# A geometric morphometric analysis of wing shape variation in monarch butterflies *Danaus plexippus*

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***Abstract***

Shape is an important element in biological systems because it provides a link between genotype and environment. Morphological variation may be the result of genetic differences or environmental factors, depending on the degree of phenotypic plasticity. In this experiment we explored the effects of diet and gender on both forewing and hindwing shape of monarch butterflies *Danaus plexippus.* The butterflies were reared on five different species of milkweed plant (*Asclepias curassavica, Asclepias erosa, Asclepias fascicularis, Asclepias speciosa,* and *Asclepias incarnata*), each varying in cardenolide concentration and toxicity. We found that gender influenced the shape of both monarch butterfly forewings and hindwings, but that diet influenced the shape of only forewings. The effect of diet on forewing shape was maintained even after correction for dietary effects on wing size. There is strong evidence that the total cardenolide concentration and cardenolide composition of milkweed hosts have a significant effect on forewing shape. Our results suggest that the wings of monarch butterflies are sexually dimorphic, and that diet and cardenolide concentration have significant effects on monarch butterfly forewing shape. However, further research is still needed to determine how these wing shape variations affect monarch butterfly flight ability, ecological interactions, and reproductive success.

***Introduction***

The analysis of shape is crucial in the field of biology. Throughout history, the study of shape has been used for taxonomic classification, analysis of different developmental forms, defining functional and evolutionary relationships, and exploring environmental

effects on organisms, in addition to many other uses (Adams 1999; Ricklefs & Miles 1994; Adams et al. 2004). Shape provides information from homologous points when variation in size, location, scale, and orientation are removed (Zelditch et al. 2012; Rohlf & Slice 1990; Adams 1999). Shape is a morphological trait that provides a phenotypic link between genotype and environment (Ricklefs and Miles 1994). Previous research has examined how both genotypic and environmental factors create variation in shape and how these factors may affect overall performance.

Specifically, if environmental factors strongly affect a phenotypic trait, such as shape, then traits are said to exhibit phenotypic plasticity (Via & Lande 1985; Schlichting & Pigliucci 1998). Traits with higher levels of plasticity are more sensitive to changes associated with environmental changes (Via & Lande 1985). Depending on an organism's life history, there may be optimal levels of plasticity to maximize overall fitness. Increased levels of plasticity may allow an organism to fill more niches and occupy a broader range of environments (Via et al. 1995). The ability to vary morphologies may also be a result of differential interactions with other organisms (Agrawal 2001). Predatory or mutualistic interactions between organisms may drive the expression of phenotypes (Thompson 1988). Ultimately, a lower level of phenotypic plasticity may lead to negative outcomes for two organisms competing or being introduced into a novel environment (Pellmyr & Huth 1994; Agrawal 2001).

Phenotypic plasticity can be adaptive (Via et al. 1995). For example, many previous studies have examined how shape variations correlate with changes in environmental factors (Merckx & Van Dyck 2006). It is common for animals to alter their morphology or life history behaviors in different environments (Schlichting 1986; Harvell 1994;

Kingsolver & Huey 1998; Schlichting & Pigliucci 1998; Tollrian & Harvell 1999). Seasonal polyphenism is a type of phenotypic plasticity in which phenotype changes with the environmental variations by season. Brakefield et al. (1996) conducted an experiment altering the temperature and photoperiod in a laboratory setting where caterpillars were raised. He found that butterfly wing color changed with the environmental conditions experienced by larvae. Similarly, water temperature during the larval stages of certain fish species affects phenotype, specifically shape, throughout the remainder of the life of the fish (Georgakopoulou et al. 2007). Damselflies also exhibit significant wing shape variation in different landscape habitats. Broader wing bases are more common and beneficial for damselflies living in open landscapes (Outomoro et al. 2012). Additionally, sea urchin larvae have been shown to change the lengths of their arms, or ciliary bands based on the amount of food present (Miner & Vonesh 2004). It is therefore common for organisms to exhibit adaptive phenotypic plasticity in response to environmental changes.

While plasticity in shape may be adaptive, it also may be a sign of stress. In biology, symmetry is often studied as a measure of health. The more symmetrical certain characters are, the fitter the individual is said to be. This theory is known as fluctuating asymmetry, where morphological traits are observed under different levels and types of environmental stress. Increased levels of stress supposedly disrupt developmental processes and correlate with increased levels of asymmetry (Parsons 1992). For example, larval crowding, a stress inducing environment, causes wing asymmetry in speckled wood butterflies (Gibbs & Breuker 2006). Shape is an important factor that affects symmetry, and a preliminary analysis of shape and the factors that affect it will provide valuable information on the conditions that influence individual fitness.

While some experiments show that organisms vary morphologically depending on their environment, this is not always the case. A study using geometric morphometric methods examined the wing shape of *Synneuria* (Lepidoptera: Geometridae) and the factors that affect it. The moths were sexually dimorphic, but showed no significant differences in shape between localities (Benitez et al. 2011). Sexual dimorphism is common in many organisms because each gender is under different selection pressures to maximize reproductive success, and each gender has different life history traits. For example, sexual dimorphism is often seen in insects, as females have to allocate resources towards producing and ovipositing eggs, while some males divert resources towards creating spermatophores (Breuker et al. 2007). The life history of some insect species leads males to never even eat as adults (Shine 1989). Differences in resource allocation may often play a significant role in sexual dimorphism and variation in shape (Shine 1989). Additionally, sexual selection may also lead to sexual dimorphism. Intersexual selection within species leads to mate preference, driving the traits of one gender to change in response to that preference (Lande 1980).

The current study examines phenotypic plasticity and morphological shape variation in the specialist herbivore, the monarch butterfly, *Danaus plexippus.* Monarchs are holometabolous insects that feed only on milkweed plants (genus *Asclepias*) in their larval stages. *Danaus plexippus* have been of significant interest to biologists for centuries. They are a unique and interesting species, both ecologically and aesthetically. Every year at the end of summer and beginning of autumn, they migrate from North America to either Mexico or Southern California (Urquhart & Urquhart 1978; Brower & Malcolm 1991; Calvert & Lawton 1993). Monarchs are the only known Lepidopteran species to make a

migration of this length, similar to bird migrations. Wing shape, and the factors that affect it, are very important for monarchs because of this long distance flight. Previous research has shown that increased parasite load on migratory monarch populations decrease flight distance and flight speeds (Bradley & Altizer 2005). Knowledge of ideal wing shape, and ultimately symmetry, may eventually assist in the conservation of the species as they continue their annual migration. In this experiment, we used geometric morphometric analysis to examine how sex, diet, butterfly cardenolide concentration, and cardenolide composition affect monarch butterfly wing shape using five different species of milkweed as the larval diet. We found that butterfly gender affects both monarch forewing and hindwing shape, but that only forewing shape was affected by larval diet. Our results also provide evidence that total cardenolide content and composition, an important variable in monarch diet, may be responsible for the dietary effect on forewing shape.

