for. Samples for hydrogen isotopic analysis were converted to CO and H_2 with a pyrolysis elemental analyzer (TC/EA, ThermoFinnigan, Bremen); these two gases are separated with a GC column (0.6m x ¼" x 4.0 mm, molecular sieve 5A, Varian) and analyzed with a continuous flow isotope ratio mass spectrometer (Delta PlusXP, Thermofinnigan, Bremen).

Stable isotope ratios were calculated relative to reference standards (Vienna Standard Mean Ocean Water (VSMOV) for hydrogen and Vienna Pee Dee Belemnite (VPDB) for carbon) using the following formula:

δD or $\delta^{13}C$ (‰) = $[(R_{sample}/R_{standard}) - 1] \times 1000$

where R = D/H (deuterium/hydrogen) or $^{13}C/^{12}C$.

Statistical Analyses

Wing Length Measurements

To test the hypothesis that there is directional selection for size in monarch butterflies during the fall migration, I performed several statistical analyses. In order to determine whether mean wing sizes were different among sampling localities, I carried out a Kruskal-Wallis Test with FWL of all monarchs as the response variable and state of collection, including Mexico, as the predictor variable.

Next, I regressed FWL against latitude of collection site and FWL against longitude of collection site. Previous studies had not taken into account that there may be differences in forewing length among migration phases. In order to correct for these possible differences, I divided the forewing length according to the three migration phases and repeated the regressions of FWL against latitude and FWL against longitude in each group.

Last, I repeated the above analyses with the data from PA included in the dataset. Only monarchs collected on dates with AA of 57.0 or smaller were considered, since this AA is correlated with the beginning of the migration (O. R. Taylor, pers. com.).

After that, I compared the FWL of monarchs sampled in the U.S. during the fall migration of 2006 to the FWL of monarchs photographed in the overwintering site Cerro Pelon in February 2007. In order to avoid bias due to different sampling sizes, I used the mean FWL of each collection site and tested for differences between samples from the U.S. on the one hand and Mexico on the other hand with a one-way ANOVA.

In order to test the hypothesis of stabilizing selection on FWL, I compared the variability in FWL among states. I used the coefficient of variation as a measure of variability (CV = (standard deviation*100)/mean). It allows the comparison of variation, even when dealing with samples having different means, because it is thought to be independent of sample means (Sokal and Rohlf, 1995; Arango Velez, 1996).

Forewing length among the three migration phases of all monarchs collected during the fall migrations of 2001, 2003, 2006 and 2007 were compared. Several Kruskal-Wallis tests and one-way ANOVAs assessed whether FWL differed among migration phases for the entire migration and among states. A simple linear regression showed how much of the variation in forewing length could be explained by variation in AA, which is a measure of migration phase. The analyses were performed first with and then without the data from PA. I performed a one-way ANOVA and a nonparametric Kruskal-Wallis test in each case where one of the assumptions of an ANOVA (normality, homogeneity of variances) was violated. Both tests resulted in the same outcome at a 5% significance level, which justifies why I accepted the results. In cases where both assumptions were violated, I used the Kruskal-Wallis test only.

Last, a Student's t-test assessed whether the FWL of resident monarchs, i.e., monarchs collected at an AA of 57.1 or below, had a significant different mean wing size from migrants in the PA 2001 and PA 2003 samples. Even though it is possible that some of the monarchs assigned as residents were migrating, the AA is probably the most reliable means to time the start of the migration when observations are not possible. The Student's t-test tested whether mean wing lengths differed between male and female monarchs.

Stable Isotopes

A one-way analysis of variance (ANOVA) tested whether the δD values were different among states. Then, I divided FWL in groups according to δD values, with the most negative δD values indicating origin at higher latitudes and the least negative δD values origin at lower latitudes. A one-way ANOVA assessed whether there were differences in mean FWL among groups for the entire migration and within each migration phase. Moreover, I calculated the CV and used a one-way ANOVA to test for differences in mean FWL among migration phases in each δD group. Next, I performed simple linear regression analyses with FWL as predictor variable and δD value as response in order to test the hypothesis that butterflies are selected for larger size during the fall migration. Moreover, I compared FWL and δD within the samples from KS and OK and between the TX, and TX2007 samples with a Student's t-test. Last, I compared the mean FWL of monarchs among groups divided according to δD values in the KS sample and the OK sample respectively with a one-way ANOVA.

In order to evaluate the relationship between hydrogen stable isotope and latitude or longitude respectively, I regressed the δD value of monarchs sampled in 2007 against the latitude of their place of emergence and against the longitude of their place of emergence respectively. I repeated this procedure with the δD value of milkweed collected in 2007. Next, I regressed the δD value of monarchs sampled in 1996 against the latitude of their place of emergence. These values were the basis for the original 1996 base map of monarch hydrogen stable isotopes across North America. Next, I compared the slopes of the two regression lines as follows (Sokal and Rohlf, 1995):

$$F_s = \frac{(b_1 - b_2)^2}{\frac{\sum x_1^2 + \sum x_2^2}{\left(\sum x_1^2\right)\left(\sum x_2^2\right)} \bar{s}^2_{Y \cdot X}} = 0.1560$$

where $x^2 = (X - \overline{X})^2$ and $\bar{s}^2_{Y \cdot X} = \frac{SS_{error(1)} + SS_{error(2)}}{b_{0(1)} + b_{0(2)} - 4}$

Since $F_s < F_{0.95[1, -52.4]}$ the two groups of data were sampled from populations of equal slopes. In order to estimate $F_{0.95[1, -52.4],}$ I read the following values from and F-table: $F_{0.95[1, 52]} = 4.0266$ (downloaded at Learning by Simulations: http://www.vias.org/simulations/simusoft_distcalc.html).

Moreover, since hydrogen stable isotope values in precipitation are influenced, among other things, by temperature, the amount of precipitation, altitude, and distance from the coast (Dansgaard, 1964), I explored whether these variables contribute in explaining a variation in the δD value of monarch wings and milkweed. Three sets of data were considered:

(1) the isotope data of monarchs sampled in 2007 at places of natal origin,

(2) isotope data of milkweed collected in 2007, and

(3) the hydrogen stable isotope data as published by Hobson et al. in 1999, with which the 1996 base map was created (Figure 2).

First, I looked up the mean temperature and mean precipitation amount from June to August of the U.S. states in which the butterflies and milkweed had been sampled in the summer of 1996 and the fall of 2007 respectively (National Climatic Data Center: http://lwf.ncdc.noaa.gov/oa/climate/research/cag3/state.html Accessed June and July 2008). Samples from seven locations in Canada were not included into the analyses because no mean summer temperature and precipitation could be obtained. Second, I obtained elevation information from the Geographic Names Information System (http://geonames.usgs.gov/pls/gnispublic/f?p=136:1:11627204772379933580::NO::: Accessed July, 2008) and with Google Earth (downloaded at http://earth.google.com/). The distance to the nearest coast was measured with the geographic information system software DIVA-GIS 5.2 (downloaded at http://www.diva-gis.org/down.htm). This included uploading the coordinates of the sampling sites and measuring the distance between sampling site and nearest coast with the distance tools. This approach did not take the direction of the movement of air masses into account. Next, I regressed the δD value of monarchs sampled in 2007 against each of the five obtained variables separately and repeated the same with the data from 1996.

After that, I performed a multiple linear regression with latitude, longitude, mean summer temperature, mean summer precipitation amount, elevation, and distance to the nearest coast as predictor variables and δD as response variable on each of the three datasets. I used a best subset regression to choose the regression equation with the variables which explained variation in δD best. Last, I tested whether temperature and latitude as well as precipitation amount and latitude were linearly related.

All statistical analyses were performed in the statistical software program Minitab 14 with a maximal Type I error rate of 0.05. The non-parametric Kruskal-Wallis test was used instead of an analysis of variance in all cases where the residuals of forewing length neither had equal variance, nor followed a normal distribution, and could not be transformed to have one.

Gliding Simulations

Computational Fluid Dynamics (CFD) analyses were carried out with the computer program FLUENT 6.2.12[9], which is used in aerospace engineering. These analyses illustrated how wing size influences lift production during gliding. Furthermore, manipulating parameters such as the angle of attack (AT) and wind velocity (V) allowed me to compare potential flight performance of monarchs under varying conditions. The angle of attack α is the angle between the wing’s chord and

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[9] Fluent Software Package, Ver. 6.2.12, 2005, Fluent Inc. Lebanon, NH.

the direction of movement, and lift increases as the angle of attack increases (Alexander, 2002) (Figure 3). The chord is the length between the forward most point of a wing and the farthest rearward point, in a plane parallel to the animal's long axis (Alexander, 2002) (Figure 7). For these experiments, aspects which influence lift production such as wing scales, which increase lift, hairs, which decrease lift (Nachtigall, 1967), the effect of the butterfly body (including head, thorax, and abdomen), wing venation, and dihedral could not be factored in. The dihedral is the upward angle of the wing as it goes further away from the body (Gibo, 2000). However, the relative effects of different wing sizes, angles of attack, and wind velocities can be compared.

I obtained 35 coordinate points of the right wing of a monarch butterfly by tracing its outline on millimeter paper. As a stencil I used the picture of a monarch with its wings in gliding position. The coordinates allowed Wonjin Jin, Ph.D. candidate in aerospace engineering, to generate 3-dimensional unstructured grids for all butterfly wing geometries at three different angles of attack using GAMBIT 2.2.30[10] software. A grid is an arrangement of discrete points throughout the flow field over which calculations are made (Anderson Jr., 1995). A total of 1,031,663 tetrahedral cells with 2,101,939 triangular faces were dedicated for each grid generation (Figure 8). The outer walls were defined and the walls were located far from the wing model in order not to cause wall effects on the model. The grids were opened in FLUENT 6.2.12 [3d, segregated, lam].

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[10] Gambit Software Package, Ver. 2.2.30, 2004, Fluent Inc. Lebanon, NH.

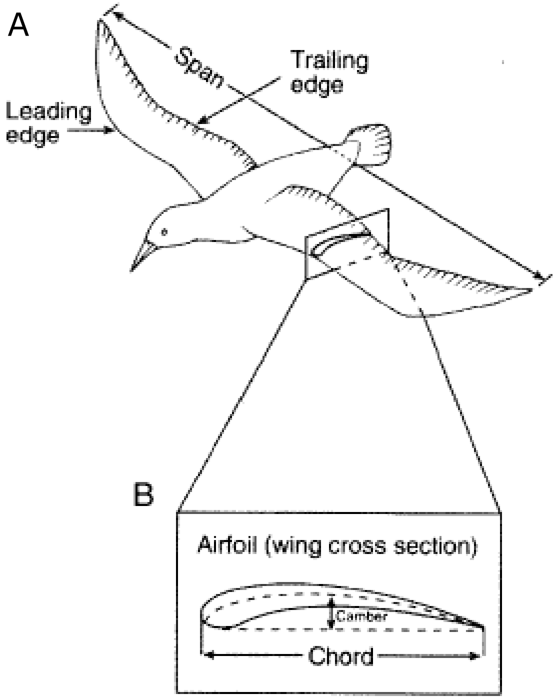


Figure 7. Wing and airfoil terminology. The cross section of a wing is shown in B (Alexander, 2002).

Wonjin Jin and I chose the following values for the three parameters of the flight simulations, where all possible combinations were tested, giving a total of 27 (3^3) simulations. Angle of attacks of 5º, 7º, and 10º were tested. Nachtigall (1967; in Goldsworthy and Wheeler, 1989) found that the best gliding numbers of butterflies and moths seem to be 2.3 to 4 at angles between 5 and 15 degrees. Even though FLUENT can theoretically simulate glide with wings held at an AT of 15º, the error at this angle is too big, and no meaningful lift coefficients could be obtained.

For size we assigned forewing lengths of 51.21 mm, the mean wing length of all monarchs sampled in 2006 and 2007, 39.9 mm, the forewing length of the smallest monarch sampled, and 57.9 mm, which was the largest monarch from the migration collections. FLUENT calculated the projected surface areas, i.e., the wing areas (A) with a little deduction for the angle of attack, of these three wing lengths for both wings (Table 5). The areas were similar to values which Arango Velez (1996) measured with a leaf-area meter. She found a mean wing area of 34.81 cm^2 at a mean wing length of 52.26 mm for migratory monarchs and an area of 31.94 cm^2 at a wing length of 49.13 mm for residents.

As headwind velocities, we used 5 m/s, 10 m/s, and 15 m/s. In his aerodynamic measurements on butterflies in a wind tunnel, Nachtigall used velocities of 1-3 m/s (1967). For Scarce Swallowtails (*Iphiclides podalirius*), he observed gliding velocities of 1-10 m/s in nature. Again, due to a large error in our simulations at a wind velocity of 1 m/s, no lift coefficients for this velocity could be obtained. Ambient temperature for the simulations was set to 297 Kelvin or 23.85°C, which lies in the middle of the temperature range in which monarchs migrate. Wonjin Jin chose several other parameters, and I set them as well (Table 6).

Table 5. Wing measurements and scaling factors for the three body sizes.

Relative Forewing Length	**78%**	**100%**	**113%**
Forewing Length (mm)	39.9	51.21	57.9
Chord = Width of Wing from Front to Back (mm)	39.12	50.2	56.77
Upper Wing Area for Both Wings (cm^2)	21.47	35.36	45.22
Wing thickness (mm)	0.031	0.040	0.045
Scale Factor for Scaling Grid to Size of Monarch Wing	0.000401647 * 0.7793	0.000401647	0.000401647 * 1.1309

Table 6. Parameters set in FLUENT for gliding simulations.

Energy	Energy Equation on	
Viscous Model	k-epsilon (2 eqn), realizable	
Residual Monitors	continuity	
	x-velocity	Convergence Criterion: 0.0001
	y-velocity	Convergence Criterion: 0.0001
	z-velocity	Convergence Criterion: 0.0001
	energy	Convergence Criterion: 0.0001
	k	Convergence Criterion: 0.001
	epsilon	Convergence Criterion: 0.001
Force Monitors	Wall Zones	wing surface
	Drag Coefficient	Force Vector X: 1
		Force Vector Y: 0
		Force Vector Z: 0
	Lift Coefficient	Force Vector X: 0
		Force Vector Y: 0
		Force Vector Z:1
Boundary Conditions	Zone: inlet	Velocity Magnitude (m/s): 5 or 10 or 15
	Type: velocity-inlet	Temperature (K): 297
		Turb. Kinetic Energy (m^2/s^2): 0.1
		Turb. Dissipation Rate (m^2/s^3): 1
	Zone: outlet	Gauge Pressure (pascal): 0.1
	Type: pressure-outlet	Temperature (K): 297
		Turb. Kinetic Energy (m^2/s^2): 0.1
		Turb. Dissipation Rate (m^2/s^3): 1
Reference Values	Area (m^2)	0.002147 or 0.003536 or 0.004522
	Length (mm)	39.12 or 51.2 or 56.77
	Temperature (K)	297
	Velocity (m/s)	5 or 10 or 15

The calculation for each simulation case was run for half an hour to six hours, or between 150 and 850 iterations, until all residuals for the lift coefficient and several other parameters became very small (approaching 0.001) and approached constant values (Figures 9 and 10). For each calculation, the PCs at the Eaton Hall computer lab in the KU School of Engineering had been used. Each computer equips a 3.4 GHz-Intel® Pentium® 4 processor and 2.047 GB of RAM.

