Hostplant-derived cardenolide fingerprints as labels of spring migration strategy and a test of the "oogenesis-flight syndrome" in monarch butterfly arrivals to Michigan

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Abstract

Each March, tens of millions of monarchs leave their overwintering sites in Mexico in a highly synchronized fashion, to re-colonize milkweed habitat across their geographic range in North America, east of the Rocky Mountains. This re-colonization occurs by successive broods of migrants that move north as they generate new generations through time. The overwintered generation exploits the southern milkweed species *Asclepias viridis* as the primary resource for their larval offspring, which on emergence as adults, continue the migration north to where *A. syriaca* is the most abundant milkweed resource. As larvae, monarchs sequester toxic cardenolides from their host-plant milkweed species as a natural chemical defense against predators. Because each species of *Asclepias* has a characteristic geographical and seasonal distribution and contains its own unique range or "fingerprint" of cardenolides we can determine the host-plant origin of wild-caught monarchs and use this to describe migration in space and time as well as shifts in monarch generation synchrony throughout the course of a summer. Monarch butterflies are also thought to engage in the oogenesis-flight syndrome in which migrants use energy for flight and not egg production and are in a state of reproductive diapause. Here we test this hypothesis and determine the sources of adult monarchs that arrive in southwest Michigan in May and subsequent wild-caught monarchs during the summer to determine whether first arrivals in spring are offspring of overwintered butterflies and whether migration and voltinism are synchronized. Based on cardenolide fingerprints and chorionated egg counts of dissected, wild-caught females our data suggest that female monarchs do not enter reproductive diapause during migration. Instead, monarch butterflies are reproductively active as soon as they leave the southern United States, laying eggs as they re-colonize their northern range. Preliminary evidence also suggests that monarch arrivals in spring are derived from the southern milkweed *Asclepias viridis* and later adults during the summer are derived from the northern *A. syriaca.*

Introduction

The monarch butterfly, *Danaus plexippus,* is an iconic example of insect migration by virtue of its predictable, long-distance annual migration in North America between overwintering locations in Mexico and breeding habitat distributed across the USA and southern Canada east of the Rocky Mountains (Urquhart, 1960; Johnson, 1969; Urquhart and Urquhart, 1976, 1978).

From early wing-tagging studies by Nora and Fred Urquhart in Canada (Urquhart, 1960; Urquhart and Urquhart, 1976, 1978) and later studies coordinated by Orley Taylor of Monarch Watch at the University of Kansas (www.monarchwatch.org) we know adult monarchs fly each autumn to 12 locations in oyamel fir forests above 3,000 m altitude in the Sierra Transvolcanica mountains of central Mexico, west of Mexico City (Calvert and Brower, 1986; Brower, 1995, 1996). These high-altitude habitats not only provide a temperate and moist environment for the migrants to overwinter, but they also provide excellent protection from winds and cool temperatures to lower metabolic rates and reduce rates of stored lipid use. We know that the butterflies will remain over winter in aggregated clumps for up to 5 months until the end of March at the spring solstice when they begin the journey north once again to the southern United States, primarily Texas and Louisiana. At this point the long-lived migrating generation will mate, lay eggs on local milkweed (*Asclepias*) species, and die (Cockrell *et al.,* 1993; Malcolm *et al.,* 1993). The offspring of these migrants from Mexico then feed on southern hostplant species and upon emergence as adults continue the migration north to recolonize the full spatial extent of their milkweed hostplants across North America, east of the Rocky Mountains.

Like all butterflies in the genus *Danaus*, monarch larvae are specialist feeders on milkweed plants in the Apocynaceae, especially the genus *Asclepias.* They have been recorded to lay eggs on 27 of the 108 North American *Asclepias* species described by Woodson (1954), all of which contain species-specific toxic steroids known as cardenolides (Malcolm and Brower, 1986). Monarch larvae sequester cardenolides as a chemical defense used by adults against natural predators such as birds (Brower and Moffitt, 1974; Fink and Brower, 1981) and mice (Glendinning and Brower, 1990).

Each species of milkweed contains its own pattern, or “fingerprint”, of cardenolides that are chemically distinct in structure from the cardenolides in other

milkweed species. Malcolm *et al.* (1993) pioneered the use of these fingerprint patterns to describe the "successive brood" migratory strategy of monarchs during spring migration and recolonization. These authors found that 92% of overwintering monarchs in Mexico had fed as larvae on the abundant northern, summer milkweed, *A. syriaca* and these were the same, worn butterflies that arrived in the southern USA in early spring as they migrated north into the USA to lay eggs on milkweeds. However, Malcolm *et al*., found that the spring arrivals two months later in the northern USA were fresh butterflies that had fed as larvae on the southern, spring milkweed, *A. viridis,* which is the most abundant milkweed resource through the southeastern USA (Woodson, 1954). Hence they were able to discriminate between two migratory hypotheses by which monarchs could recolonize their entire range through either a "single sweep" of migrants, or the offspring of overwintered migrants could recolonize by "successive broods."