### *Materials and Methods*

The monarchs used in this experiment were the non-inbred descendants of butterflies collected in St. Marks, FL, U.S.A. Individual larvae were reared from larval eclosion to adulthood on one of five species of milkweed: *Asclepias curassavica, A. erosa, A. fascicularis, A. speciosa,* and *A. incarnata*. Because the butterflies were subsequently caged and mated for additional experiments (unrelated to the study presented here), many individuals suffered wing damage during the course of their adult lives. Unfortunately, any damage to the wing disqualified them from further use in the study. Even the smallest bit of morphological damage made the specimen unusable. Our sample sizes are therefore limited. Nonetheless, we were able to obtain forewing samples from monarchs reared from all five milkweed

species, and hindwing samples from those reared on *A. curassavica* and *A. incarnata*. The sample size of monarch caterpillars on each diet is shown is Table 1a and Table 1b.

Milkweed plants contain secondary defensive chemical compounds, toxic steroids known as cardenolides. Cardenolides can be toxic to animals by disrupting the Na+/K+ ATPase system in cell membranes (Agrawal et al. 2012). Generally, *A. erosa* and *A. curassavica* have high foliar cardenolide concentrations, *A. speciosa* and *A. fascicularis* have an intermediate concentration, and *A. incarnata* has low foliar cardenolide concentrations (Agrawal & Malcolm 2002; Sternberg et al. 2012). Additionally, the more toxic milkweed plants have a greater proportion of non-polar cardenolides (Steinberg et al. 2012). The non-polar structure allows the chemicals to more easily cross cell membranes and compromise cell function (Agrawal 2011).

Monarch larvae were reared individually in plastic containers at 25 °C, 16:8 L:D and fed their assigned plant diets ad libitum until pupation (about 16 days). The plants used to rear monarchs were grown from seed (Butterfly Encounters, Inc.) at 25 °C, 16:8 L:D. Larvae were provided with cut leaves that were renewed every 2 days during the first 8 days of larval growth and daily thereafter. Monarchs grew too large to receive all of their food from single milkweed plants, and each larva received food from 3 to 5 individual plants during the larval period. Therefore, we cannot match the cardenolide chemistry of individual plants to the chemistry of specific adults. However, we measured the cardenolides sequestered by each individual butterfly.

**Table 1a**: Forewing samples of monarchs reared on each milkweed diet

| | *A. curassavica* | *A. incarnata* | *A. erosa* | *A. fascicularis* | *A. speciosa* | Total |
|---|---|---|---|---|---|---|
| Male | 0 | 4 | 3 | 3 | 1 | 11 |
| Female | 2 | 8 | 4 | 4 | 5 | 23 |
| Total | 2 | 12 | 7 | 7 | 6 | N=34 |

**Table 1b**: Hindwing samples on monarchs reared on each milkweed diet

| | *A. curassavica* | *A. incarnata* | Total |
|---|---|---|---|
| Male | 16 | 15 | 31 |
| Female | 15 | 14 | 29 |
| Total | 31 | 29 | N=60 |

After death, monarch butterflies were placed into glassine envelopes and frozen at -23.33 °C. Subsequently, forewings and hindwings were detached from the thorax using forceps. The wings were then returned to individual glassine envelopes and stored in the freezer at -23.33 °C until scanning. As noted previously, the intended sample size for this study was much larger, but the majority of wing samples were damaged and removed from the study.

*Geometric Morphometrics*

Because of the unique nature of biological shapes, landmark-based geometric morphometric analyses are more useful than past methods that relied on measuring angles and linear distances between specimen characters (Adams et al. 2004). Geometric analyses are much more powerful and complete than simple qualitative observations (Bookstein 1978; Rohlf & Marcus 1993). The current methods of geometric morphometric analysis are capable of distinguishing subtle differences in shape (Adams et al. 2004).

The usable monarch wings were scanned into a Windows computer using a HP ScanJet 6300C. The wings were placed next to a ruler in the image to later scale the image. Each image was then saved as a TIFF file and loaded into a digitizing program, tpsDig2.17 (Rohlf, F. J. 2013. SUNY Stonybrook. www.life.bio.sunysb.edu/morph) The hindwings and

forewings were scanned to separate folders, and were analyzed separately for the remainder of the experiment.

Images were individually scaled by digitizing two points 1 cm apart on the ruler. Then a cross-hairs tool was used to digitize landmarks on each wing specimen. The 19 landmarks on the forewings are shown in Figure 1a and the 24 landmarks on the hindwings are shown in Figure 1b. Each landmark is a distinct anatomical point found on every butterfly wing, generally at vein intersections or ends. On the forewings, the landmarks 1 through 5 start at the base of the wing and enclose the discal cell at vein intersections. Landmarks 6 through 19 start at the bottom outside edge of the wing and mark each vein as it reaches the perimeter of the wing. The hindwings contained 24 landmarks. Landmarks 2 through 9 outlined the discal cell, while landmarks 10 through 24 marked veins on the outer perimeter of the wing.