Then, lift coefficients and contours of static pressure were obtained and lift production was calculated using the formula (Young et al., 1997):

$$L = C_L \text{ x } 0.5 \text{ x } \rho \text{ x } v^2 \text{ x } S$$

where L = lift force (N)
C_L = lift coefficient (non-dimensional)
ρ = the density of the air at sea level = 1.225 (kg/m^3)
v = velocity (m/s)
S = projected surface area (m^2)

The resultant lift forces were plotted against AT and wind velocity at three different wing areas.

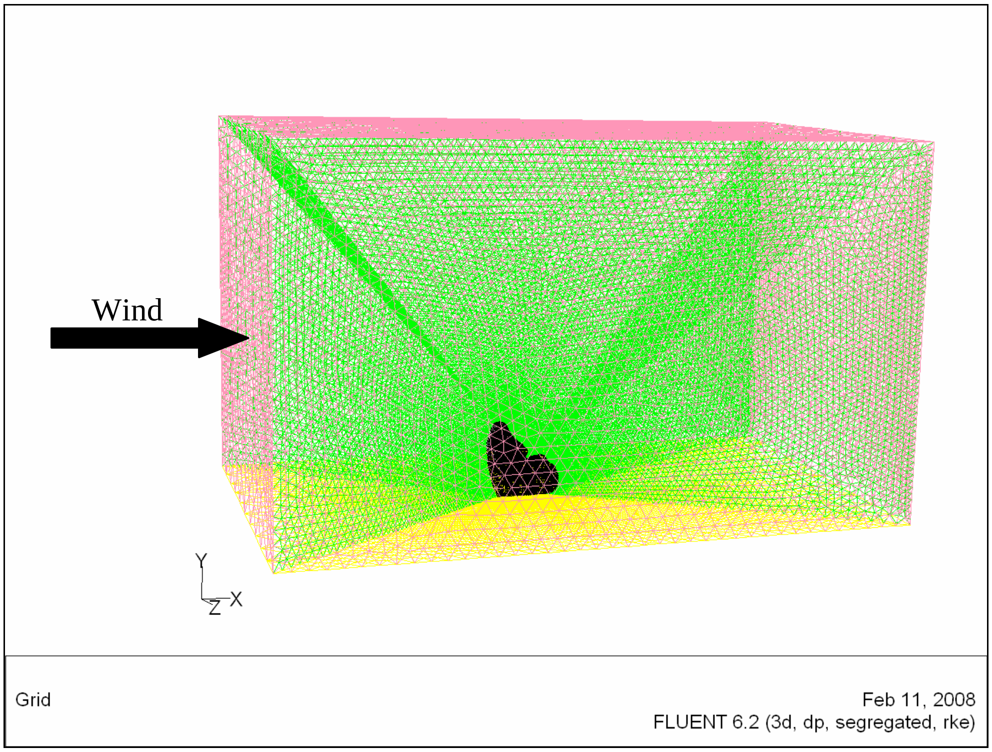


Figure 8. Mesh generation of the right monarch wing.

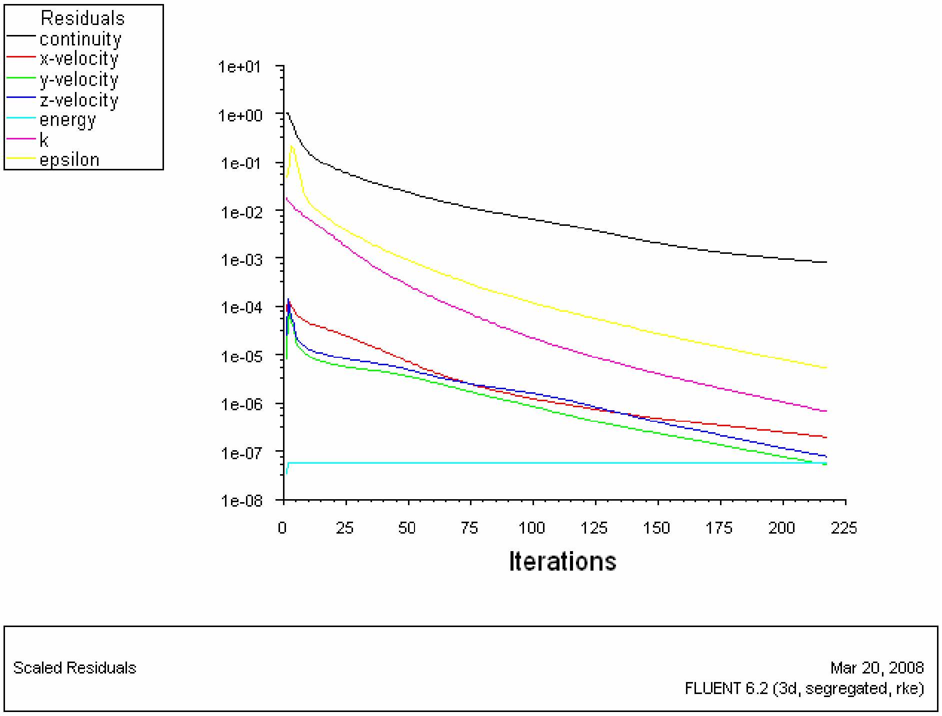


Figure 9. Residuals, i.e., error, of several parameters plotted against number of iterations.

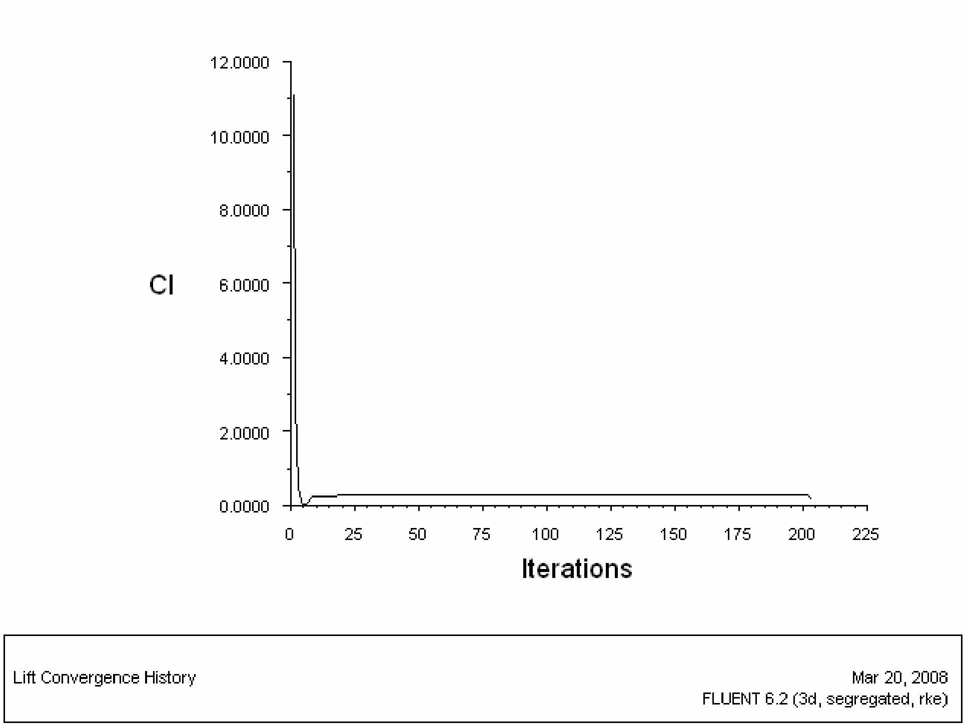


Figure 10. Lift Convergence History. The value for the lift coefficient (Cl) converges against a constant value. This is an example for the case of AT = 5º, velocity = 15 m/s, and area = 113%.

RESULTS

Wing Length Measurements

Directional Selection

The first prediction of an increase in mean size of monarchs at lower latitudes was not well supported. The mean FWL in monarchs photographed in Mexico in 2007 was 51.79 mm and slightly longer than the average of the mean FWL of monarchs sampled in the six sites in the U.S. during the fall migration of 2006 (51.3 mm). The difference, however, was not significant (adjusted $R^2 = 0.0\%$, $F_{1,5} = 0.09$, $p < 0.776$).

There were highly significant differences in FWL of butterflies among sample sites, including a comparison with the Pennsylvania and Mexican monarchs (Kruskal-Wallis test, adjusted for ties: without PA: $H_6 = 87.57$, $p < 0.001$; with PA: $H_8 = 236.64$, $p < 0.001$; without PA, with MX: $H_7 = 96.71$, $p < 0.001$, with PA and MX: $H_9 = 242.47$, $p < 0.001$).

There was a significant association between FWL and latitude as well as between FWL and longitude in several groups of migration phases (Table 7 and 8). In general, there seemed to be a slight decrease in FWL with decreasing latitude and a slight decrease in wing size with increasing longitude (compare equations in Table 7 and 8). For the entire migration, the relationship was always significant, but not when the regression was performed on butterflies from each migration phase separately. Excluding the very late, coastal TX and TX2007 monarchs from the regression of FWL of monarchs sampled in 2006 against latitude led to a nonsignificant result (adjusted $R^2 = 0.0\%$, $F_{1,697} = 1.31$, $p = 0.252$). When the data from PA were included, there was a positive relationship between FWL and latitude for monarchs from the middle and late migration phase respectively but no relationship for monarchs from the early migration phase. Without the data from PA, there was a relationship between FWL and latitude for late migrants but not for early and middle phase migrants. However, the low adjusted R^2 values showed that variation in FWL was neither well explained by longitude of the collection site nor by latitude (Table 7 and 8). A concern with these analyses may be a departure of the residuals from normality and heterogeneity of variances, as the results of the Anderson-Darling test indicate (Table 7 and 8).

Despite the strong correlation between mean FWL and latitude ($p < 0.001$), mean FWL did neither show a consistent pattern of change with latitude for the entire migration nor for each migration (Table 9). Overall, the on average largest monarchs with 53.3 mm had been collected in Arkansas. The next largest sample was the middle phase migrants from Iowa and PA2003. Butterflies collected during the late migration phase in Texas, 2006 and 2007, and Kansas were the smallest ones on average. A frequency distribution of forewing length from the whole migration shows the relationship among states (Figure 11 and 12).

Stabilizing Selection

Forewing lengths of the TX and TX2007 monarchs had the highest coefficient of variation, i.e., the highest variability in forewing length among states. The least variable samples were from South Carolina and Arkansas (Table 9). No pattern between latitude and CV could be observed; however, two of the three lowest CVs were found at the east coast, in the SC and the PA2003 samples.

Table 7. Regression of forewing length of monarchs collected in the United States versus latitude of collection site.

	N	**Mean Forewing Length (mm)**	**STDEV**	**df**	**F value**	**p value**	**adjusted R²**	**Anderson-Darling (AD) statistic**	**p value for AD**	**Regression equation**
All monarchs without PA	876	51.09	2.817	1, 874	14.06	< 0.001	1.50%	8.615	< 0.005	FWL = 48.3 + 0.0783 latitude
All monarchs without PA and with MX	1064	51.21	2.719	1, 1062	0.28	0.598	0.00%	9.834	< 0.005	FWL = 51.4 - 0.0060 latitude
Early migration phase without PA	100	51.85	2.349	1, 98	0.03	0.864	0.00%	1.078	0.008	FWL = 50.9 + 0.025 latitude
Middle migration phase without PA	156	51.21	2.527	1, 154	2.04	0.155	0.70%	0.951	0.016	FWL = 47.9 + 0.0919 latitude
Late migration phase without PA	620	50.93	2.935	1, 618	7.78	0.005	1.10%	6.826	< 0.005	FWL = 48.6 + 0.0664 latitude
All monarchs with PA	2513	51.92	2.664	1, 2511	115.36	< 0.001	4.40%	41.753	< 0.005	FWL = 45.86 + 0.1582 latitude
All monarchs with PA and MX	2701	51.91	2.63	1, 2699	44.75	< 0.001	1.60%	45.28	< 0.005	FWL = 49.8 + 0.0566 latitude
Early migration phase with PA	582	52.2	2.465	1, 580	1.89	0.17	0.20%	10.706	< 0.005	FWL = 47.7 + 0.115 latitude
Middle migration phase with PA	362	51.73	2.383	1, 360	12.24	0.001	3.00%	3.345	< 0.005	FWL = 45.5 + 0.162 latitude
Late migration phase with PA	1569	51.87	2.788	1, 1567	90.76	< 0.001	5.40%	29.217	< 0.005	FWL = 45.9 + 0.158 latitude

Table 8. Regression of forewing length of monarchs collected in the United States versus longitude of collection site.

	N	**Mean Forewing Length (mm)**	**STDEV**	**df**	**F value**	**p value**	**adjusted R^2**	**Anderson-Darling (AD) statistic**	**p value for AD**	**Regression equation**
All monarchs without PA	872	51.09	2.817	1, 874	22.28	< 0.001	2.40%	6.305	< 0.005	FWL = 57.7 - 0.0713 longitude
All monarchs without PA and with MX	1064	51.21	2.719	1, 1062	7.85	0.005	0.60%	8.967	< 0.005	FWL = 54.6 - 0.0366 longitude
Early migration phase without PA	99	51.85	2.349	1, 98	0.03	0.864	0.00%	1.078	0.008	FWL = 55.5 - 0.039 longitude
Middle migration phase without PA	156	51.21	2.527	1, 154	9.27	0.003	5.10%	0.64	0.093	FWL = 58.9 - 0.0846 longitude
Late migration phase without PA	617	50.93	2.935	1, 615	20.2	< 0.001	3.00%	4.619	< 0.005	FWL = 58.6 - 0.0829 longitude
All monarchs with PA	2444	51.92	2.664	1, 2511	167.12	< 0.001	6.20%	37.779	< 0.005	FWL = 58.3 - 0.0776 longitude
All monarchs with PA and MX	2701	51.91	2.63	1, 2699	135.77	< 0.001	4.80%	39.407	< 0.005	FWL = 57.0 - 0.0607 longitude
Early migration phase with PA	579	52.2	2.465	1, 580	2.41	0.121	0.20%	10.799	< 0.005	FWL = 53.9 - 0.0212 longitude
Middle migration phase with PA	362	51.73	2.383	1, 360	23.49	< 0.001	5.90%	2.703	< 0.005	FWL = 57.2 - 0.0663 longitude
Late migration phase with PA	1503	51.87	2.788	1, 1567	149.54	< 0.001	8.70%	24.459	< 0.005	FWL = 59.64 - 0.09418 longitude

Table 9. Mean (mm), standard deviation (STDEV), and coefficient of variation (CV) of forewing length.