Asclepias syriaca is the most abundant and widespread milkweed species in eastern North America and is therefore the most important hostplant for monarch larvae (Woodson, 1954, Seiber *et al.,* 1986; Malcolm *et al.,* 1989) and it is likely that this larval food resource is an important factor in selection for monarch migration. The geographic distribution of this plant coincides with the distribution of summer breeding monarch butterflies and the plant remains as a food source throughout the summer allowing the monarch population to generate up to three successive generations (Borkin, 1982; Malcolm *et al.,* 1987). This allows the summer population of monarchs to establish the large numbers of butterflies that fly to Mexico each autumn.

Even though much is known about migration, mating behavior, physiology, hostplant use, and the operation of defense against natural enemies in monarch butterflies, we still know little about summer population dynamics of monarchs in the Great Lakes region. For example, we do not know whether monarch arrivals to Michigan are the result of a single colonization event in May or if monarchs arrive at a steady rate from the southern United States. For this reason we used cardenolide fingerprints to determine the changing nature of spatial and temporal synchrony of monarch generations through the summer as measured by changing frequencies through time of host plant-derived chemical fingerprints.

In addition to population dynamics of monarch butterflies in Michigan little is known about the reproductive trade-offs during migration that female monarchs may undergo. Migration is a costly strategy in terms of energetics and reproductive output (Rankin *et al.*, 1986; Rankin and Burchsted, 1992) with migrating insects showing a distinct trade-off between flight and reproduction so that migrating individuals are in reproductive diapause and reproductive individuals do not migrate. This trade-off, known as the "oogenesis-flight syndrome," is thought to occur in southward migrating monarchs, in which most males and females enter a stage of reproductive diapause at the autumn equinox near the end of September before traveling to the overwintering sites in Mexico (Herman and Barker, 1977; Herman, 1985). During this time, female adult monarchs allocate less energy to the production of eggs and more energy to flight and acquisition of lipids through nectaring (Gibo and McCurdy, 1993). Monarchs remain in reproductive diapause through the overwintering period in Mexico until day lengths increase substantially in March and individuals start to migrate north. However, it is not known whether spring migrating monarchs are in reproductive diapause or exhibit the "oogenesis-flight" syndrome. Like Thomas and Showers (1992), we suspect that this is not the case for northerly migrating monarchs in spring and we propose to determine whether spring arrivals in Michigan carry chorionated eggs.

In our research we used cardenolide fingerprints of field-caught monarchs to: 1) determine hostplant origin of adults and patterns of monarch generation synchrony in southwest Michigan and 2) test whether the oogenesis-flight syndrome occurs in spring monarch arrivals to Michigan using cardenolide concentrations, fingerprint patterns and chorionated egg counts.

Methods

Adults and eggs of the monarch butterfly, *Danaus plexippus*, were collected from spring arrival in late May until autumn departure in late September 2011 at 4 locations across southwest Michigan (Table 1). The sites varied in habitat and included a range of genet sizes of the common milkweed *Asclepias syriaca* as well as occasional plants of the milkweeds, *A. incarnata* and *A. tuberosa.* We collected adults and observed immatures at each of the four sites once per week. At each site we randomly checked individual

milkweed ramets within genets for eggs and larvae while we searched for adult monarchs. The location of collected monarch adults and eggs, and observed larvae was determined with a Garmin (eTrex Legend HCx) GPS unit and recorded in both field notebooks and a GIS database in addition to date, time, and habitat, as well as butterfly sex and behavior. All samples were stored frozen at -20°C at the Pierce Cedar Creek Institute near Hastings, Michigan.