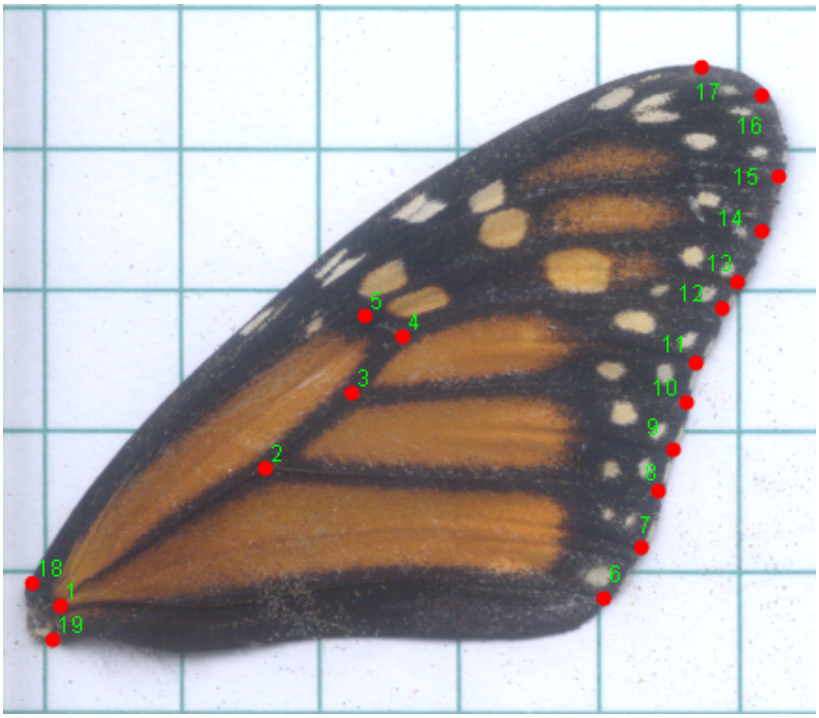


**Figure 1a**: Landmark digitization of a monarch forewing.

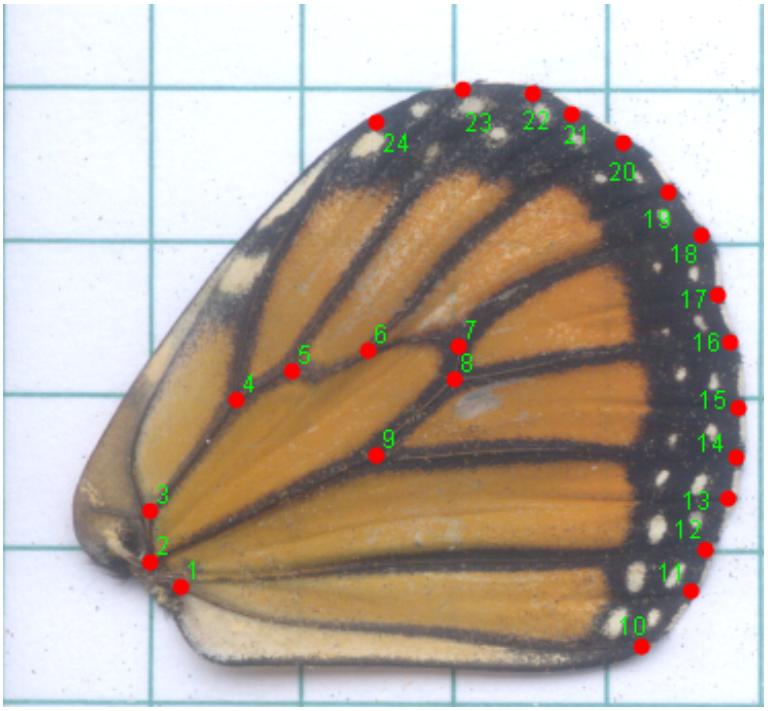


**Figure 1b**: Landmark digitization of a monarch hindwing.

Following landmark digitization, semilandmarks were added to each specimen. A curve-tracing tool was used to trace the outside of the wing not covered by the landmarks. Beginning at the base of the forewing at landmark 18, the curve traced upwards until landmark 17 was reached. Another curve was then traced from landmark 19 until landmark 6 was reached. The same process was used for semilandmark digitization of hindwings. Once each curve was traced, the "resample by length" function was used with 25 as the number of semilandmarks for both top and bottom curves on the forewings, and 30 as the number of semilandmarks on the hindwings. Individual points were then adjusted as needed to ensure their placement along the perimeter of the wing. The semilandmarks were then converted to landmarks using TpsUtil1.58 (Rohlf, F. J. 2013.

SUNY Stonybrook. www.life.bio.sunysb.edu/morph) using the "append tps curve to landmarks" function.

This new file was then loaded into TpsUtil1.58 to create a sliders file. The sliders file was used to remove the arbitrary variation caused by differences in positioning of the semilandmarks on different individuals; unlike landmarks, semilandmarks are not biologically corresponding points so their positioning along the curve is another component of non-shape (Zelditch et al. 2012).

*Cardenolide Analysis*

After scanning and digitizing the wings, they were weighed individually on a microbalance in preparation for chemical analysis. We measured cardenolides in the butterflies using methods described by Zehnder & Hunter (2007). Each wing sample was ground and then extracted in methanol. The supernatant from samples in methanol was evaporated at 45 °C until dry. Samples were then resuspended in 150 µL of methanol containing 0.15 mg/mL digitoxin as an internal standard and analyzed using reverse phase high-performance liquid chromatography (UPLC, Waters Inc., Milford, MA, USA). Running time for each sample was 9 min. Peaks were detected by absorption at 218 nm using a diode array detector, and absorbance spectra were recorded from 200 to 300 nm. Peaks with symmetrical absorption maxima between 217 and 222 nm were recorded as cardenolides. Total cardenolide concentration was calculated as the sum of all separated cardenolide peaks, corrected by the concentration of the internal standard (digitoxin) and the estimated sample mass.

To visualize differences in the cardenolide composition of wing specimens we used metaMDS in Vegan for Nonmetric Multidimensional Scaling (NMDS) (McCune & Grace 2002), stepping down from a six-dimensional model to a one-dimensional model, with 999

permutations per model run and a maximum of 20 runs per dimension. Inspection of the screen plot illustrated that model stress declined rapidly from a one-dimensional to a two-dimensional model, declining only slightly thereafter. We therefore used a two-dimensional model for visualization (model stress = 0.0624) well within the range that is typical of ecological data (McCune & Grace 2002). We used the NMDS coordinates from this analysis to plot the position of butterfly wings in multidimensional cardenolide space.

*Statistical Analysis*

Both the original TPS data file with landmarks and the sliders file were loaded into R (3.2.0). A Generalized Procrustes Analysis (GPA) was used to remove variation unrelated to shape (position, scale and orientation of the specimens). As of yet, there is no consensus on the best method of semilandmark superimposition, so two were used in this study (Appendix A). "SuperA" removes variation in the positioning of semilandmarks along the curve by minimizing the Procrustes distance, and "SuperB" reduces variation along the curve by minimizing bending energy (Bookstein 1989; Goodall 1991; Perez et al. 2006). Results were qualitatively similar between methods (Appendix A) and we present only results from "SuperB." The mean shape was determined for each data set (forewings or hindwings) using the mshape function "geomorph" package, and variation in each set was examined by first converting the data to a two-dimensional array then using a principal components analysis (PCA) so the dataset could be visually examined and any outliers determined (Zelditch et al. 2012).

The "vegan" package in R was then used to investigate wing shape variation based on size, gender, diet, and cardenolide content and composition. Again, there is not yet a consensus regarding the best statistical test to use, so we conducted both Procrustes

ANOVA and MANOVA (Appendix A). A Procrustes ANOVA is equivalent to a permutational Manova of the distance matrix (Anderson 2001). The MANOVA is a fully multivariate procedure that takes the sample variance-covariance into account. This method requires inverting the variance-covariance matrix, but covariance matrices for shape are not invertible both because there are far more coordinates than there are degrees of freedom (due to those lost by superimposition) and because there are more coordinates than individuals. To do the MANOVA, it is necessary to reduce the dimensionality of the data, which is done by replacing the coordinates by scores on principal components. Two different methods were used for determining the number of principal components to use. "PCA1" used N/4 for the number of principal components because using too many PCs relative to sample size can exaggerate the separation between samples but using too few can underestimate the error variance. The other method, "PCA2" used the broken stick model to determine the number of principal components. Tests were run on both wing sets for both sets of principal components (Appendix A).

Additionally, canonical variates analyses were performed on the dietary and gender data for each wing set. These analyses displayed shape variation between dietary and gender groups by giving the equivalent number of standard deviations between each group. These numbers, or equivalent standard deviations, are known as the Mahalanobis Distances (D).

For both forewings and hindwings, we used the previously mentioned statistical methods to explore (1) the effects of gender and diet on shape, (2) the effects of gender and diet on shape after accounting for effects of diet on size, (3) the effects of gender and

cardenolide concentration on shape, and (4) the effects of gender and cardenolide composition (NMDS axis 1 and NMDS axis 2) on shape.

***Results***

For all statistical tests run, the models provided qualitatively consistent results. In our examination of the tests, we chose to use the Procrustes ANOVA tests with the superimposition minimizing bending energy (SuperB) because these data provided the clearest distinction of diet related effects for all hypotheses. The results for this test were consistent with all of the other models, with the exception of one test run: test one examining the relationship between shape diet and sex of hindwings (Appendix A).

The shape of monarch butterfly forewings varied with gender ($F_{1, 28} = 4.2570$, $p < 0.001$) ($D = 4.4385$) (Figure 2 and Figure 3) and diet ($F_{4, 28} = 2.5184$, $p < 0.001$) (Figure 4 and Figure 5). The greatest difference in mean shape between dietary groups was between those that fed on *A. incarnata* and those that fed on *A. erosa* ($D = 6.9130$). An effect of diet on forewing shape was maintained ($F_{4, 27} = 1.6506$, $p = 0.049$) after accounting for the effect of diet on forewing size ($F_{1, 27} = 5.5375$, $p < 0.001$).