	Entire Migration*			**Early Migration Phase**			**Middle Migration Phase**			**Late Migration Phase**		
Sample Site (Total N)	**Mean**	**STDEV**	**CV**	**Mean**	**STDEV**	**CV**	**Mean**	**STDEV**	**CV**	**Mean**	**STDEV**	**CV**
Ames vicinity, Iowa (110)	52.23	2.592	4.963	n/a	n/a	n/a	53.061	2.672	5.04	52.063	2.559	4.92
Pennsylvania 2001 (722)	52.12	2.918	5.600	52.21	2.520	4.830	51.791	2.109	4.07	52.125	3.384	6.49
Pennsylvania 2003 (1039)	52.56	2.046	3.890	52.35	2.436	4.650	52.358	2.233	4.27	52.665	1.859	3.53
Lawrence vicinity, Kansas (279)	50.73	2.732	5.386	51.88	2.159	4.160	51.230	2.728	5.32	50.094	2.761	5.51
Oklahoma City vicinity, Oklahoma (111)	50.90	2.908	5.713	51.80	2.647	5.110	49.526	1.782	3.6	51.409	3.629	7.06
Monticello vicinity, Arkansas (40)	53.31	1.938	3.636	n/a	n/a	n/a	n/a	n/a	n/a	53.305	1.938	3.64
Folly Beach and Seabrook Island, South Carolina (149)	51.73	1.742	3.367	n/a	n/a	n/a	51.963	1.752	3.37	51.648	1.738	3.37
Port Lavaca vicinity, Texas 2006 (40)	48.92	3.679	7.522	n/a	n/a	n/a	n/a	n/a	n/a	48.915	3.679	7.52
Port Lavaca vicinity, Texas 2007 (137)	50.29	3.129	6.222	n/a	n/a	n/a	n/a	n/a	n/a	50.294	3.129	6.22
Cerro Pelon, Mexico (188)	51.79	2.117	4.088	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

* according to AA of 57.0 and lower; monarchs in Cerro Pelon were collected during the overwintering period

Differences Among Migration Phases

Overall, wing length was significantly different among migration phases at each collection site (Table 10). Monarchs from the early phase were the largest, whereas monarchs from the late phase were the smallest when the PA data were not included, but middle phase migrants had the shortest wings in the dataset with the PA butterflies (Table 9). Within each state of collection, only KS and OK monarchs had significant size differences among migration phases (Table 10). Data from Arkansas and Texas could not be assessed, as all monarchs had been collected during the last migration phase.

Overall, monarchs from the early migration phase were larger than the ones from the middle and late phase (Table 9), but the size of butterflies from the middle migration phase did not significantly differ from the late migrants ($t_{270} = 1.21$, two-tailed, $p = 0.229$). The same pattern showed when the wing length measurements from Pennsylvania were included ($t_{610} = -0.95$, $p = 0.343$).

However, differences in mean FWL among migration phases were not significant for the subset of butterfly wings which were sorted by their latitude of origin according to δD value (Table 11 and 12). Only the sample of monarchs with a wing δD between –144 and –130 ‰ had significant FWL differences among migration phases (Table 11).

There was also a relationship between forewing length and AA, according to which migration phases had been determined (adjusted $R^2 = 2.3\%$, $F_{1,874} = 22.00$, $p < 0.001$). However, only 2.3% of the variation in FWL was explained by AA.

A visual inspection of the frequency distributions shows that wing length distributions of the early migration phase look most similar to the distribution found in Mexico (Figure 13).

Table 10. One-way ANOVA or Kruskal-Wallis test for forewing length of monarchs collected in the United States with migration phase as predictor.

	df	**test statistic (adjusted for ties)**	**p value**	**df**	**test statistic (adjusted for ties)**	**p value**
Ames vicinity, Iowa (110)	1	H = 2.87	0.09	1, 108	F = 2.26	0.136
Pennsylvania 2001 (722)	2	H = 3.51	0.173	2, 696	F = 0.69	0.504
Pennsylvania 2003 (1039)	2	H = 2.18	0.336	-	-	-
Lawrence vicinity, Kansas (279)	2	H = 23.07	< 0.001	2, 276	F = 11.50	< 0.001
Oklahoma City vicinity, Oklahoma (111)	2	H = 18.93	< 0.001	-	-	-
Charleston vicinity (Folly Beach and Seabrook Island), South Carolina (149)	1	H = 1.78	0.182	1, 157	F = 0.98	0.325
All data**	2	H = 11.26	0.004	2, 2510	F = 4.35	0.013
All data without PA**	2	H = 8.63	0.013	2, 873	F = 4.79	0.004

Table 11. Comparison of forewing length (FWL) among early, middle, and late migration phases at different δD ranges with one-way ANOVAs. δD ranges are an indicator of latitude.

δD Range (‰) (N)	**df**	**F value**	**p value**
-144 to -130 (10)	2, 7	16.33	0.002
-129 to -120 (28)	2, 25	0.91	0.417
-119 to -110 (38)	2, 35	1.29	0.289
-109 to -100 (54)	2, 51	0.02	0.98
-99 to -90 (60)	2, 57	0.31	0.736
-89 to -74 (25)	2, 22	0.65	0.53

Table 12. Mean forewing length (mm) of monarchs at different δD ranges. The δD ranges are an indicator of latitude with the most negative values indicating higher latitudes and least negative values indicating lower latitudes.

	Entire Migration			**Early Migration Phase**			**Middle Migration Phase**			**Late Migration Phase**		
δD Range (‰) (N)	**Mean**	**STDEV**	**CV**	**Mean**	**STDEV**	**CV**	**Mean**	**STDEV**	**CV**	**Mean**	**STDEV**	**CV**
-144 to -130 (10)	52.89	1.832	3.460	54.12	0.896	1.650	49.95	0.778	1.560	52.80	0.872	1.650
-129 to -120 (28)	51.59	2.361	4.580	51.76	1.868	3.610	52.18	1.872	3.590	50.86	2.965	5.830
-119 to -110 (38)	51.24	2.343	4.570	52.30	3.250	6.220	51.89	2.027	3.910	50.73	2.441	4.810
-109 to -100 (54)	51.69	2.754	5.330	51.81	2.875	5.550	51.75	2.681	5.180	51.62	2.849	5.500
-99 to -90 (60)	49.68	3.348	6.740	49.93	3.490	6.160	50.22	8.505	5.810	49.42	3.520	7.120
-89 to -74 (25)	48.74	2.807	5.760	50.47	3.110	6.990	48.78	5.380	4.750	48.43	2.933	6.060

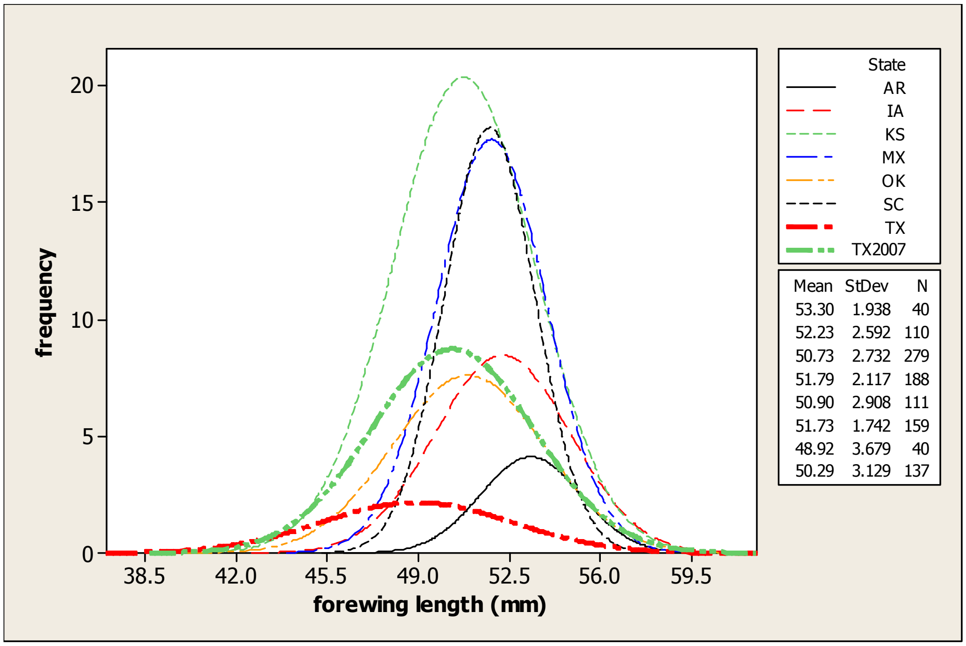


Figure 11. Frequency distribution (with fit) of forewing length of monarch butterflies sampled in six U.S. states during the fall migration of 2006, of monarchs collected in Texas during the fall 2007 migration, and of monarchs from the overwintering site in Mexico.

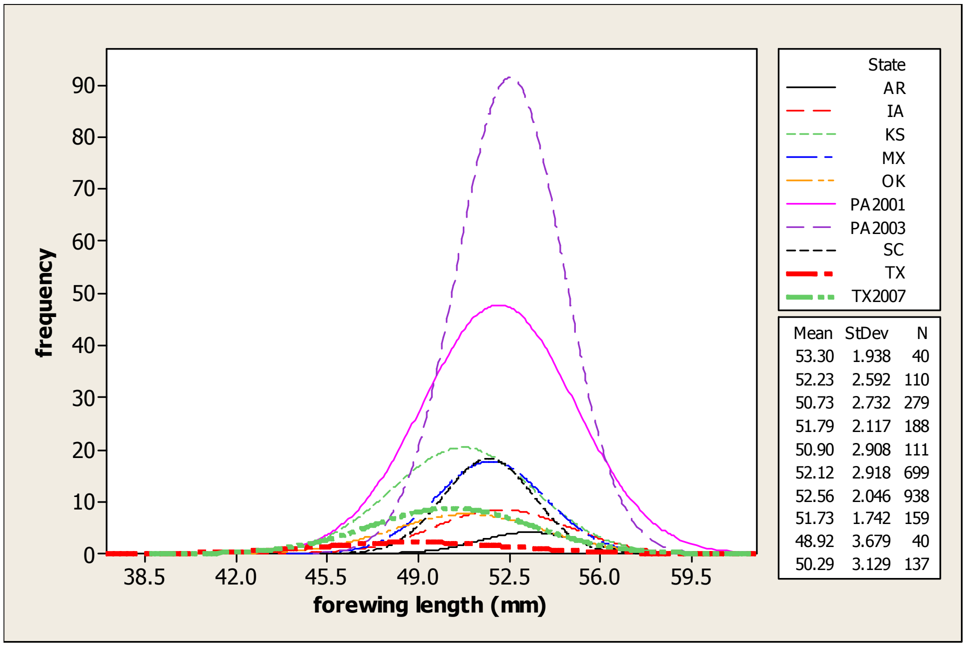
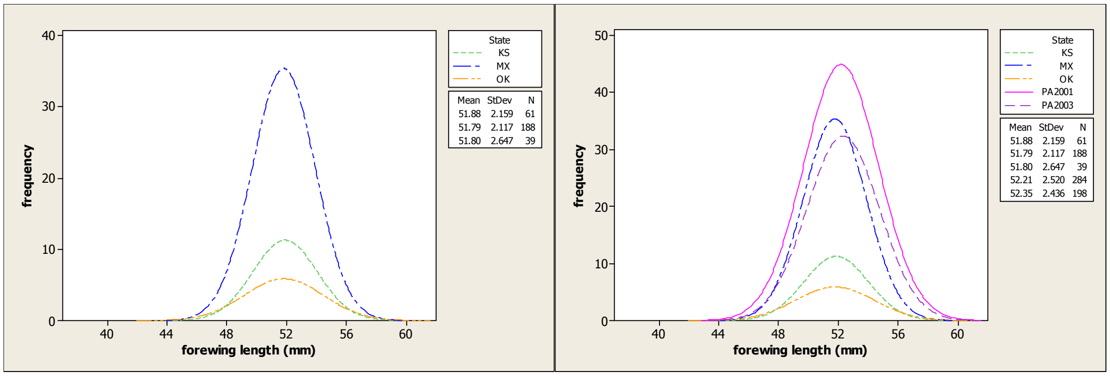
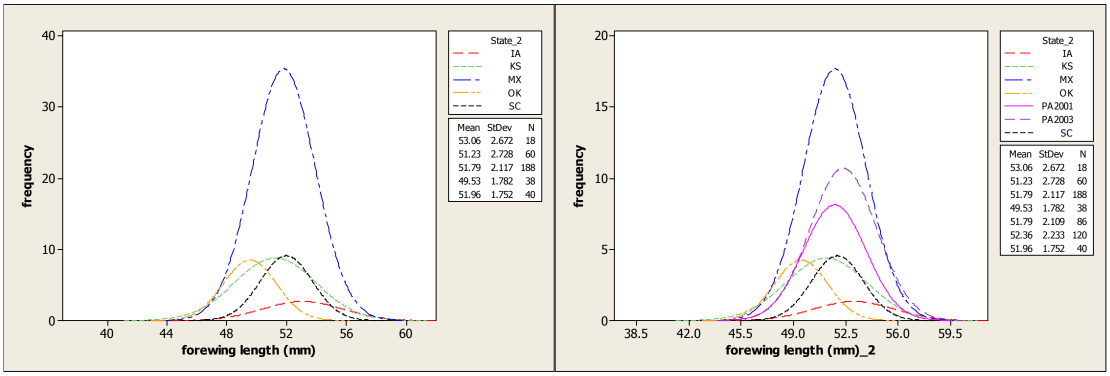


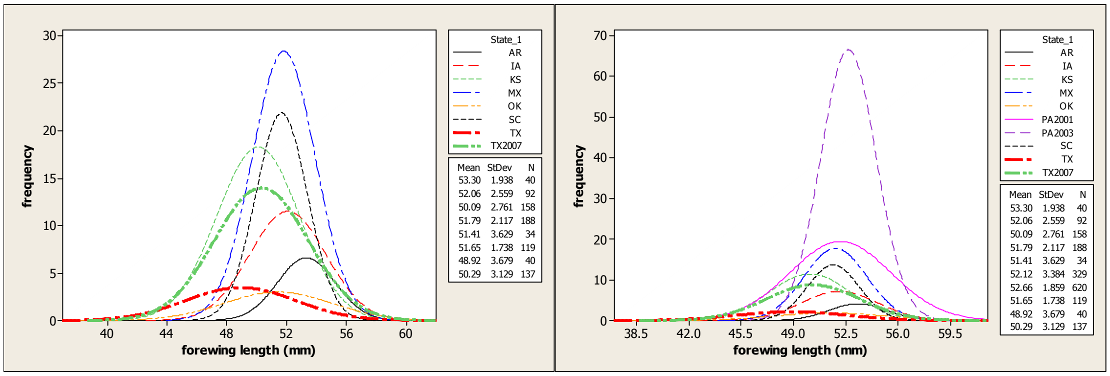
Figure 12. Frequency distribution (with fit) of forewing length of monarch butterflies sampled in six U.S. states during the fall migration of 2006, of monarchs collected in Texas during the fall 2007 migration, of monarchs collected in Pennsylvania during the fall 2001 and 2003 migration, and of monarchs from the overwintering site in Mexico.