To determine the cardenolide fingerprints of butterflies reared from different *Asclepias* species present in southwest Michigan, we collected mating monarch pairs in the field and placed them in nylon mesh cages (61x61x183 cm, Live Monarch Foundation, Boca Raton, FL) with a single, potted *Asclepias* species on which they laid eggs. We reared the larvae produced on *Asclepias incarnata, A. syriaca, A. tuberosa, A. viridis,* as well as the neotropical milkweed, *A. curassavica*. *A. curassavica* was included because commercial releases of this monarch butterfly species at weddings may influence captures of wild monarchs. Mated females laid eggs on the leaves of the milkweeds and the larvae were allowed to feed *ad libitum* on the potted plants until they pupated in the cage. Cages were placed in a laboratory at Pierce Cedar Creek Institute where temperature, duration of light, and light intensity could be held constant. Mating pairs were kept in each cage for 2 days and were fed with aqueous sucrose solution. When a sufficient number of eggs had been laid on each plant the mating pairs were sacrificed and the monarch larvae were allowed to mature. Emerging adults were allowed to eject their meconium and harden their wings before being collected and frozen in glassine envelopes at -20°C.

Monarchs collected from the field were frozen, weighed, the four wings were separated from the thorax and both the dorsal side and the ventral side of each monarch was photographed with a scale for wing size and shape measurement, color analysis and wing wear determination. The length of the right forewing was measured for another comparison of size, and a 5 mm diameter circle was cut from the discal cell of the right hindwing with a cork borer and mounted on a labeled microscope slide for digital wing wear analysis. Wing wear was determined on a scale of 0-5, with 5 representing very worn butterflies and 0, freshly emerged, completely intact butterflies. The neogregarine sporozoan, protist pathogen, *Ophryocystis elektroscirrha* was also sampled from each

butterfly using a circle of clear sticky Scotch™ tape ($1cm^2$) placed on the ventral surface of the abdomen. The tape containing the abdomen scale sample was then placed on the microscope slide next to the wing bore sample. The processed butterflies were placed in the freezer in sealed bags to prevent desiccation until ready for dissection.

Females were dissected from the ventral surface of the abdomen so that the number of chorionated eggs in the abdomen around the bursa copulatrix could be counted and the bursa cut free once fat had been removed. The bursa was then weighed and extracted for cardenolide content to determine male contributions to both the female cardenolide fingerprint and male contributions to eggs laid by females. For each female the butterfly body and bursa were treated separately in analyses for fat and cardenolide content.

For males no dissections were performed. Instead the bodies were placed in a preweighed and recorded tube and with females and bursae all material was freeze dried in a LabConco lyophilizer. After drying, all samples were reweighed before extraction for fat content determination and cardenolide analysis and these weights were used to calculate cardenolide and fat concentrations.

Fat and cardenolide extraction via high performance liquid chromatography (HPLC) analysis

Freeze-dried butterflies were ground with 4 ml petroleum ether in an 18 x 150 mm glass tube using a motorized homogenizer. A further 2 ml of petroleum ether was used to rinse any remaining insect material on the homogenizer into the tube containing the thoroughly ground butterfly material. Each tube was for 10 minutes and the supernatant decanted into a preweighed, labeled 12 x 75 mm glass tube and left in the fume hood overnight to evaporate the ether, leaving behind the fat extract. The extracted fat weight was then recorded. After fat extraction, 4 ml of 100% methanol was added to the insect residue in the original tube, which was then vortexed and sonicated in a heated waterbath at 55°C for 10 minutes. The extract was centrifuged for 10 minutes and the supernatant was poured into a 13 x 100 mm glass tube. A further 2 ml of methanol was then added to wash the residue from the previous step and pooled to give a 6 ml extract. The methanol extract was dried under nitrogen in a water bath at 55°C. Once dry, the

methanol extract was resuspended in 1 ml acetonitrile, vortexed, and filtered through a 0.45 µm Luer-lock syringe filter on a 3 ml plastic syringe and placed into a 1 ml, labeled autosampler vial, ready for HPLC.

The extraction of cardenolides from the dissected bursae followed the same procedure, except that ether extraction of fat was not performed because fat had been removed in the dissection process. Instead, the bursae were ground with the homogenizer in 4 ml methanol and the procedure described above was followed.

Cardenolide analyses of butterfly and bursae extracts were performed using the method of Wiegrebe and Wichtl (1993) on a Waters gradient HPLC system with WISP autosampler, 600E pump, 996 diode array detector and Millennium 2010™ chromatography software. The reverse-phase elution gradient was acetonitrile:water at 1.2 ml·min^{-1} at 40˚C, with 20% acetonitrile at start, 32% after 35 min., 40% after 45 min., 50% after 55 min., then back to 20% at 61 min., and 20% at 65 min., on a 250-4 LiChroCART® RP-18 column packed with LiChrospher® 100, 5µm (E. Merck) with a 10 mm guard column. The 20 µl sample injections were separated over 65 minutes with 10 min. equilibration between samples and cardenolides were detected at 218.5 nm and identified by their symmetrical spectra between 205 and 235 nm and a λ_{max} of between 214 and 224 nm. Cardenolide concentration for each peak (µg/0.1g sample DW) was calculated from a calibration curve with the external cardenolide standard digitoxin (Sigma, St Louis, Missouri). Only cardenolide peaks reported by Millennium 2010® software as consistently pure were considered for analysis.