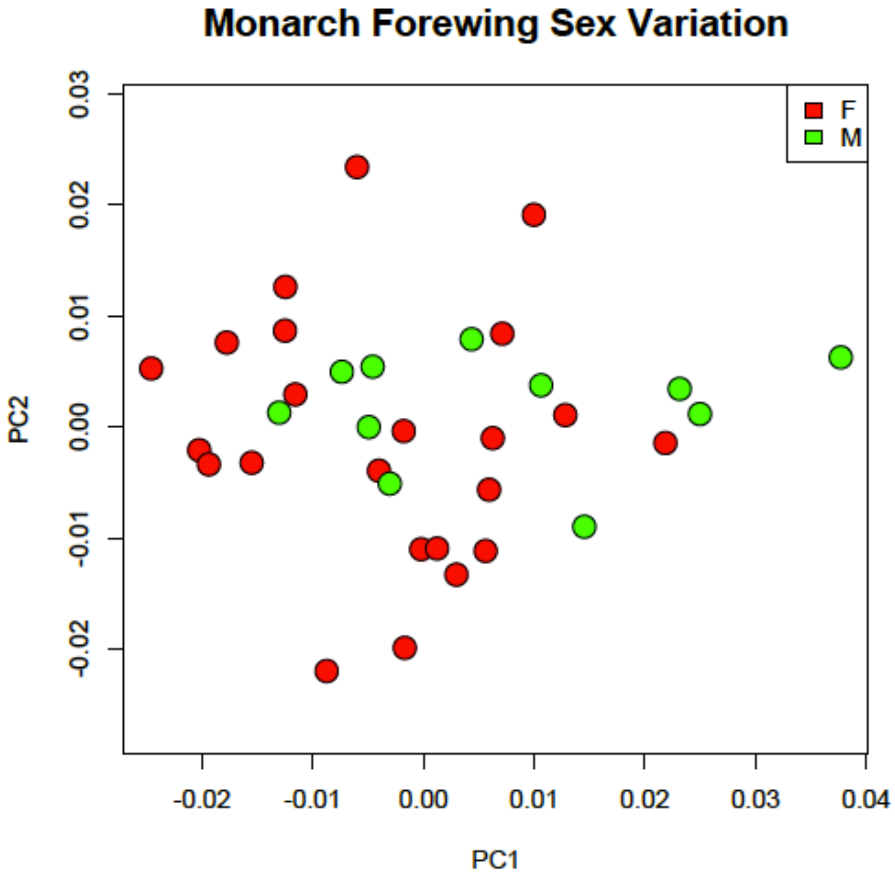


**Figure 2**: A principal components plot of forewing shape distinguished by gender.

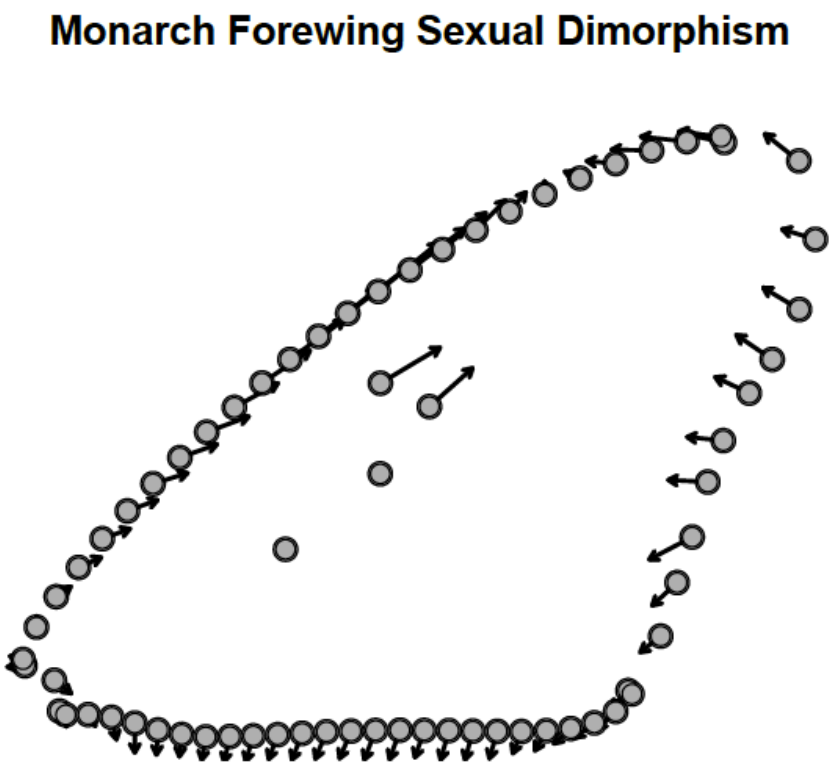


**Figure 3:** Mean forewing shape variation between females and males exaggerated three times. The gray dots are representative of the female means and the arrows point in the direction of the male means.