Early migration phase (left: KS, MX, OK; right: KS, MX, OK, PA2001, PA2003).



Middle migration phase (left: IA, KS, MX, OK, SC; right: IA, KS, MX, OK, PA2001, PA2003, SC).



Late migration phase (left: AR, IA, KS, MX, OK, SC, TX, TX2007; right: AR, IA, KS MX, OK, PA2001, PA2003, SC, TX, TX2007).

Figure 13. Frequency distributions (with fit) of forewing length of monarch butterflies sampled in the U.S. during the three different phases of the 2006 and 2007 fall migrations and of monarchs from the overwintering site in Mexico. Note that Y-scale is not consistent. List of sampling sites from top to bottom (black: AR, red: IA, green: KS, blue: MX, yellow: OK, pink: PA2001, violet: PA2003, black: SC red: TX, green: TX2007).

Residents versus Migrants and Male versus Female

In the PA 2001 sample, resident monarchs had significantly shorter wings than migrants ($t_{111} = 2.60$, two-tailed, $p = 0.011$; 52.12 for migrants, 51.74 mm for residents) but not in the PA 2003 sample ($t_{24} = 0.75$, two-tailed, $p = 0.459$; 52.56 mm for migrants, 51.8 mm for residents).

Male butterflies had longer wings than females in the samples from all but one state (Table 13). Overall, differences in wing size between sexes were not significant, although male wings were 0.33 mm longer on average ($t_{821} = -1.76$, two-tailed, $p = 0.079$). However, with the Pennsylvania butterflies included, differences between sexes were highly significant ($t_{1569} = -5.41$, two-tailed, $p < 0.001$). Within each state of Arkansas, South Carolina, and Pennsylvania (2001 and 2003), male and female monarchs were significantly different in size, whereas butterflies from the other states did not differ. Male PA2001 and PA2003 monarchs assigned as residents had on average longer wings than females (males: 51.91 mm, females: 51.58 mm), but the differences were not significant ($t_{120} = -0.73$, two-tailed, $p = 0.467$). Since sexes could not be determined for a large portion of monarchs photographed in Mexico, wing length between males and females could not be compared.

Table 13. Mean forewing length of female and male monarch butterflies.

State	**Mean Forewing Length (mm)**	
	Female	**Male**
IA	52.08	52.34
PA2001	51.60	52.32
PA2003	52.17	52.64
KS	50.58	50.88
OK	50.59	51.15
AR	52.61	54.07
SC	51.24	51.95
TX	49.25	48.77
TX2007	49.96	50.42
All data without PA	50.89	51.22
All data	51.54	52.12

Stable Isotope Analyses

Directional Selection

The stable isotope analyses addressed the prediction that large monarchs at any one location originate from farther north than small butterflies. Wing size increased with decreasing δD value, i.e., the farther north the monarchs originated (Table 12 and 15, Figure 14). There was a significant relationship between FWL and δD (Table 14). The adjusted R^2 suggests that 16.5% of the variation observed in forewing length can be explained by δD, as a surrogate of latitude of natal origin (Figure 14). For all the stable isotope data pooled, 13.7% of the variation in FWL could be explained by variation in δD (Table 14). The same trend of increasing wing size with increasing latitude was observed when the measurements were divided into the three migration phases (Table 14). The coefficient of variation of FWL in the groups according to δD value tended to increase at less negative δD, i.e., at lower latitudes (Table 12). The decrease in variability at lower latitude is obvious in the frequency distribution of FWL from the late migration phase (Figure 15), but not so much in the frequency distribution of FWL for the entire migration (Figure 16).

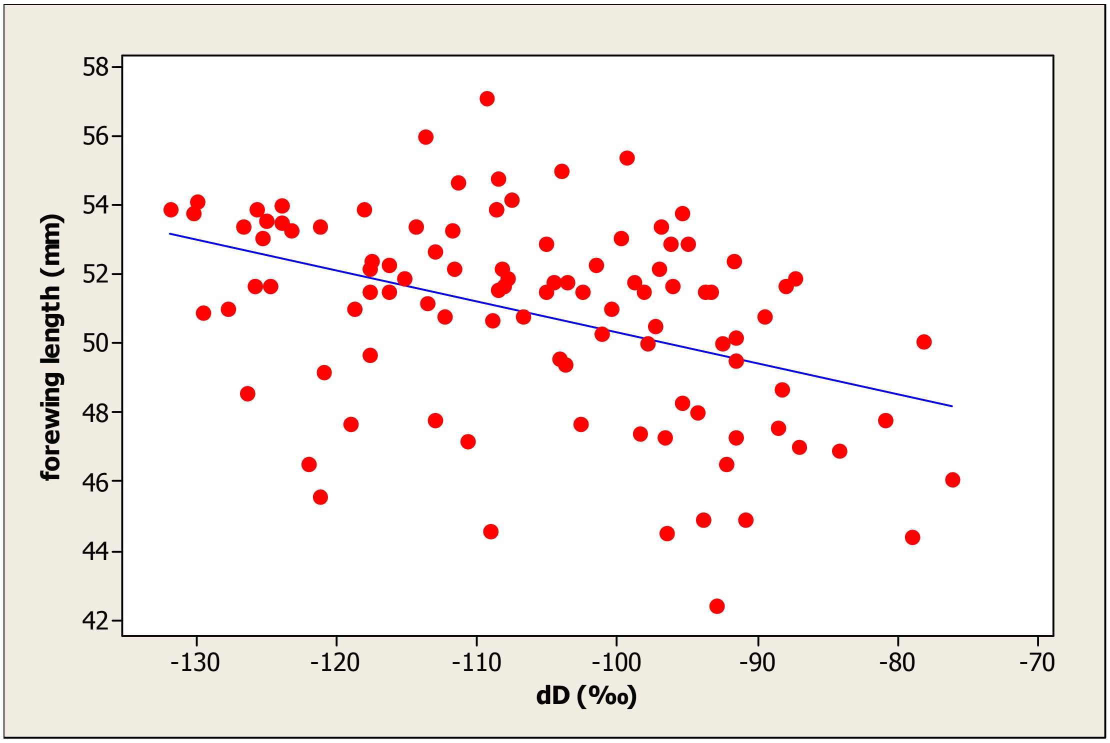


Figure 14. Linear regression of forewing length against δD with 10 samples from each IA, OK, AR, SC and TX 2006 and 50 samples from KS. The TX outlier (δD = -46.98) was removed.

Table 14. Regression of forewing length (FWL) versus δD.

Monarch Sample	df	F value	p value	Adjusted R²	Regression Equation
53 specimens from KS, 10 specimens from IA, OK, AR, SC, and TX each	1, 100	20.91	< 0.001	16.50%	FWL = 41.4 - 0.0898 δD
All isotope data*	1, 213	34.86	< 0.001	13.70%	FWL = 41.9 - 0.0835 δD
All isotope data from early migration phase	1, 30	9.88	0.004	22.30%	FWL = 41.90 - 0.08977 δD
All isotope data from middle migration phase	1, 58	7.08	0.01	9.20%	FWL = 44.49 - 0.06211 δD
All isotope data from late migration phase	1,120	15.08	< 0.001	10.40%	FWL = 41.54 - 0.08448 δD
Kansas 1st and 2nd half	1, 38	0.69	0.411	0.00%	FWL = 47.4 - 0.0310 δD
Oklahoma 1st and 2nd half	1, 38	8.31	0.006	15.80%	FWL = 40.8 - 0.0998 δD

* 93 monarchs from KS, 50 from OK, 23 from TX2006, 19 from TX2007, 10 from IA, AR, and SC

Table 15. Mean forewing length (mm) of monarchs at different δD ranges in the two states with the highest sampling sizes in δD (N). The δD ranges are an indicator of latitude with the most negative values indicating higher latitudes and least negative values indicating lower latitudes.

δD Range (‰)	OK (50)	KS (93)
-144 to -130	54.18	51.90
-129 to -120	51.38	51.11
-119 to -110	51.66	50.24
-109 to -100	52.97	51.93
-99 to -90	50.18	49.68
-89 to -74	47.37	49.84

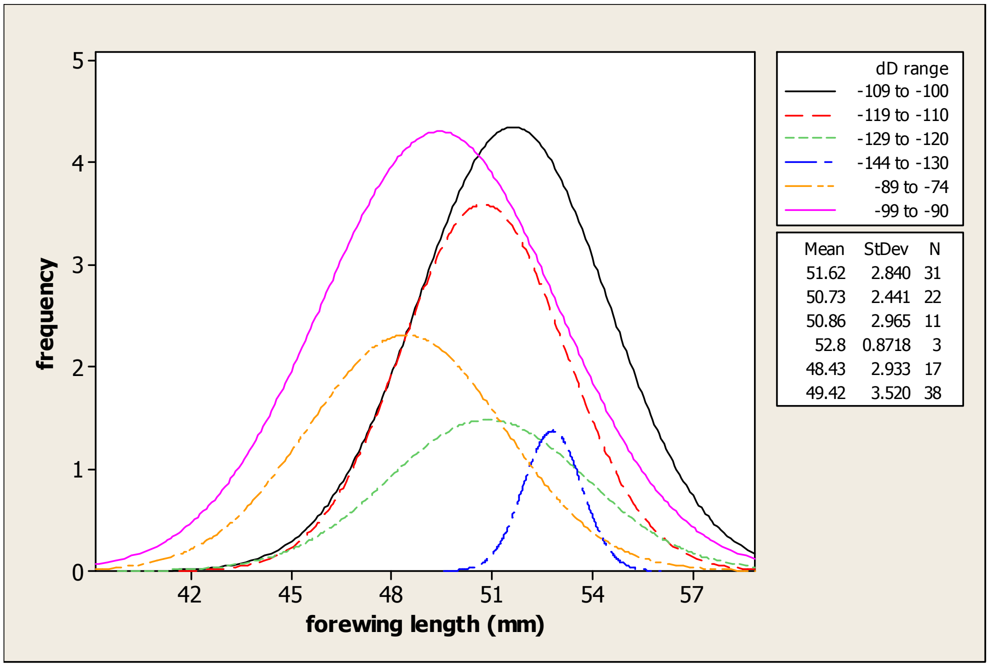


Figure 15. Frequency distribution (with fit) of forewing length of monarch butterflies sampled in the late migration phase and grouped in different ranges of δD values. δD values are an indicator of latitude.

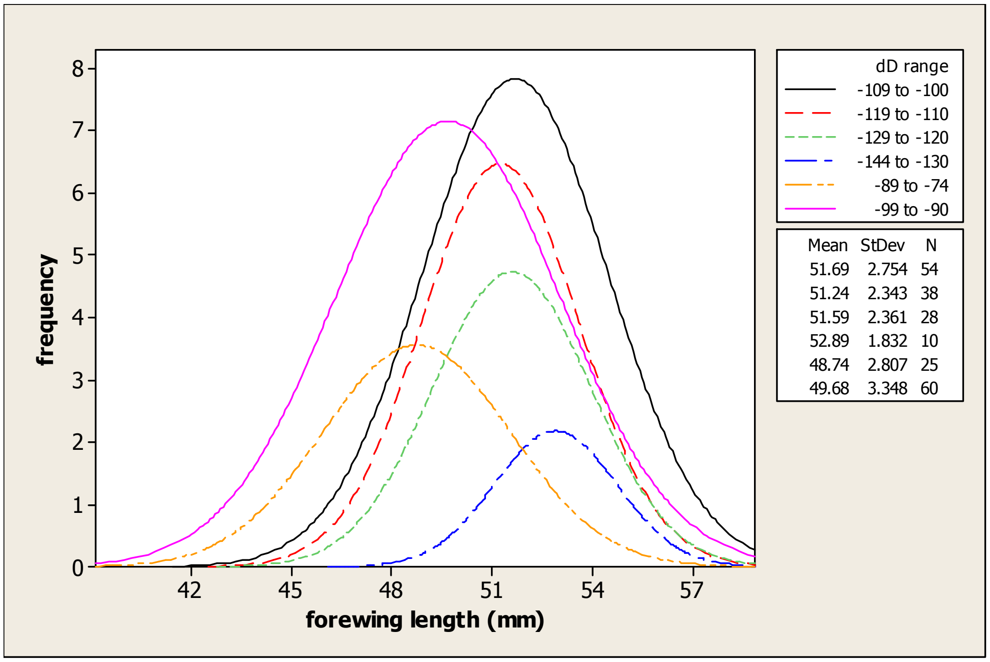


Figure 16. Frequency distribution (with fit) of forewing length of monarch butterflies sampled during the entire migration and grouped in different ranges of δD values. δD values are an indicator of latitude.

Comparisons between migration halves in Kansas and Oklahoma showed that monarchs from the first half had longer wings than monarchs from the second half (Table 16). The difference was significant for the data from Kansas (Table 17). 15.8% of variation in forewing length seen in Oklahoma could be explained by variation in δD if a linear relationship was assumed (Table 14). Including δD^2 and δD^3 as predictors improved the model and yielded an adjusted R^2 of 20.7% ($F_{3,36} = 4.39$, $p < 0.001$) (Figure 17). None of the variation in FWL seen in Kansas could be explained by δD (Table 14). Differences in FWL or δD value between KS and OK for first or second migration half were not significant (Table 17).

Mean wing δD value was more negative in the first half in both states, i.e., monarchs originated from farther north on average. The minimum δD value was found in the first migration half as well (Table 16). However, the difference in the hydrogen stable isotope ratio between migration halves was only significant in the OK monarchs (Table 17).

Table 16. Comparison of forewing length and δD value of samples from Kansas, Oklahoma, and Texas.

Location of Random Subsample	**Migration Half (N)**	**Mean FWL (mm)**	**STDEV**	**CV**	**Mean δD Value (‰)**	**Minimum δD Value (‰)**	**Maximum δD Value (‰)**	**Range δD Value (‰)**	**STDEV**	**CV**
Kansas	1st (20)	51.78	2.41	4.66	-109.64	-136.82	-89.34	47.48	13.62	-12.42
	2nd (20)	49.66	2.94	5.93	-103.13	-121.81	-86.05	35.76	10.23	-9.92
Oklahoma	1st (19)	51.62	3.06	5.93	-112.7	-144.01	-86.65	57.36	16.09	-14.28
	2nd (21)	51.45	3.47	6.73	-102.1	-116.42	-88.74	27.68	8.91	-8.73
Texas 2006 (20)		48.83	3.43	7.02	-92.68	-122.91	-74.7*	48.21**	11.96	-12.91
Texas 2007 (19)		49.97	2.63	5.26	-104.31	-132.96	-88.45	44.51	12.6	-12.08

* the actual maximum from this dataset was -46.98; however, it was considered an outlier because it was more than 2 standard deviations from the mean

** the actual range from this dataset was 67.42; however, the maximum value was considered an outlier and replaced by the next highest value

Table 17. Student's t-test evaluated differences in forewing length between samples and differences in δD value between samples.