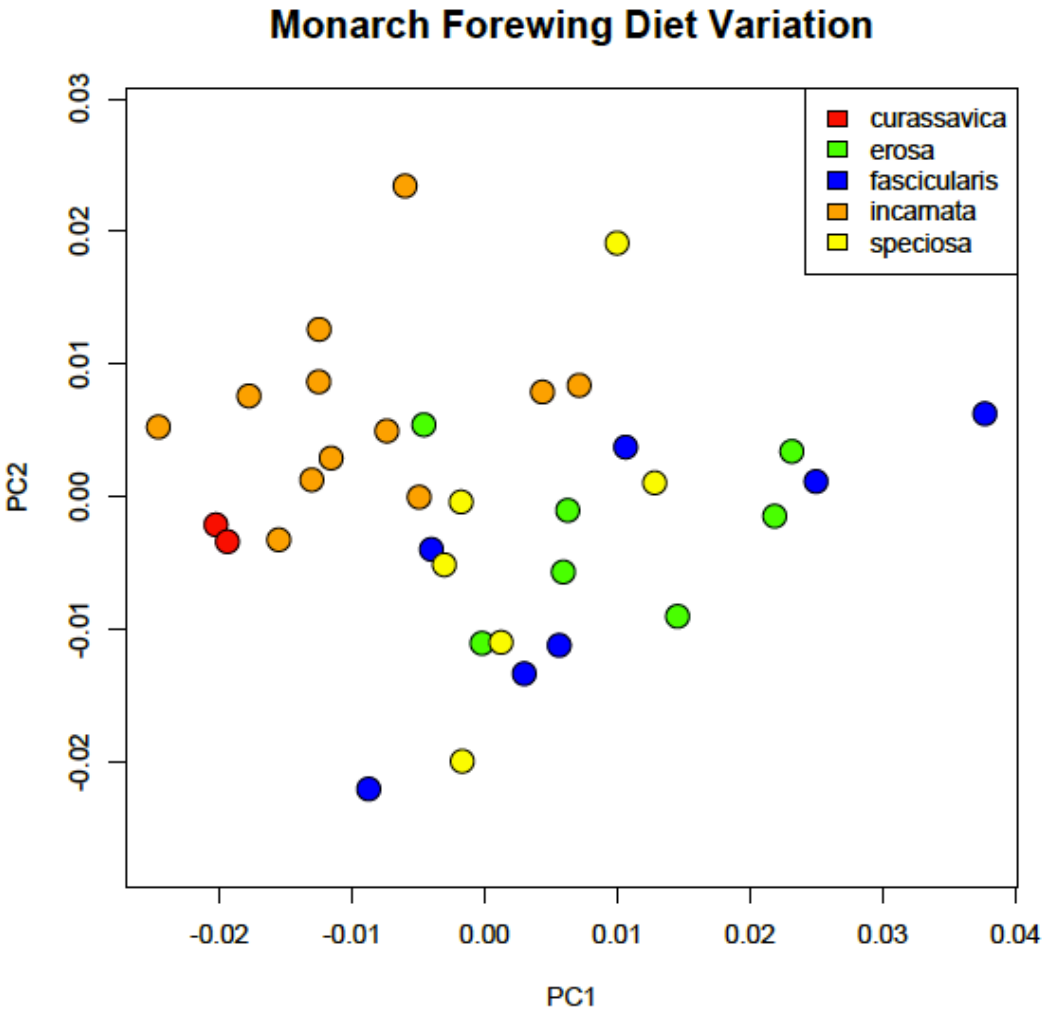


**Figure 4**: A principal components plot of forewing shape distinguished by species of milkweed used as larval diet.

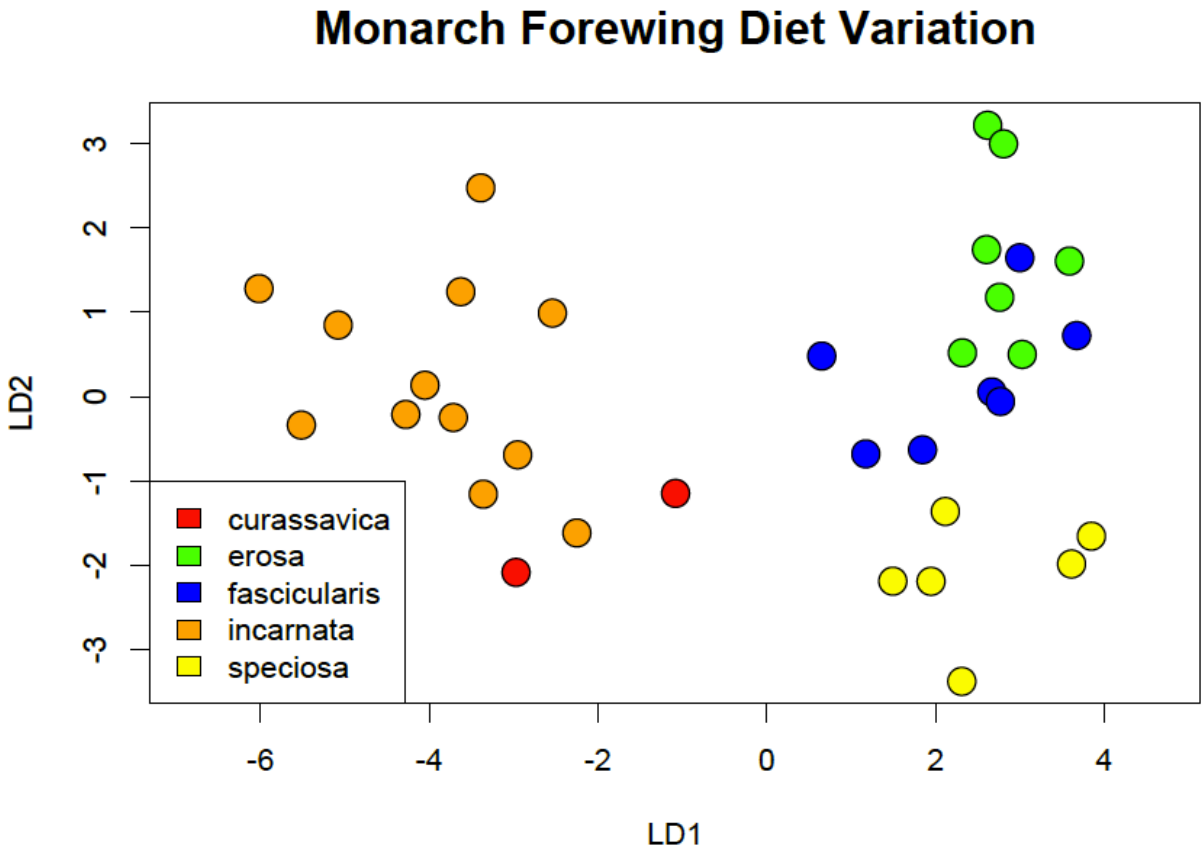


**Figure 5**: A canonical variates analysis plot of between group forewing variation of monarchs that fed on different species of milkweed.

The total concentration of cardenolides in butterfly wings was associated with the shape of monarch forewings ($F_{1, 30}$ = 2.1441, p = 0.007). In addition to the total cardenolide concentration, the composition of the cardenolides (NMDS 1) was associated with monarch forewing shape variation ($F_{1, 29}$ = 4.9722, p < 0.001). These provide two independent lines of evidence that cardenolides are a component of monarch diet that affect forewing shape. NMDS 2, another axis describing cardenolide composition, did not affect forewing shape ($F_{1, 29}$ = 0.4883, p = 0.904).

In addition to its effect on forewing shape, gender also influenced the shape of monarch butterfly hindwings ($F_{1, 57} = 2.1441$, $p = 0.039$) ($D = 2.7575$) (Figure 6 and Figure 7). Effects of diet on hindwing shape are equivocal. Using ANOVA Super B, diet had a significant affect on hindwing shape ($F_{1, 57} = 2.3749$, $p = 0.018$) ($D = 1.3367$) (Figure 8 and Figure 9), but this result was inconsistent among statistical methods (Appendix A), calling into question any clear effect of diet on hindwing shape. A weak or inconsistent effect of diet on hindwing shape is further supported by additional analyses; total cardenolide concentration ($F_{1, 54} = 1.2807$, $p = 0.239$), NMDS 1 ($F_{1, 53} = 1.3330$, $p = 0.196$), and NMDS 2 ($F_{1, 53} = 1.5771$, $p = 0.092$) were all unrelated to hindwing shape. Additionally, while size had an affect on hindwing shape ($F_{1, 56} = 2.6970$, $p = 0.012$), there was no independent effect of diet ($F_{1, 56} = 1.5831$, $p = 0.112$) on hindwing shape after accounting for size. In short, it appears that the shapes of monarch forewings, but not hindwings, are associated with larval host plant use and the cardenolides that the plants contain.

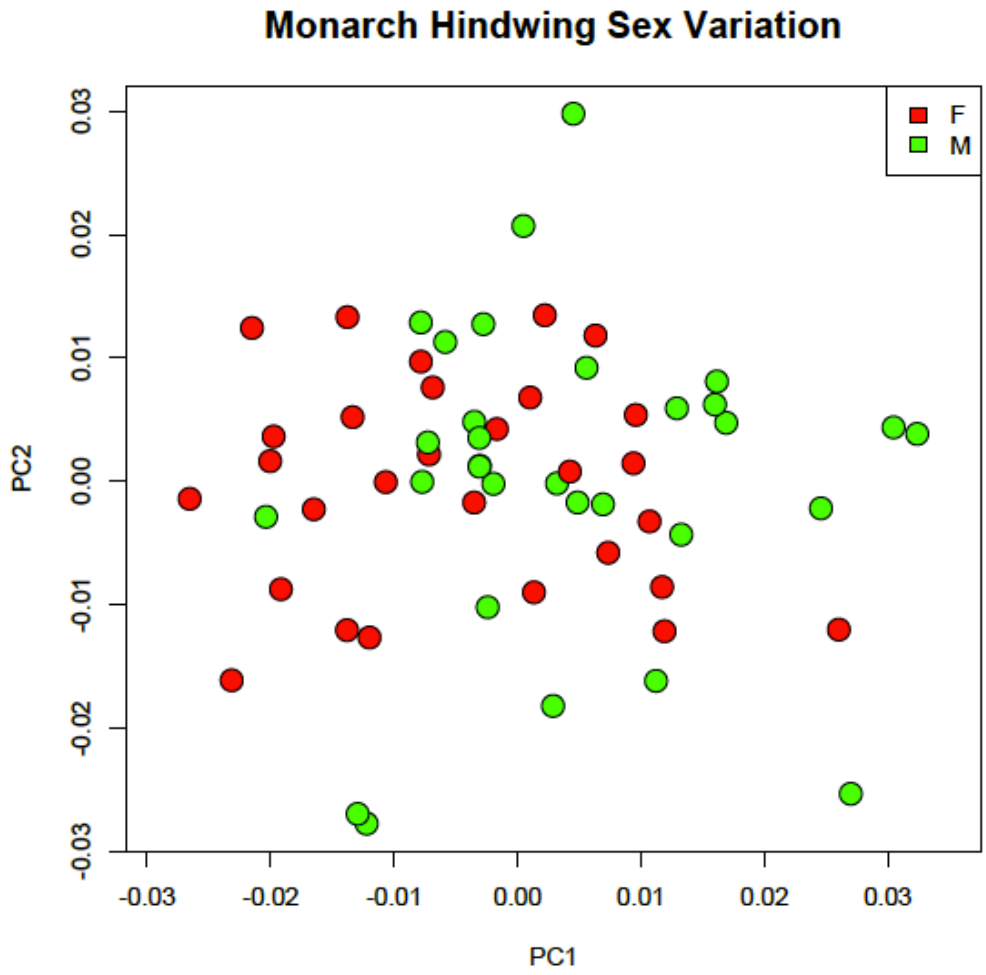


**Figure 6**: A principal components plot of hindwing shape distinguished by gender.

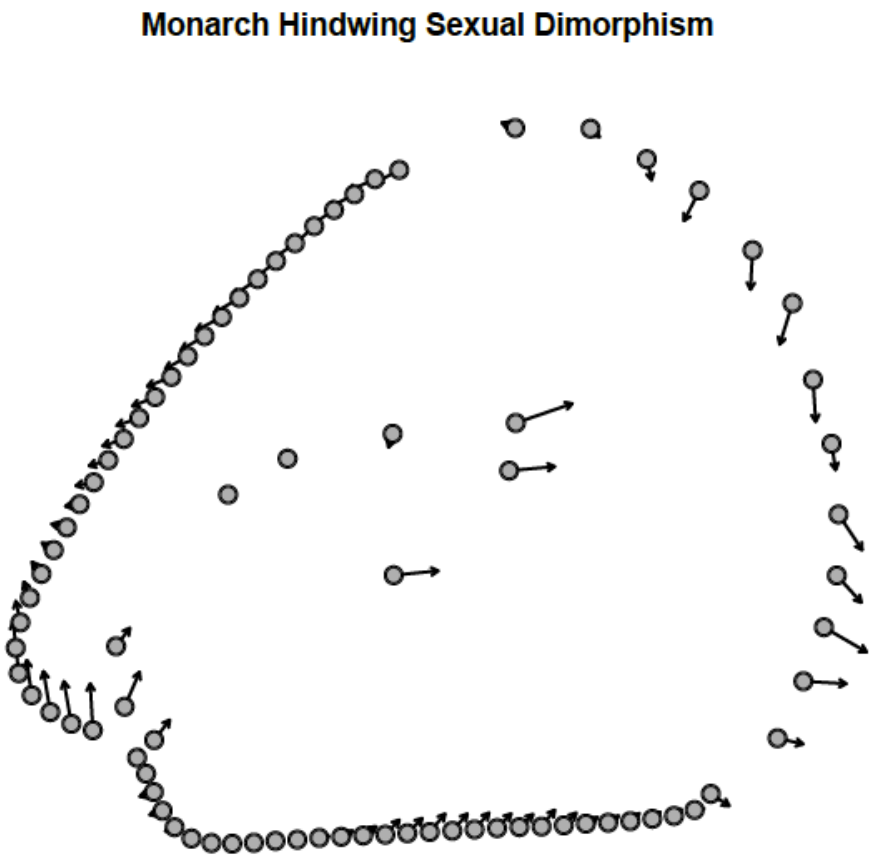


**Figure 7:** Mean hindwing shape variation between females and males exaggerated five times. The gray dots are representative of the female means and the arrows point in the direction of the male means.