Samples	**Forewing length**			**δD Value (‰)**		
	df	**t value**	**p value**	**df**	**t value**	**p value**
Kansas 1st versus 2nd half	36	2.49	0.017	35	-1.71	0.96
Oklahoma 1st versus 2nd half	37	0.16	0.871	27	-2.54	0.017
Texas 2006 versus 2007	35	-1.17	0.249	36	2.95	0.006
KS 1st half versus OK 1st half	34	0.18	0.859	35	0.64	0.526
KS 2nd half versus OK 2nd half	38	1.79	0.082	37	0.34	0.734

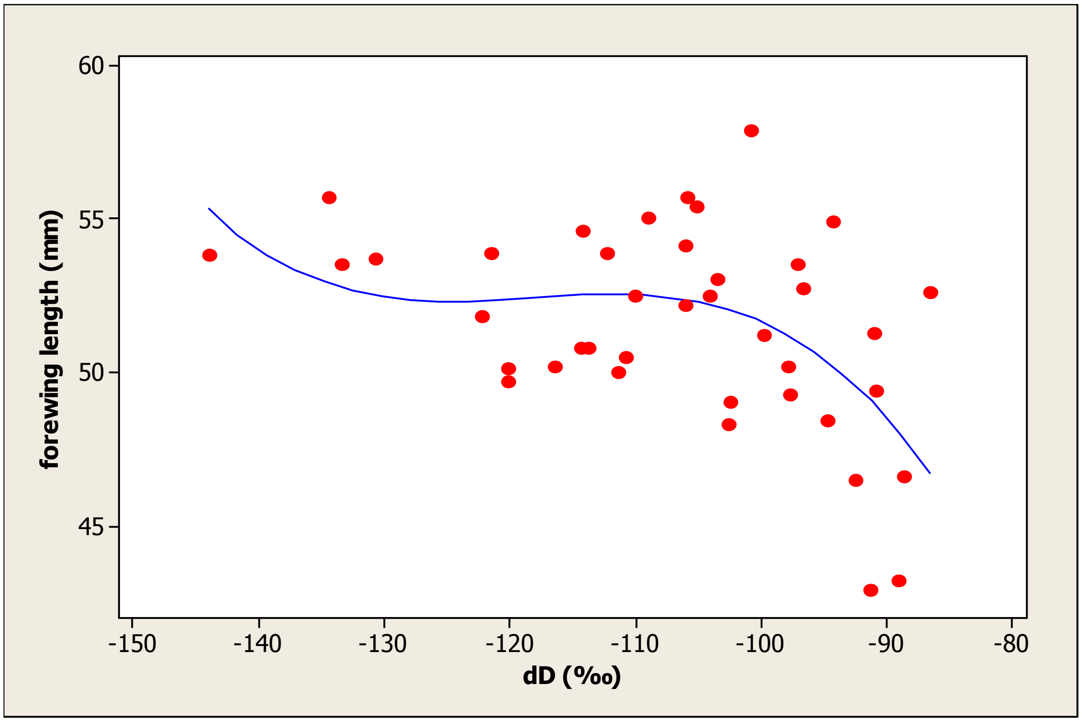


Figure 17. Nonlinear regression of forewing length against δD with 19 samples from the first migration half and 21 samples from the second migration half in OK, sampled in 2006.

As to a comparison among years, monarchs collected in Texas in 2007 were on average larger than the ones collected in 2006 (Table 16). This difference, however, was statistically not significant (Table 17). Texas 2007 monarchs had a more negative mean hydrogen stable isotope ratio. Moreover, the minimum δD from

2007 was about 10 ‰ more negative than the one from 2006 (Table 16). This indicates that monarchs collected in Texas in 2007 had a longer average FWL and originated from higher latitudes than those sampled at the same location in the previous year. The difference in δD was significant (Table 17).

The Stable Isotope Technique

I used the 1996 base map to evaluate the hydrogen stable isotope data of tagged monarch butterflies recovered in Mexico. In most cases, the tagging location was south of the latitude of natal origin or at the same latitude, as interpreted by the map (Table 4, Figure 2). Only a few locations were north of the place of origin according to the δD value, for instance, monarchs tagged in Tripoli, IA, or Green Bay, WI. Replicates of the wing isotopic ratio of three butterflies show that the δD value of the same individual varied between 2.7 and 5.6‰.

There was a significant relationship between the δD value of monarchs and their latitude of natal origin as well as between the δD value of milkweed and the latitude of collection (Table 18). In both cases, the δD value increased with decreasing latitude (Figure 18 and 19). This was true as well for the linear regression performed on the monarch hydrogen stable isotope data published by Hobson et al. in 1999 (Table 18). Adding the quadratic variable latitude^2 improved the fit of the model (adjusted $R^2 = 68.0\%$, $F_{2,30} = 35.05$, $p < 0.001$, $\delta D = -158 + 4.96$ latitude $- 0.0892$ latitude^2). However, for better comparison, I plotted the linear regression line of both the data from Hobson et al. (1999) and my own monarch butterfly data (Figure 18).

The slopes of the two regression lines were not significantly different ($F_s = 0.156 < F_{0.95[1, -52.4]}$; Table 18).

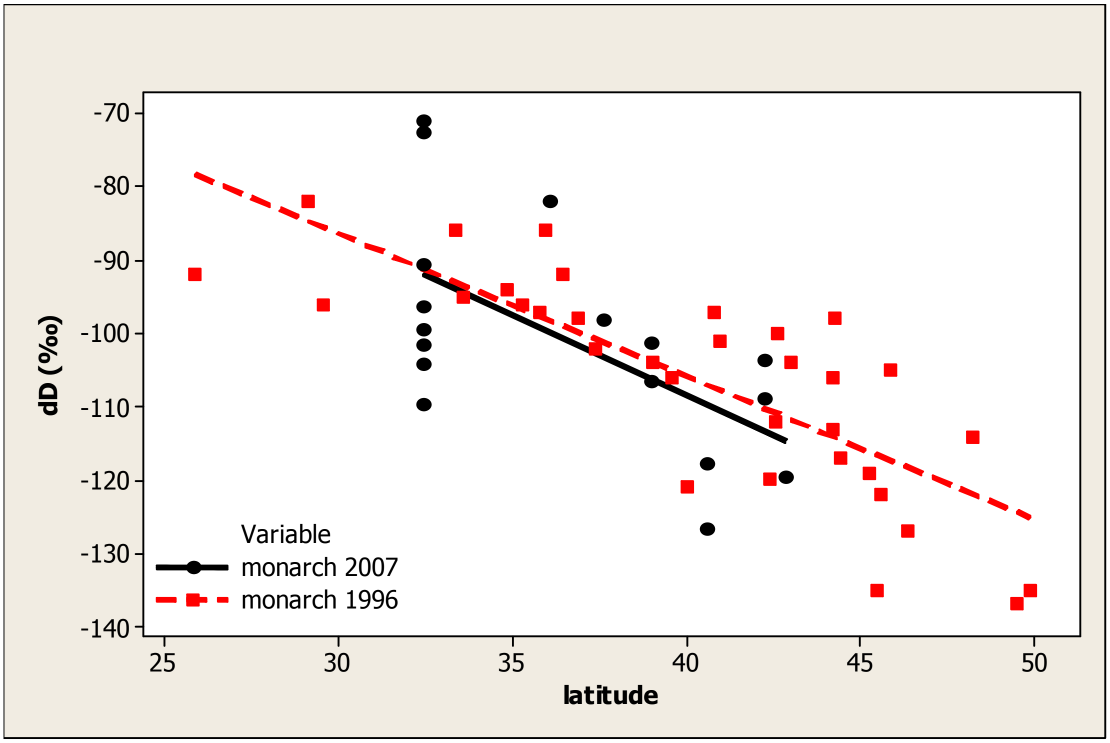


Figure 18. Linear regression of hydrogen stable isotope values against latitude. Black: Values of monarchs sampled during the fall migration in 2007. Red: Values of monarchs sampled during the summer of 1996, as published by Hobson et al. (1999).

Table 18. Linear and multiple regressions of different variables versus δD of monarchs and milkweed with known natal origin. Predictor variables were latitude (lat), longitude (lon), elevation (el), and distance to the nearest coast (el) of the location of origin, and temperature (T) and precipitation amount (P) between June and August of the sampling state in the respective year.

Dataset	**df**	**F value**	**p value**	**Adjusted R^2**	**Regression Equation**
(1) Monarch 2007 δD*	1, 15	8.89	0.009	30.0%	δD = - 20.53 - 2.197 lat
(2) Milkweed 2007 δD*	1, 15	15.94	0.001	48.3%	δD = 1.0 - 3.30 lat
(3) Monarch δD from Hobson et al. (1999)*	1, 31	54.64	< 0.001	62.6%	δD = - 27.9 - 1.95 lat
(1) Monarch 2007 δD*	1, 15	0.15	0.705	0.0%	δD = - 119 - 0.234 lon
(2) Milkweed 2007 δD*	1, 15	0.3	0.592	0.0%	δD = - 102 + 0.311 lon
(3) Monarch δD from Hobson et al. (1999)*	1, 31	1.3	0.263	0.9%	δD = - 115 - 0.101 lon
(1) Monarch 2007 δD**	1, 15	9.55	0.007	34.8%	δD = - 180 + 3.28 T 07
(2) Milkweed 2007 δD**	1, 13	1.17	0.298	1.2%	dD = - 174 + 2.04 T 07
(3) Monarch δD from Hobson et al.(1999)**	1, 25	36.04	< 0.001	57.4%	δD = - 199 + 1.34 T 96
(1) Monarch 2007 δD**	1, 15	0.07	0.79	0.0%	δD = - 91.8 - 0.030 P 07
(2) Milkweed 2007 δD**	1, 13	1.05	0.323	0.4%	δD = - 99.9 - 0.0848 P 07
(3) Monarch δD from Hobson et al.(1999)**	1, 25	0.36	0.556	0.0%	δD = - 105 + 0.0127 P 96
(1) Monarch 2007 δD**	1, 15	1.18	0.295	1.1%	δD = - 97.7 - 0.0222 el
(2) Milkweed 2007 δD**	1, 15	1.45	0.247	2.7%	δD = - 123 - 0.0182 el
(3) Monarch δD from Hobson et al.(1999)**	1, 31	2.02	0.166	3.1%	δD = - 109 + 0.00910 el
(1) Monarch 2007 δD*	1, 15	0.76	0.398	0.0%	δD = - 98.5 - 0.0096 dis
(2) Milkweed 2007 δD*	1, 15	3.92	0.066	15.4%	δD = - 117 - 0.0174 dis
(3) Monarch δD from Hobson et al. (1999)*	1, 31	3.75	0.062	7.1%	δD = - 101 - 0.00761 dis
Best Subset Regression					
(1) Monarch 2007 δD**	3, 13	6	0.009	48.4%	δD = 116 - 3.83 lat - 0.278 P 07 + 0.0346 el
(2) Milkweed 2007 δD**	6, 8	8.36	0.005	75.9%	δD = 1712 - 24.6 lat + 5.84 lon - 21.8 T 07 + 0.104 P 07- 0.123 el + 0.213 dis
(3) Monarch δD from Hobson et al.(1999)**	3, 29	20.87	< 0.001	65.1%	δD = - 24.6 - 2.14 lat + 0.00522 el + 0.00368 dis

* these datasets include the sampling locations from Canada; without the Canadian sites, the adjusted R^2 was generally lower

** these datasets exclude the sampling locations from Canada because mean summer temperature and precipitation could not be obtained in the same fashion as for U.S. states

The relationship between δD value and temperature was significant in two of the three datasets, but the relationship between δD and amount of precipitation was significant only in the dataset from 1996 (Table 18). There was no significant relationship between δD value and elevation of sampling site, distance to the nearest coast, or longitude in any of the three datasets. Nevertheless, for dataset (2), milkweed sampled in 2007, the regression line indicates a negative relationship between δD and distance to the nearest coast (Figure 19).

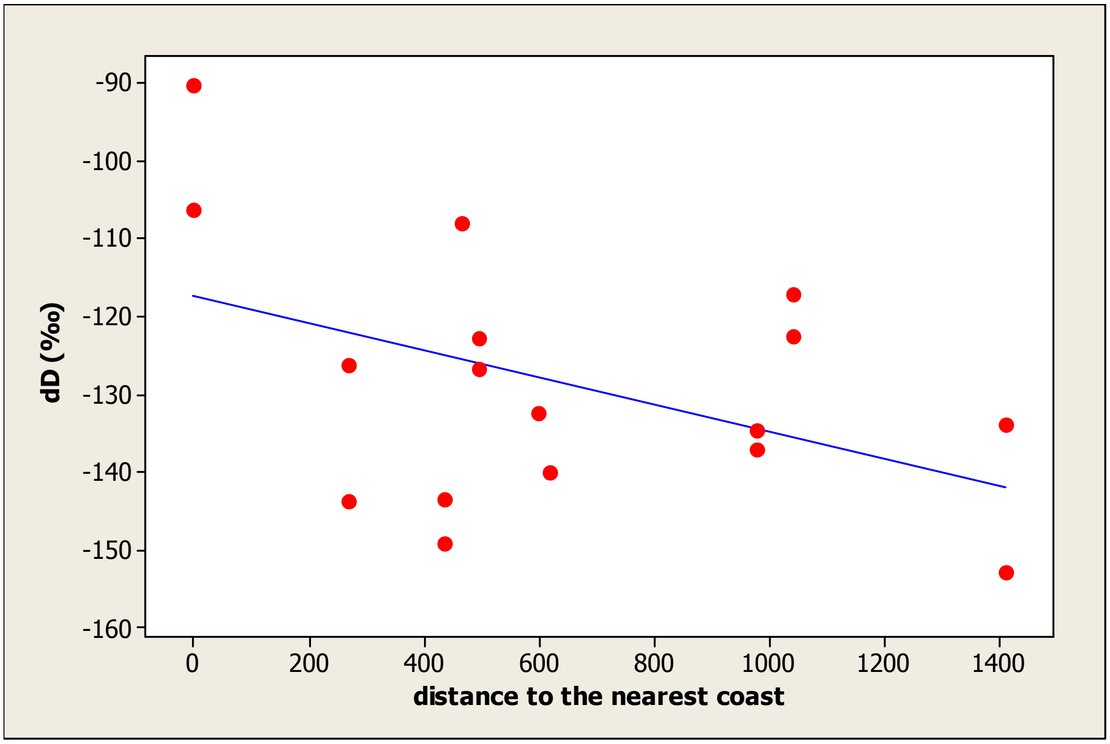


Figure 19. The relationship between δD and distance of milkweed sampling site to the nearest coast was significant at the 0.1% significance level (Type I error rate of 0.1) but not at the 0.05% significance level (p = 0.066).

Latitude and elevation entered as predictor variables for δD in all of the multiple linear regression equations chosen with the best subset regression. Precipitation amount and distance to coast were predictors in two of the three regression equations. For the δD in milkweed, all predictor variables entered in the equation (Table 18).

The multiple linear regression analysis showed that mean summer temperature and precipitation amount helped explain variation in δD. Mean summer temperature was included in the best multiple regression equation for the hydrogen stable isotopes in milkweed, and precipitation amount was one variable of the best equation for δD of monarchs collected in 2007 (Table 18). Mean summer temperature alone explained 34.8% of the variation in δD of monarchs from 2007. As to the isotope data published by Hobson et al. (1999), mean summer temperature was the best single predictor in the dataset which excluded isotope values sampled in Canada. It explained 57.4% of the variation in the wing δD (Table 18).

The relationship between latitude and temperature was highly significant, whereas latitude and amount summer precipitation showed a significant relationship only in one of the three datasets (Table 19).

Table 19. Linear regression of different variables versus latitude (lat) of origin of monarchs and milkweed. Predictor variables were temperature (T) and precipitation amount (P) between June and August of the sampling state in the respective year. The datasets exclude the sampling locations from Canada because mean summer temperature and precipitation could not be obtained in the same fashion as for U.S. states

Dataset	**df**	**F value**	**p value**	**adjusted R^2**	**Regression Equation**
(1) Monarch 2007 δD	1, 15	96.27	< 0.001	85.60%	lat = 69.1 - 1.36 T
(2) Milkweed 2007 δD	1, 13	21.72	< 0.001	59.70%	lat = 66.9 - 1.20 T
(3) Monarch δD from Hobson et al. (1999)	1, 25	104.65	< 0.001	79.90%	lat = 73.1 - 1.52 T
(1) Monarch 2007 δD	1, 15	3.05	0.101	11.40%	lat = 50.8 - 0.0486 P
(2) Milkweed 2007 δD	1, 13	2.48	0.139	9.60%	lat = 30.5 + 0.0264 P
(3) Monarch δD from Hobson et al. (1999)	1, 25	7.73	0.01	20.60%	lat = 47.6 - 0.0282 P

Gliding Simulations

The contours of static pressure showed that there was less pressure on the upper side of the wing and more pressure on the lower side (Figure 20). The low pressure on top pulls up, the high pressure on the bottom pushes up, and adding the pressures together over the wing's surface gives the lift force (Alexander, 2002).

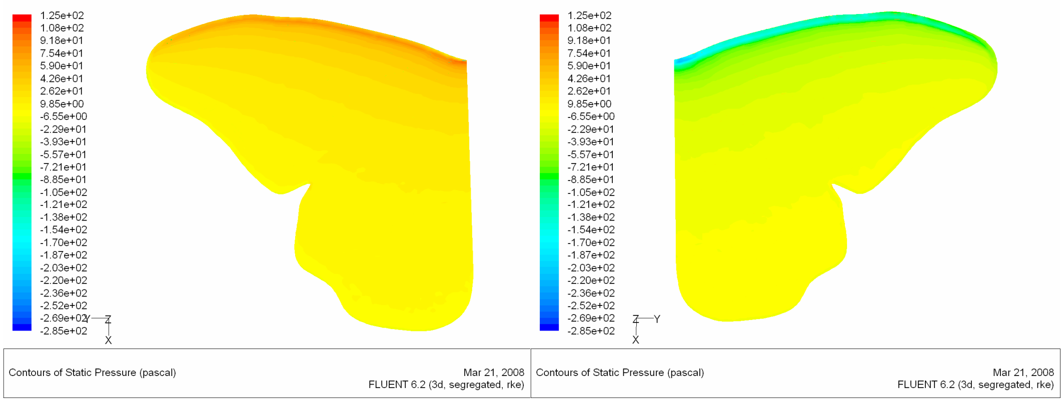


Figure 20. Static pressure contours for the case of AT = 5º, velocity = 15 m/s, and area = 113%. The lower underside of the wing is shown on the left; the upper surface is on the right. Red indicates high pressure, whereas blue stands for low pressure.

Increases in any of the variables led to a rise in lift production (Figure 21 through 23). Lift increased proportional to wing area or angle of attack increased and proportional to velocity-squared (Table 20).

Table 20. Changes in lift force with changes in wing area (A), wind velocity (V), and angle of attack (AT).

V and AT constant			
absolute change in A (m^2)	absolute mean change in lift (N)	% change in A	% mean change in lift
0.00215	0.046	100.0	100.0
0.00354	0.076	164.7	164.6
0.00452	0.097	210.6	211.0
AT and A constant			
absolute change in V (m/s)	absolute mean change in lift (N)	% change in V	% mean change in lift
5	0.015	100.0	100.0
10	0.062	200.0	406.2
15	0.142	300.0	926.6
V and A constant			
absolute change in AT (degree)	absolute mean change in lift (N)	% change in AT	% mean change in lift
5	0.05	100.0	100.0
7	0.071	140.0	140.1
10	0.099	200.0	196.3

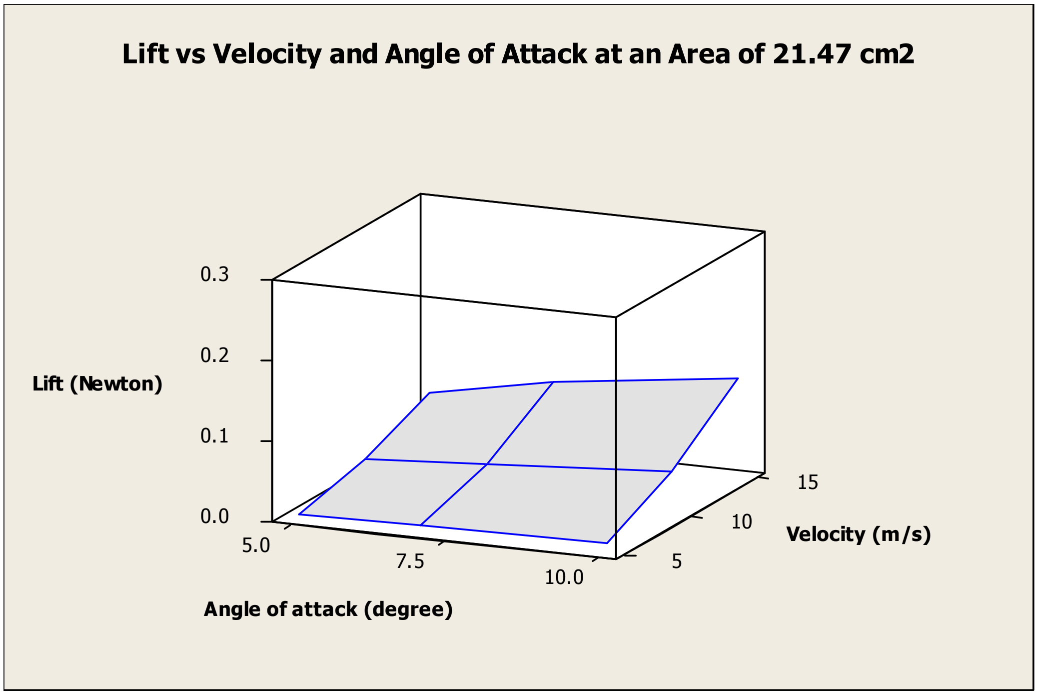


Figure 21. Lift versus velocity and angle of attack at the smallest area simulated.

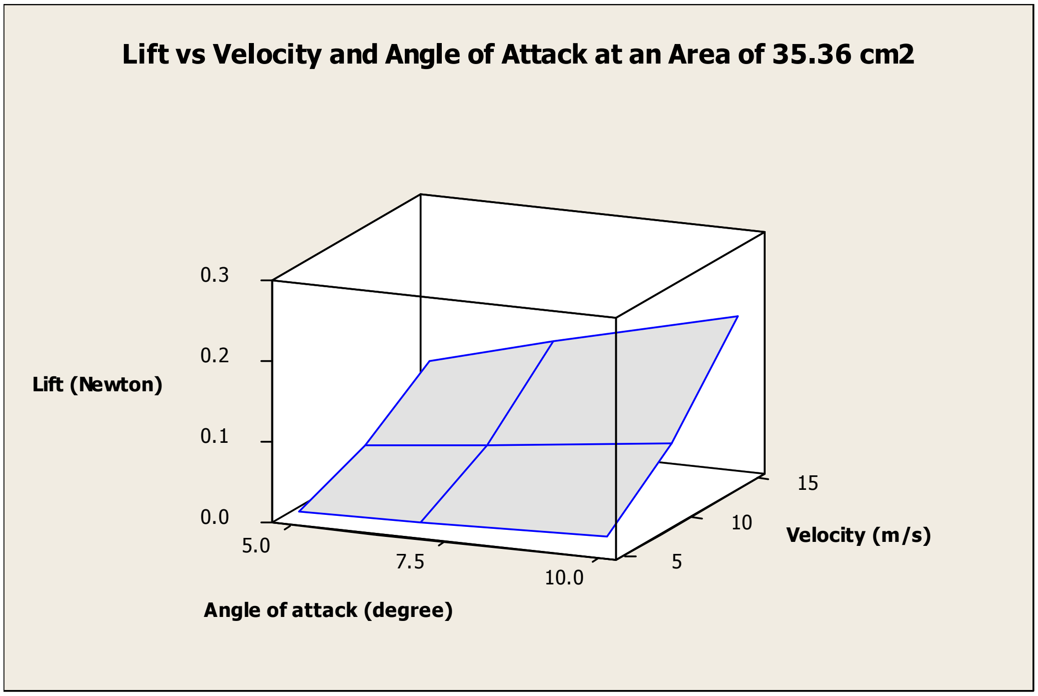


Figure 22. Lift versus velocity and angle of attack at average wing area.

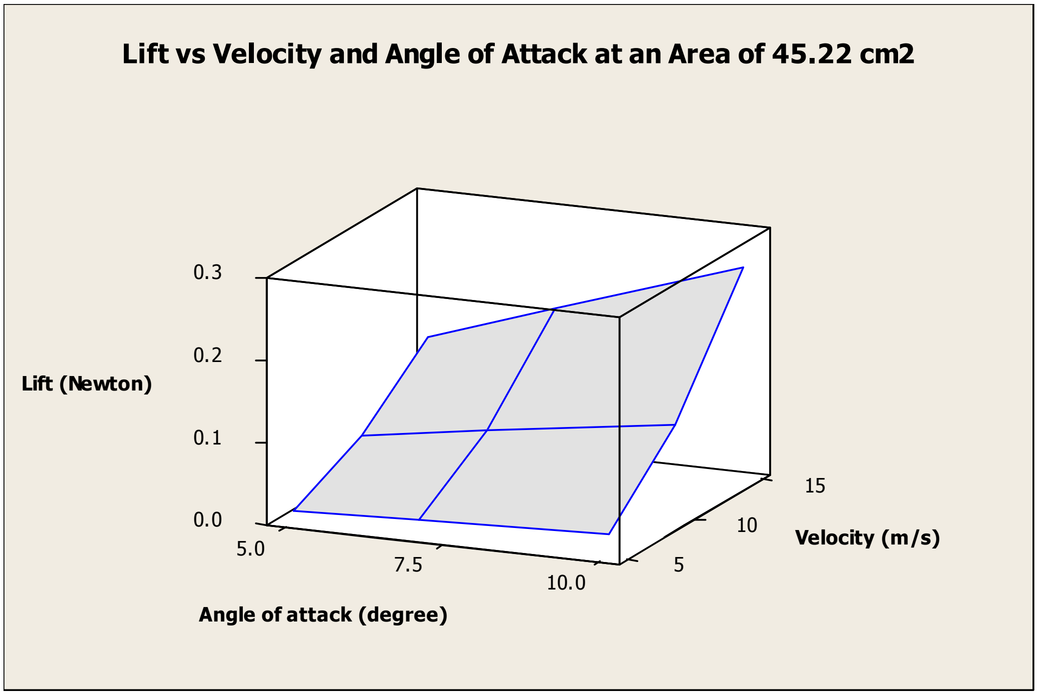


Figure 23. Lift versus velocity and angle of attack at the largest area simulated.

DISCUSSION

Directional Selection

In this study, I tested the hypothesis that directional selection favors monarch butterflies with longer wings during their annual fall migration from southern Canada and the United States to the overwintering sites in Mexico. Stable isotope analysis provided evidence for the presence of directional selection. It addressed the prediction that monarchs with longer wings at any location originate from higher latitudes than smaller butterflies. In general, monarchs with a higher FWL had more negative hydrogen stable isotope values, which indicates a more northern natal origin than less negative values. This pattern could be observed for all isotope data pooled, a subset of all isotope data, as well as in a comparison of a subsample taken from Oklahoma. 13.7% to 16.5% of the variation in δD could be explained by forewing length. There was, however, no relationship between these two variables in a subsample from Kansas.

Moreover, the same general trend was observed in monarch butterflies from Oklahoma. In addition, the TX2007 monarchs had a higher mean forewing length than the TX ones and a more negative mean δD value. These findings indicate that there is year to year variation in the origin of butterflies reaching coastal Texas and that butterflies from higher latitude, as shown by the δD values, tend to have longer wings than butterflies from lower latitudes. Since wing size tends to decrease with date of capture (Borland et al., 2004; Gibo McCurdy, 1993), another possible explanation is that the sampling of the TX2007 monarchs had started 24 days earlier than the collection of the TX butterflies (Table 1).

These observations that monarchs with longer forewings originate from farther north would seem to suggest that monarchs with larger wings may have a higher survival rate than those with shorter wings, assuming that the wing length distribution is the same at the beginning of the fall migration in the entire breeding range of the eastern North America monarch butterfly. A differential survival based on wing size differences probably leads to the bias of monarchs from higher latitudes having longer wings and those from lower latitudes having shorter wings. Individuals with longer wings would thus be selected for, and on average more larger butterflies could potentially reproduce after the overwintering period and pass on their genes to offspring.

Selection Pressures

Aerodynamic and metabolic advantages during the fall migration predict that monarchs with longer wings should be more successful in progressing south and in reaching their overwintering sites. First, monarch butterflies rely heavily on soaring and gliding during the migration (Gibo, 1981). A larger wing area produces more lift, as illustrated by the CFD analyses, and thus allows larger monarchs to travel longer distances. Another possible aerodynamic advantage of longer wings was demonstrated by the analyses of Beall (1948), who measured the wing length of 47 monarchs lost in crossing Lake Erie on 13 September 1943. These drowned monarchs had statistically significantly shorter wings than those taken from clusters around the same area within the same week. This outcome suggests that the smaller monarchs suffered the greater loss. Possibly, the butterflies with longer wings withstand adverse weather conditions better than the ones with shorter wings or make better use of rising winds and survived crossing Lake Erie. The low fat content of the drowned monarchs may have also played a role in this outcome.