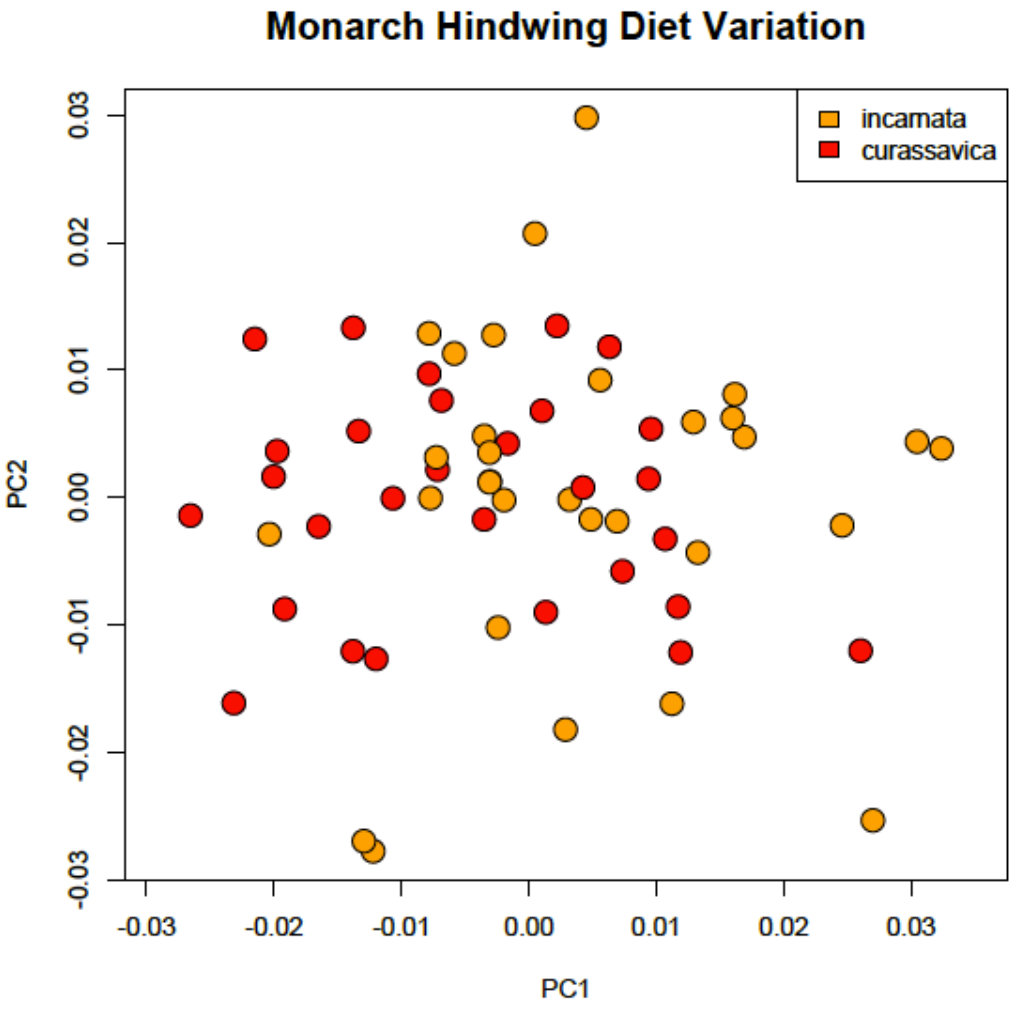


**Figure 8**: A principal components plot of hindwing shape distinguished by species of milkweed used as larval diet.

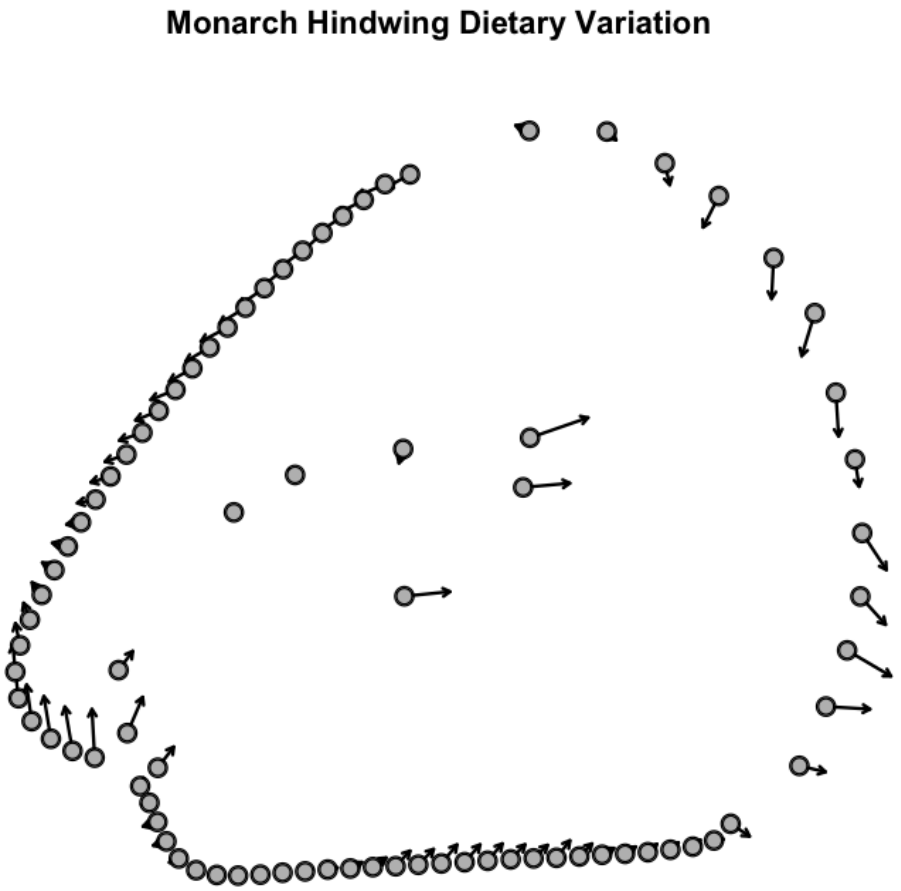


**Figure 9:** Mean hindwing shape variation between larvae fed on *A. curassavica* and *A. incarnata* exaggerated five times. The gray dots are representative of the means of larvae fed on *A. curassavica* and the arrows point in the direction of the means of larvae fed on *A. incarnata.*

***Discussion***

Our results illustrate that, in monarch butterflies, gender influences the shape of both forewings and hindwings. These findings are interesting because visual differences in insect wing shape are not obvious and are often difficult to detect (Benitez et al. 2011). Our

results support previous research that examines sexual dimorphism in many organisms. Males and females have different life history patterns and because of this are subjected to differential resource allocation (Shine 1989). Monarch females must devote energy and resources towards producing eggs and oviposition (Breuker et al. 2007). The females have chemo-sensors that sense the cardenolide concentration of the milkweed plant where they are ovipositing eggs (Stadler 2002; Zhan et al. 2011). Additionally, previous research on Lepidoptera has reported that females are often larger than males (Daly 1985). The size difference is possibly the result of an adaptive advantage for greater fecundity and increased parental care by females (Bentiez et al. 2011; Forrest 1987; Andersson 1994). The differential resource allocation may partially explain the wing shape variation between genders.

Both hindwings and forewings show significant shape difference between genders, but forewing samples show greater variation. This may be a result of the smaller forewing sample size (N=34) skewing our estimate of variance, but it also likely to have ecological importance. The forewing samples show non-uniform shape variation with both expansions and contractions in different sections of the wing (Figure 3). Expansions or contractions in the top corner of the wing make the forewing corner sharper or rounder, possibly affecting monarch flight ability. For the different life histories of each gender, selection should act on wing shape to optimize flight capabilities. Lepidopteran females have longer wings than males, on average, because of their need to fly and find the optimal host plant to oviposit eggs (Betts and Wooton 1988). Forewings play a greater role in flight than hindwings, so this is one possible reason for the greater difference in wing morphology (Johanson et al. 2009). However, given that both genders migrate, flight

distance is unlikely to be the cause of forewing shape variation between genders. We can speculate that the differences in maneuverability during oviposition or mating may act as selective forces between genders.

Our results add to an already existing pool of research on insect wing morphology supporting the idea that forewing shape varies with differences in life history. Previous studies have found variation in forewing shape based on whether individual Lepidoptera disperse or migrate (Betts & Wooton 1988; Outomoro 2012). Since monarch butterflies migrate annually, it is expected that migratory versus non-migratory populations vary in forewing shape. Longer more slender wings are seen in Lepidoptera that fly longer distances, mainly migratory populations (Betts & Wooton 1988) because it is known that slimmer wings enhance lift production (Wooton 1992; Grabow & Ruppell 1995). Monarch migratory populations from Cuba have longer more slender forewings than do non-migratory resident populations (Dockx 2007).

Similarly, dragonfly populations exhibit hindwing shape variation associated with different migratory patterns and forewing shape variation associated with male mate guarding and mating displays. Dragonfly males have shorter and broader forewings than females, which they use for sexual displays (Outomoro et al. 2011). Gender based variation of both monarch forewings and hindwings may be due to sexual selection or differences in behaviors between the genders (Outomoro & Johansson 2011), in addition to differential resource allocation.