A second advantage during migration may be the ability of larger butterflies to store more lipid reserves. In a study of the lepidopteran family Olethrutidae, Miller (1977) showed that biomass increases with increasing forewing length, suggesting that larger individuals can potentially store more fat. Considering the long migration of monarch butterflies, it is important for the butterflies to have enough fuel in form of lipids, and larger monarchs are therefore thought to be more successful than smaller ones during the migration cycle (Arango Velez, 1996). Moreover, the bigger butterflies have a lower metabolic rate per gram tissue as compared to smaller ones, which may give them a relative energetic advantage. Gordon Plague (1992) demonstrated the effect of body size on metabolic rate of monarchs by measuring oxygen consumption of monarch butterflies (Chaplin and Wells, 1982; Silverthorn, 2007) in an experimental ecology class. He found a negative relationship between body weight and O_2 consumption per gram weight in non-reproductive (migratory) monarch butterflies. Ten to 26% of the variation in body weight was explained by O_2 consumption, depending on the collection site of the butterflies (Linares, Mexico, or Lawrence, KS). In mammals and birds, Speakman (2005) showed a negative relationship between lifetime expenditure of energy per gram body tissue and body mass.

Considering the whole animal, a reduction in size reduces the overall metabolic costs of flight (Angelo and Slansky Jr., 1984). However, this aspect is most important in powered flight. For a butterfly which uses a lot of soaring and gliding during a long migration the advantage of having a large wing area, which increases lift production, and the ability to store lipids as fuel probably outweighs the increased metabolic cost of a higher body mass during short periods of flapping flight (Arango Velez, 1996).

What Prevents Wings from Getting Too Long?

Aerodynamic constraints during mating and predator avoidance may prevent selection for yet even longer wings. In the overwintering sites in Mexico, non-random mating has been observed, where preferentially small and lightweight males with wings in poor conditions mated with heavy females with large wings in good condition. These mating patterns do not seem to be the results of female choice; rather, small males captured large females during aerial pursuit (Van Hook, 1993). This non-random mating may suggest that males choose larger, heavier females which might ensure a higher survival rate of offspring. However, it has been observed that male butterflies at the overwintering sites attempt to mate with females of any size, and even males (O. R. Taylor, pers. com.). A laboratory experiment by Orley R. Taylor (pers. com.) suggested that the non-random mating pattern observed in

Mexico is not due to male choice but is instead a result of differential maneuverability based on wing size and body weight. Lightweight, male monarchs with on average short wings mated with heavy females with large wings when male and female monarchs of all size- and weight-ranges were placed in a mating cage at a time of reproductive activity. Females have been described to use resistance to mating (Van Hook, 1993; Solensky, 2004). The bias toward large female monarchs mating may be due to the fact that they cannot escape male capture attempts as easily as females with shorter wings. Large wings may incur disadvantages in maneuverability, as indicated by Wickman's (1992) study on butterfly mating systems. In the perching system, males typically sit and wait, and rapidly take off towards passing object, whereas males of patrolling species actively search in flight for females. Male butterflies of perching species had, among other variables, higher aspect ratios (wing span squared divided by wing area, with wing span = two times wing length) and wing loadings (fresh body weight divided by wing area) than patrolling species. This indicates that their wing area was smaller, which may, together with more flight muscle mass, promote rapid acceleration ability, speed, and maneuverability (partly a series of changes in acceleration) as opposed to flight endurance, which is important in patrolling species. Rapid acceleration and maneuverability may therefore explain why smaller female monarch butterflies can better avoid mating than females with larger wings. It remains to be seen whether such a non-random mating implies differential reproductive advantages and can influence mean wing length of the North American monarch butterfly population.

Greater maneuverability of monarchs with smaller wings may also present a survival advantage with respect to escaping from predators. It has been demonstrated that some palatable butterflies have shorter wings and smaller wing areas as well as shorter and stouter bodies with more flight muscle mass than unpalatable ones (Wickman, 1992). The palatable, smaller butterflies can more easily evade predatory birds. Similarly, hummingbirds with shorter wings may have better acceleration and maneuverability (Feinsinger et al., 1979). Although monarch butterflies are toxic, two bird species and a mouse species prey on them at the overwintering sites in Mexico (Alonso-Mejía et al., 1998). Alonso-Mejía et al. (1998) did not find a difference in wing length between monarchs preyed on by birds and live inactive ones collected from trees in one overwintering area. However, since most monarchs at the overwintering sites are attacked by birds while the monarchs are inactive in clusters in the early morning (O. R. Taylor, pers. com.), maneuverability and wing size does not seem to play a role in this type of predation. On the other hand, Pinheiro (1996) established the general rule that larger neotropical butterflies tended to escape bird attacks more frequently than small ones due to their high flight speed and sometimes unusual aerial maneuvers. Further observations or experiments are necessary in order to determine whether wing length plays a role in the escape of monarchs from predators, what wing size may be optimal, and what other morphological properties of the monarchs play a role.

A Possible Alternative Explanation

The observation that larger monarch butterflies originate from higher latitudes could be explained by cooler temperatures and unfavorable feeding condition in the northern parts of the breeding ground. Temperature and latitude are related, with cooler temperatures generally found farther north (Table 19). It has been demonstrated that cooler temperatures and starvation during development lead to an increased size of some insects and specifically increased wing size of butterflies at higher latitudes (Arango Velez, 1996; Chown and Gaston, 1999). Previous studies have found both this pattern during the fall migration (Beall and Williams, 1945; Borland et al., 2004) as well as the opposite pattern of small monarchs being collected at higher latitudes and monarchs with longer wings being sampled at lower latitudes (Beall and Williams, 1945). My own data does not show a pattern of size increase or decrease with latitude of collection site (Table 9). However, it is difficult to interpret data of monarchs sampled during the migration because they probably represent a mixture of butterflies originating from various places north of the sampling location, monarchs freshly emerged at the site of collection, as well as nonmigratory monarchs.

The pattern that monarchs originating from the north had longer wings than those from the south (Table 12 and 15) allows two interpretations. It can be explained by directional selection for longer wings during the fall migration, by a temperature-dependent size gradient across latitude, or both. Since variability in FWL tended to increase at lower latitudes (Table 12), the pattern is possibly due to selection for longer wings. If all sizes of monarchs were equally successful in the migration and the pattern is due to a temperature-dependent size gradient, I would expect the variation in FWL and frequency distribution in each δD group to be similar with just the mean FWL increasing at less negative δD values, i.e., at lower latitudes. However, variation at less negative δD increases, which indicates that only a certain group of monarchs around a high mean FWL may survive, and monarchs of all size ranges join the migration as the monarchs move south. These monarchs joining may not have been subject to selection pressures yet or local monarch may have been caught, which adds to the variability in FWL at lower latitudes. Even though it is possible that monarchs originating in the south have longer wings on average, it is likely that there is a directional selection for longer wings during the migration. Sampling freshly emerged migratory monarchs at different latitudes would resolve which explanation for the observed relationship between FWL and latitude is most probable.

Wing Length Measurements

The wing length measurement analyses considered alone did sufficiently support the hypothesis of directional selection on wing size during the fall migration. Even though monarchs from Mexico had longer wings than monarchs sampled in the U.S., the difference was not significant. It is not certain how well the butterflies photographed in Cerro Pelon represent the wing length of monarchs arriving in the overwintering sites, which would be the more reliable group of monarchs for the comparison. However, it has been shown that mean FWL of monarchs in the overwintering sites remain the same until mid-February and decline afterwards

(Calvert and Lawton, 1993). Since the sample of monarchs from Cerro Pelon had probably been killed by cold temperatures in January (O. R. Taylor, pers. com.; Monarch Watch: http://monarchwatch.org/blog/2008/03/21/deforestation-and-monarch-conservation Accessed June 14, 2008), FWL measurements are likely to represent the FWL of monarchs arriving in Mexico. A greater sample size may give more evidence, especially since only the mean forewing lengths was compared in order to avoid bias due to different sampling sizes.

There was no increase in mean size of monarchs at collection sites with lower latitude. In fact, the sample with the smallest wing size came from Texas, collected in 2006, the southernmost collection site in the U.S. This corroborates previous studies which found smaller monarchs in southern states (Beall and Williams, 1945; Borland et al., 2004). Rather, the simple linear regression indicated that there was a decrease in wing length with decreasing latitude. Yet, this relationship was more often than not non-significant in the forewing length data collected in 2006 and 2007 and latitude explained only 1.5% or less of the variation in forewing length (Table 7). The significance was generally higher when the PA2001 and PA2003 data were included. However, since it is not certain that the wing length of these butterflies had been measured the exact same way I did, a direct comparison of these wing length data with the other data is difficult.

The reason that no wing size pattern can be detected may lie in the problem that the butterflies used for FWL measurements represent a mix of monarch groups with differences in wing length due to several different reasons. Migratory monarchs could not be distinguished from freshly emerged or local monarchs. Depending on the distance traveled, migratory monarchs may have already been subject to selective pressures, thus shifting the mean wing length. In contrast, the size of freshly emerged monarchs is determined only by genetic, environmental, and developmental factors, and not by selective pressures. Monarch butterflies still breed during the migration until the late migration phase (Urquhart, 1987), when the environmental factors influencing wing size can be very different from the summer. In Lawrence, KS, monarch larvae can be found until the first week of October (O. R. Taylor, pers. com.), which falls into the late migration phase (Table 1). On October 4, 2001, in Cape May, NJ, local monarchs were still mating, laying eggs, and dying, while migrant monarchs were coming through with force (New Jersey Audubon Society homepage: http://www.njaudubon.org/Tools.Net/Sightings/Sightings.aspx?rt=NaturalHistory&rd=10/4/2001&tl=&tk=&ss= Accessed May 11, 2008.). The altitude angle on that day was 46.5 (U.S. Naval Observatory), which indicates the late migration phase. Breeding during the fall migration seems to be common (Borland et al., 2004). A possible decrease in food availability and the nutrient value of milkweed (Langvatn et al., 1996) during development time can influence body and wing size of monarchs emerging during migration and lead to different mean sizes from the summer population or butterflies emerging at the beginning of the migration. Borland et al. (2004) speculated that late migrants in Texas may have shorter wings because they were compromised during their larval development and sacrificed wing length to rapid development or lipid storage. This may be the general case in monarchs that develop when the migration is already on its way. Moreover, the butterfly samples from the early migration phase might contain summer monarchs, which have shorter wings than migratory monarchs (Herman, 1988).

Monarchs sampled further east, i.e., at the Atlantic coast, had longer wings than monarchs sampled at western longitudes. This relationship was significant in most cases (Table 8). It is possible that monarchs are blown off course on the Atlantic ocean and drown (Campesino, 2003; Urquhart, 1987), and monarchs with small wings may have more difficulties withstanding winds or flying back to land if blown out to sea or attempting to cross from peninsulas to mainland areas, e.g. Cape May, N.J., as Beall’s (1948) observation on monarchs that crossed Lake Erie suggests. Here, drowned monarchs had on average shorter wings than living ones sampled in the same area. This may explain why butterflies sampled closer to the east coast had on average longer wings. However, the TX monarchs collected at the coast of the Gulf of Mexico had small average wing lengths with 48.9 mm in 2006 and 50.3 mm in 2007. Those butterflies may have had a low mean wing length *a priori* because they were sampled at the very end of the fall migration, as discussed above. Moreover, this observation supports the idea that the smaller TX and TX2007 monarchs were outflown by larger ones that migrate faster. Completing the fall migration earlier in the season may be of advantage as more nectar sources are available earlier in fall and weather conditions may be better.

Stabilizing Selection

The hypothesis of stabilizing selection on wing length during the monarch fall migration was not supported. The highest variation in wing length was found in Texas, the southernmost sampling site, where low variability was expected. Even though the samples from Mexico, the destination of the monarchs, had a comparably low CV, samples from South Carolina and Arkansas were less variable. In the case of the SC butterflies, selection may be acting to reduce wing variation because there may be an optimal wing size for monarchs to deal with the migration along the coast. I speculate that monarchs with short wings may not be able to fly back to shore once blown out on the ocean, whereas monarchs with very long wings may be blown out farther on the ocean due to the increased contact surface. An optimal wing size may be a crucial advantage in survival since Shannon (1954; in Brower, 1995) speculated that it was unlikely that a group of monarchs that had been reported 24 km at sea ever regain the land.

The monarchs sampled in Arkansas likely originated from far north since there is little local monarch reproduction within 300 miles north of the sampling site (O. R. Taylor, pers. com.). Therefore, these monarchs probably present a group of migrants on which selection pressures have already acted and reduced variation.

Dockx (2007) determined that monarchs found in Cuba with the lowest CV (3.4 and 5.7 in two collection sites) were migrants, whereas the wing length of residents on Cuba were more variable (CV = 5.4 and 8.1 in two collection sites). Arango Velez (1996) observed similar trends both in wild caught and lab-reared monarchs. Migratory butterflies from various localities, extending from Wisconsin to central Mexico, collected over several years, were less variable in wing length and had longer wings than their resident counterpart. She concluded that stabilizing selection has eliminated the production of significant variation in migrant monarchs.

Differences among Migration Phases

Mean wing size of monarchs tended to decrease at the site of collection as the migration progressed through time. This pattern has also been observed by Borland et al. (2004) and Gibo and McCurdy (1993).

There are two possible explanations for this pattern. First, the monarchs emerging during the late phase of the migration may have shorter wings due to decreasing nutrient values of milkweed and environmental changes during larval development time, as discussed above.

Second, the butterflies with on average longer wings may have outflown individuals with shorter wings due to their improved gliding and soaring abilities. Monarchs with longer wings would arrive at any one location on their migration earlier than smaller monarchs and form the leading edge of the migration, i.e., the first butterflies to arrive at any one site during migration. In contrast, monarchs with shorter wings would lag behind and form the trailing edge of the migration, i.e., the last butterflies to arrive at any one location during migration.

The second explanation for the observed pattern is likely but possibly both apply. Mean FWLs among migration phases were not different at the latitude of origin, as determined by δD value, but monarchs at each collection sites in the late phase of the migration had smaller wings than monarchs sampled earlier in the migration at each site. Mean FWL may have decreased in the late migration phase because larger monarchs migrate faster, outfly smaller monarchs, and arrive in Mexico earlier.

A determination of the age of the butterflies would give further evidence for one of the explanations. If all monarchs took the same time to travel, there would be no age difference between monarchs with longer and shorter wings, which would support the first explanation. On the other hand, if monarchs with longer wings sampled at any one location were younger than butterflies with shorter wings, larger individuals possibly outfly smaller ones.

Curiously, the frequency distributions of the monarchs sampled during the earliest phase of the migration look similar to the wing length distribution in Mexico (Figure 13), giving rise to speculation that earlier phase migrants determine the distribution of monarch wing lengths at the overwintering sites. This may be the case. An earlier study (Taylor and Gibo) showed that the probability to arrive in Mexico is highest for early migrants which were tagged at AAs between 53 and 59. Since early migrants have a higher mean FWL, the successful arrival in the Mexican overwintering sites seems to depend on size and timing of migration or an interaction thereof.