In addition to gender differences, we find that larval diet influences monarch butterfly forewing shape, but not hindwing shape. There is strong evidence that cardenolide content and composition of the milkweed diet contribute to the variation in

forewing shape. Our experiment tests two independent factors related to diet, and we find that both total cardenolide content and NMDS axis 1 (relative abundance of cardenolide types) are correlated with forewing shape variation. There is also good evidence that diet has an effect on shape independent to dietary effects on size. Three of six statistical tests indicate an additional affect of diet on shape, even after correcting for the dietary affect on size (Appendix A). These results indicate a level of phenotypic plasticity for monarch forewings associated with host plant use. Forewing shape is not just a function of evolutionary change, but also a phenotype influenced by environmental factors such as larval diet.

It is possible that the number of different diets for each wing set (forewing or hindwing) influence the results and conclusions of the experiment. There are five different species of milkweed in the forewing group, but only two milkweed species in the hindwing group. Perhaps increasing the number of different diets in the hindwing group would have illuminated a shape variation based on larvae diet as well. *A. curassavica* generally have high foliar cardenolide concentration and *A. incarnata* have low foliar cardenolide concentration. By using more species in the hindwing group, in addition to these two milkweed species with extreme cardenolide concentrations, a shape difference might have been illuminated. Again, it is possible that the combination of small sample forewing sample size and higher number of larval diets contribute to the outcome of our results, stating that diets affect forewing shape.

Since total cardenolide content and NMDS axis 1 of the monarch forewings are both correlated with shape variation, it is likely that cardenolides are the component of diet altering forewing shape. However, future research may elucidate how different

components of larval diets (protein, carbohydrate, lipids) influence monarch wing shape and the monarch's ability to complete their annual migration. Cardenolides are toxic secondary compounds sequestered by monarchs as a defense against predators, but they can also have deleterious effects on monarchs at high concentration (Zalucki et al. 2001). High levels of non-polar cardenolides, the most toxic type, decrease the survival and growth rate of monarch larvae (Sternberg et al. 2012; Fordyce & Malcolm 2000; Zalucki et al. 2001). In contrast, previous research has shown that higher concentration of cardenolides increase protection against parasites (De Roode et al. 2011). It is known that cardenolides can have an antibiotic effect, and future studies should investigate how cardenolide content may be able to alleviate any wing asymmetry that is generated by parasite infection.

As stated previously, while most statistical tests performed had qualitatively consistent results, this was not universally true (Appendix A). Although there is not yet a consensus on the best method of superimposition or which statistical test to use, we note that the statistical method chosen affects the conclusions of our work. The hindwings in particular are very sensitive to the number of principal components used in MANOVA. It is possible that the results are so sensitive due to the small sample size of both forewing and hindwing datasets. The intended sample size for this study was much larger, but the majority of wing specimens had to be discarded from the experiment due to morphological damage before scanning. Future studies should increase the statistical power of the results by increasing the monarch sample size and implementing a more equal distribution of genders and diets. Additionally, future research should examine how sex, diet, and cardenolide content and composition affect wing symmetry. Wing symmetry is particularly

important for monarchs, and has large implications for monarch fitness and ability to complete the migration. Geometric morphometric analyses will be useful in determining an optimal diet or cardenolide concentration for monarchs with regards to wing symmetry. Future research on symmetry will show how different diets and cardenolide concentrations ultimately affect monarch flight speed and distance.

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***Appendix A***

**Table 2a**: Forewing compiled data with all statistical tests performed.
SuperA = superimposition of data with "ProcD=FALSE"
SuperB= superimposition of data without "ProcD=FALSE"
PCA1 = number of principle components = N/4 = 8
PCA2 = number of principle components determined using the broken stick model = 5

Key: x < 0.05, x x < 0.01, x x x < 0.001

1: shape~sex+diet

| | Sex | Diet | Size | NMDS 1 | NMDS 2 | Log (total cardenolides) |
|---|---|---|---|---|---|---|
| ANOVA SuperA | x x x | x x | | | | |
| ANOVA SuperB | x x x | x x x | | | | |
| MANOVA SuperA PCA1 | x x x | x | | | | |
| MANOVA SuperA PCA2 | x x x | x | | | | |
| MANOVA SuperB PCA1 | x x x | x x x | | | | |
| MANOVA Super B PCA2 | x x x | x | | | | |

2: shape~size+sex+diet

| | | | | | | |
|---|---|---|---|---|---|---|
| ANOVA SuperA | x x x | | x x | | | |
| ANOVA SuperB | x x | x | x x x | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| MANOVA SuperA PCA1 | x x x | x | x x x | | | |
| MANOVA SuperA PCA2 | x x | | x x x | | | |
| MANOVA SuperB PCA1 | x x x | x | x x x | | | |
| MANOVA Super B PCA2 | x x | | x x x | | | |

3: shape~sex+cards

| | | | | | | |
|---|---|---|---|---|---|---|
| ANOVA SuperA | x x x | | | | | x |
| ANOVA SuperB | x x | | | | | x x |
| MANOVA SuperA PCA1 | x x | | | | | x |
| MANOVA SuperA PCA2 | x x x | | | | | |
| MANOVA SuperB PCA1 | x x x | | | | | x |
| MANOVA Super B PCA2 | x x x | | | | | x |

4: shape~sex+NMDS 1 + NMDS 2

| | | | | | | |
|---|---|---|---|---|---|---|
| ANOVA SuperA | x x x | | | x x | | |
| ANOVA SuperB | x x x | | | x x x | | |
| MANOVA SuperA PCA1 | x x | | | x x | | |
| MANOVA SuperA PCA2 | x x x | | | x x x | | |
| MANOVA SuperB PCA1 | x x x | | | x x | | |
| MANOVA Super B PCA2 | x x x | | | x x x | | |

**Table 2b**: Hindwing compiled data with all statistical tests performed.
SuperA = superimposition of data with "ProcD=FALSE"
SuperB= superimposition of data without "ProcD=FALSE"
PCA1 = number of principle components = N/4 = 15
PCA2 = number of principle components determined using the broken stick model = 5

Key: x < 0.05, x x < 0.01, x x x < 0.001

1: shape~sex+diet

| | Sex | Diet | Size | NMDS 1 | NMDS 2 | Total Cards |
|---|---|---|---|---|---|---|
| ANOVA SuperA | | | | | | |
| ANOVA SuperB | x | x | | | | |
| MANOVA SuperA PCA1 | x x x | | | | | |
| MANOVA SuperA PCA2 | x | | | | | |
| MANOVA SuperB PCA1 | x x x | | | | | |
| MANOVA Super B PCA2 | x x x | | | | | |

2: shape~size+sex+diet

| | | |
|---|---|---|
| ANOVA SuperA | | |
| ANOVA SuperB | x | x |
| MANOVA SuperA PCA1 | x x x | x x x |
| MANOVA SuperA PCA2 | | |
| MANOVA SuperB PCA1 | x x x | x x x |
| MANOVA Super B PCA2 | x x x | x x x |

3: shape~sex+cards

| | |
|---|---|
| ANOVA SuperA | x |
| ANOVA SuperB | x x x |
| MANOVA SuperA PCA1 | x |
| MANOVA SuperA PCA2 | |
| MANOVA SuperB PCA1 | x x x |
| MANOVA Super B PCA2 | x x |

4: shape~sex+NMDS 1+NMDS 2

| | | |
|---|---|---|
| ANOVA SuperA | | |
| ANOVA SuperB | | |
| MANOVA SuperA PCA1 | x x x | |
| MANOVA SuperA PCA2 | | x |
| MANOVA SuperB PCA1 | x x x | |
| MANOVA Super B PCA2 | x x | |