The results of stable isotope analysis demonstrate that monarchs travel in a successive sweep during the fall migration. Monarch butterflies sampled in Oklahoma during the first half of the migration originated from further north, according to δD value, than the ones sampled in the second half. Therefore, it seems probable that as the body of monarchs moves from north to south as the migration progresses, the proportion of butterflies from further north declines because monarchs from further south, particularly through latitudes north of 35ºN, join the body of butterflies as it passes through those localities.

Residents versus Migrants and Male versus Female

My comparisons between summer and fall migratory monarch butterflies corroborated that resident monarchs have shorter wings. This is the same pattern observed in Minnesota monarch by Herman (1988). It is possible that resident monarchs allocate their resources to reproduction rather than wing growth since they would not gain advantages by increased soaring abilities like migrants do. Furthermore, males had longer wings than females, which is most likely genetically determined. The difference is consistent with earlier studies (Beall and Williams, 1945; Borland et al., 2004; Monarch Watch: http://www.monarchwatch.org/class/studproj/mass.htm Accessed in 2006 and 2008; Oberhauser and Frey, 1999) and was not only seen in migrants but also in residents (Herman, 1988).

The Stable Isotope Technique

Patterns in Monarch Wing and Milkweed Isotope Ratios

The applicability of hydrogen stable isotopes to determine the latitude of natal origin of eastern North American monarch butterflies was validated. The analyses of monarch and milkweed samples of known origin clearly showed that there is a relationship between latitude of origin and hydrogen stable isotope value of the butterfly or plant tissue. The main cause of this relation may be the temperature gradient across latitude since there was a tight relationship between latitude and mean summer temperature. In the isotope dataset from Hobson et al. (1999), temperature was the best single predictor for δD (Table 18).

Temperature influences two of the factors determining δD in water, namely evaporation and condensation. Evaporation increases with higher solar radiation and higher temperature, i.e., at lower latitudes. Isotope fractionation during evaporation generally increases the δD value of the source water, since the lighter isotope evaporates more readily. Factors influencing fractionation during evaporation are atmospheric humidity, the amount of liquid, the isotopic composition of the evaporating water body and atmosphere, and other factors described in the Craig-Gordon model (Craig and Gordon, 1965). Evaporation can occur for instance from land surfaces, water bodies, and leaf surfaces (Bowen and West, 2008) and influences the isotopic composition of plant water. Moreover, water molecules containing the heavy isotope form are preferentially incorporated into water droplets or ice crystals during condensation. These droplets or crystals are removed from the cloud system, thus leaving the cloud vapor depleted in deuterium. As air masses move from tropical to polar regions, the δD value of the cloud vapor and condensed droplets in precipitation become progressively lower according to the Rayleigh equation (Bowen and West, 2008). Fractionation during evaporation and condensation explain why the hydrogen isotope composition of milkweed and thus of monarch wings became enriched at lower latitudes.

The monarch wing δD values from 1996 were comparable to the ones from 2007 when plotted against latitude of origin (Figure 18) despite differences in summer temperature and precipitation between the two years (Table 21). Since the slopes of the two linear regressions were not significantly different, I assume that the 1996 base map (Figure 2) might be used to estimate the latitude of origin of monarchs sampled in 2007. This means that the monarch collected in Texas in 2007 with the lowest δD values probably originated in southern Canada at a latitude above 45ºN, maybe above 50ºN. The butterfly with the highest δD value probably emerged at a latitude below 35ºN and may have even been local. Whether the base map could also be used to estimate the origin of monarchs sampled in 2006 is more problematical.

Table 21. Mean temperature from June to August (T) and mean precipitation from June to August (P) in 3 years in the USA and in the states in which isotope data were collected (Source: http://lwf.ncdc.noaa.gov/oa/climate/research/cag3/state.html Accessed July 2008)

Location	**T 1996 (°C)**	**P 1996 (L/m^2)**	**T 2006 (°C)**	**P 2006 (L/m^2)**	**T 2007 (°C)**	**P 2007 (L/m^2)**
USA	22.6	221.1	23.5	203.2	23.3	218.0
States in which monarchs were reared in 2007	22.0	351.2	22.8	411.2	22.6	286.8
States in which milkweed were reared in 2007	22.3	340.3	23.2	376.8	23.2	313.1
States of wild-rearing experiment by Hobson et al. (1999)	22.9	316.3	23.9	307.4	23.6	296.6

Overall, the summer of 2006 was warmer (Table 21), which may have shifted the geographic patterns south compared to the base map from 1996. This might explain why several monarchs in my samples from 2006 originated south of the collection site if their δD value was interpreted using the base map from 1996 (Figure 2). Local variability may have also played a role. Only comparisons of monarch wing δD values across a geographic range among years would help to determine how large the interannual variation of hydrogen isotope is and whether the base map of one year could potentially be used for another year, if necessary after factoring in sources of variation such as temperature.

Other Sources of Variation

Isotope data obtained from milkweed grown at the Atlantic coast in James Island, South Carolina, give evidence of large variation in wing and milkweed δD of up to 30‰, even within in the same species (Table 3). The *Seutera augustifolium* plants were probably collected in two sites. One site was on north Folly Island where the plants grew on an outer seepage slope with brackish soils where maritime forest transitions into salt shrub. The second site was on Black Island with a similar habitat, however, the plants received more sunlight and grew on probably less organic soil (Billy McCord, pers. com.). Even though it is not known which of the three *Seutera* plants grew in which site, it is possible that the different light, water, and soil conditions at the two sites account for the variation in the wing δD values of the monarchs raised on these plants. The δD values of monarchs raised on the tropical milkweed (*Asclepia curassavica*) grown in loamy, organic soils at one site were more similar with only about 5‰ difference. The result is expected since the milkweed plants grew under similar conditions. They had been seedlings in Billy McCord's yard in a loamy, organic soil with varying moisture dependent on rainfall. They had been watered from a shallow well, and while rearing the monarch larvae, they had not been watered at all (Billy McCord, pers. com.).

A number of factors may have led to the observed variability. Plants in shade are likely to have access to water which experienced less evaporation and are themselves subject to decreased evaporation, thus counteracting enrichment.

On the other hand, strong winds increase surface evaporation (Luo and Stephens, 2006) and thus lead to isotopic enrichment of water. Leaf water becomes isotopically enriched relative to source water during transpiration (Pendall et al., 2005), i.e., evaporation of water from plants. Stomatal transpiration is regulated by atmospheric humidity, light intensity, temperature, and wind velocity, and does not follow a simple linear relationship with temperature.

Moreover, the water source of a plant can influence its isotopic composition. For instance, the δD value of soil water varies with depth (Valentini and Mugnozza, 1992; Jackson et al., 1999; Newman et al., 2006), which can lead to differences in δD between shallow root and deep root plants even at the same location. Plants may even use a mixture of different water sources (Feild and Dawson, 1998). Milkweeds are generally deep-rooted; the butterfly milkweed (*Asclepias tuberosa*), for instance, has a deep taproot (Kansas Wildflowers & Grasses:

http://www.kswildflower.org/details.php?flowerID=2 Accessed July, 2008), and the rootstock of the common milkweed (*Asclepias syriaca*) may be 10 to 40 cm below the soil surface (Jeffery and Robison, 1971). Since monarch butterfly larvae exclusively feed on milkweed, any variation with respect to shallow and deep rooting plants should be largely eliminated. In fact, the common milkweed is probably the principal milkweed host of overwintering monarchs that begin their southward fall migration to Mexico in September (Malcolm et al., 1989). Only the tropical milkweed (*Asclepias curassavica*) is very shallow rooted (O. R. Taylor, pers. com) and thus would reflect the water available at the surface level rather than deeper as in other milkweeds. This might be the reason why the wings of monarchs raised on tropical milkweed had more depleted δD values than monarchs raised on other milkweed species (Table 3).

There is altitudinal depletion in δD from -1 to -4‰ per 100 m rise in elevation because deuterium in precipitation tends to rain out more at lower elevation than at higher elevations (Hobson, 2008). Elevation played a minor role in explaining the δD of monarchs and milkweed possibly because most sampling sites were on altitudes with only 400 m difference. The latitudinal effect had a much greater influence on the hydrogen stable isotope ratio.

In general, there is a negative correlation between amount of precipitation and δD. Although this effect is pronounced in most tropical areas, it can also be found at mid latitudes during the summer (Daansgard, 1964; Gat, 1996). My analyses showed no relationship between mean summer precipitation and δD (Table 19). The effect of amount of precipitation might be negligible in the latitudes at which the measurements were taken. Moreover, the measurements for the amount precipitation were obtained for the entire state and not for the specific sampling location, which might have skewed any possible effect.

The δD value in precipitation becomes generally more depleted the closer to the interior of a continent it is measured. Even though the hydrogen stable isotope value of the monarch and milkweed samples from 2007 and 1996 showed this tendency, the relationship was not significant. The measurements of distance to the nearest coast can not be used to gauge δD values since such measures do not reflect the real path which air masses travel nor the potential influence of inland water bodies.

Moreover, variation in discrimination between water source and tissue as well as between diet and tissue contribute to uncertainties with assigning an organism to its place of origin. Even though there was a tight fit between monarch wing δD and plant growth water δD in a laboratory experiment ($R^2 = 0.99$), the relationship was not as strong in field-reared monarchs ($R^2 = 0.69$) (Hobson et al., 1999).

Besides the natural variation in stable isotope ratios, there is an analytical error inherent in CF-IRMS measurements for δD of ±2‰ (Wassenaar, 2008) or even larger, as in the replicates of tagged Mexican monarch butterflies.

Employing a second stable isotope to decrease assignment errors and increase resolution is desirable. Hobson et al. (1999) measured the carbon stable isotope in monarch butterfly wings to that end. However, the geographic pattern of $\delta^{13}C$ is spatially not predictable from year to year (Len Wassenaar, pers. com.), which is why a new base map for each sampling year needs to be created.

The monarch wild-rearing experiment, on which the isotopic base maps from 1996 was based (Figure 2), excluded one factor of variation, namely plant water source. Monarchs were raised from eggs on naturally occurring milkweed whose only source of moisture was local rainwater. The volunteers participating in the experiment were instructed not to use milkweed from gardens, irrigated fields, drainage ditches, inner city lots, and other locations in which the water may have had inputs other than that of rainwater (Hobson et al., 1999).

Moreover, to improve the estimate of the true isotope value of monarchs raised in each location, the mean isotopic value of one to nine monarch butterflies in each rearing site was used. However, standard deviations in δD between 0.1 and 15.7 indicate that there can be much isotopic variation among individuals in one site, even greater variation than the monarch samples from South Carolina from 2007 show, which had a standard deviation of 14.3.

For isotope studies on wild-caught migratory monarch butterflies, it is not possible to determine whether butterflies fed on milkweed whose only water source was local rainwater and to analyze a group of monarchs from the same location of natal origin unless they were tagged at the place of their emergence. Therefore, determining the origin of a monarch is inevitably prone to errors, even if a base map is established for each year in which monarchs are sampled in order to avoid inter annual variation in the isotopic pattern.

One potential source of isotopic variation was removed in all these studies because of the tight coupling between monarch butterfly larvae and their single genus of host plants (Hobson, 2008). Differences in gas exchange, photosynthetic pathways and other physiological plant processes among groups of plants can lead to slight to pronounced differences in stable isotopic signatures (Ehleringer and Cerling, 2001; Dawson et al., 2002).

Challenges

A challenge to the application of stable isotopes as indicators of natal origin of monarch butterflies is to understand the mechanisms that drive the observed isotopic variation and to incorporate this variation in statistical methods for assigning individuals to places of origin (Kelly et al., 2008). The use of year-specific base maps is desirable, at least until the isotopic variation is better understood and maybe beyond that. The drawbacks of creating base maps involve efforts with respect to the logistics, work and cost of obtaining and analyzing year-specific tissue samples from the geographic range of interest.

It is important to understand what ecological and physiological factors determine water-tissue and diet-tissue isotopic discrimination and how that discrimination varies within and among individuals at a given location or on a given diet (Kelly et al., 2008). Moreover, knowing the ecology of the animal in question is crucial. It was prerequisite that monarchs migrate between isotopically distinct landscapes and that they incorporate the isotopic signature of their natal origin in their wing tissue. The discrepancies in the isotope data alerted me of the interannual variation in geographic patterns of stable isotopes; for instance, it is unlikely that monarch butterflies travel north during the fall migration, even though the hydrogen stable isotope values of some butterflies suggested just this.

For studies that concentrate on animals other than monarch butterflies, the choice of the tissue to be analyzed, the way of cleaning the tissue, time of sampling, isotopic variation within the tissue and variation among individuals from the same site are important points to keep in mind. Of course, the application of several stable isotopes can enhance spatial resolution, and the use of additional tracking techniques can validate findings.

Conclusion

The results of this study do not allow definite conclusions for or against my hypotheses of selection on monarch butterfly wing length during the fall migration. The isotope analysis provides evidence that directional selection increases the mean size of monarch butterflies during the fall migration. CFD simulations demonstrated that lift production increases with wing size, which may be one the causal factor of an improved migration success of larger individuals. Monarchs with longer wings probably originated from the northern part of the breeding range. A temperature-dependent size gradient across latitude might explain the observed results as well, but an increase in the variation in forewing length at lower latitudes suggests that there is selection for longer wings during the fall migration. Larger monarchs may be able to travel faster and arrive in Mexico earlier. The distributions of wing lengths of early migrants are similar to those seen at the overwintering sites in Mexico. Further, the results of recoveries of tagged monarchs shows that the probability of reaching Mexico is highest for those monarchs advancing on the leading edge of the migration (Taylor and Gibo). An implication of these findings is that the likelihood to reach the overwintering sites in Mexico may depend on size and time of migration, with larger, early migrants having advantages over smaller, late migrants.

The hypothesis of stabilizing selection on wing size was not supported by my results. More extensive studies are necessary in order to resolve the questions of directional and stabilizing selection. First, wing length measurements during the summer months and at the beginning of the migration at various latitudes in the monarch breeding range should be conducted to resolve whether wing length varies with latitude of origin. If this is not the case, the wing lengths and stable isotope ratios of monarch butterflies from a greater range of latitude should be measured. If possible, sampling should occur multiple times over the entire period of the migration, synchronized in each collection site according to altitude angle. Moreover, it is advised to create a new isotopic base map for both hydrogen and carbon stable isotopes to improve resolution.

This study demonstrated that hydrogen stable isotopes in monarch wing chitin can be utilized to determine the latitude of natal origin of the butterflies. Multiple sources of variability in δD should be researched further in order to improve the assignment of monarchs to the place of their origin. Furthermore, the use of a second and third stable isotope, such as ^{13}C, ^{87}Sr or ^{34}S should be considered (Hobson et al., 1999).

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