Statistical analyses were performed with MS Excel or JMP version 8 (SAS Institute).

Results

From late May until early August, 122 adult *D. plexippus* (37 female and 85 male) were collected. Only 10 females were caught on the property of Pierce Cedar Creek Institute (PCCI). The females caught at PCCI had the highest mean number of eggs per female compared to the females caught at other sites (Table 1).

Although there was a strong increase in the mean number of eggs per female with increasing wing wear (Figure 1) which suggests that older, more worn females contain

the most chorionated eggs, these data had high variances and so we were unable to detect significant differences (ANOVA $F = 0.88$, $p = 0.49$). Instead, the number of chorionated eggs found in all sampled females was randomly distributed through time as can be seen in Figure 2.

When wing wear was plotted against time (Julian day), wing wear was initially high (Figure 3) and then decreased through June and into July (July 1 = 182). This trend continued until mid-July and then there was a slight increase in wing wear at the end of July (July 31 = 210, Figure 3.).

There was no significant difference between the cardenolide concentrations of males and females (Figure 4, ANOVA $F = 0.02$, $p = 0.89$). However, cardenolide concentrations did vary with time (Figure 5) with highest concentrations in early June followed by a decrease and then another peak of concentrations towards the end of June (June 30 = 181). As time progressed cardenolide concentrations decreased and then slowly rose between Julian days 200 and 210 in the last 12 days of July (19-31). A similar pattern was found in the cardenolide concentrations of bursae when plotted against time (Figure 6). Bursae cardenolide concentrations were highest in early June (June 8 = 160) then decreased through the rest of June and increased again to a peak at Julian day 197 (July 16), followed by another decrease at the end of July.

Discussion

Our results support the conclusion of Malcolm *et al.* (1993) that monarch butterfly spring re-colonization of eastern North America occurs via a successive brood migration strategy. We interpret the initial peak in adult cardenolide concentrations that we found (Figure 5) as representing the first generation of spring migrants that had fed as larvae on the high cardenolide milkweed *A. viridis* in the southern United States (Malcolm, 1995). As time progressed, we found changes in adult butterfly wing wear (Figure 3) that started at intermediate wear scores and then decreased suggesting that the first arrivals to Michigan at the end of May were more worn than later butterflies. These wing wear data thus support the conclusion that first arrivals are *A. viridis*-derived monarchs that have migrated from farther south to colonize Michigan. These monarchs then laid eggs on the abundant milkweed, *A. syriaca,* with significantly lower constitutive

cardenolide concentrations (Malcolm *et al.*, 1989) to generate the subsequent, lower cardenolide concentrations shown in Figure 5. Interestingly, the smaller peak in cardenolide concentrations (Figure 5) at the end of June (June 30 = 181) may reflect the arrival of later, *A. viridis*–derived monarchs from the southern USA, perhaps derived from populations of *A. viridis* in Kansas and Oklahoma, or even as far north as southern Illinois, the northernmost distribution of *A. viridis* in North America (Woodson, 1954).

Cardenolide contributions to females from males during mating are reflected in the bursa cardenolide measures shown in Figure 6 and these also show an initial peak at the start of June followed by a decline. However, the timing of the second bursa cardenolide peak at almost 200 days (July 19) occurs when cardenolide concentrations in all adults is low (Figure 5), but starting to increase. This suggests that females are able to choose to mate with males that have high cardenolide concentrations, much as Oberhauser found for male monarch spermatophore mass and mating success (Oberhauser, 1988, 1989). Such mating selection may be important to increase offspring fitness because females with high bursa cardenolide can lay more toxic eggs, that are better defended against natural enemies, than females with little bursa cardenolide. Although the mean cardenolide concentrations did not differ between male and female monarchs (Figure 4), the range of cardenolide concentrations in males is clearly much larger in males than in females. This high level of variation also suggests that male variation might select for female choice based on cardenolide content during mating.

There was a noticeable shift in the synchrony of the summer population of monarch butterflies, as indicated by the cardenolide peaks seen in Figure 5. We see these pulses because monarchs reared on southern milkweed species have a higher cardenolide concentration than monarchs reared in the north. Generation synchrony should decrease throughout summer, until all wild-caught monarchs show a fingerprint consistent with that of monarchs reared on *A. syriaca*, the abundant milkweed species found in Southwest Michigan.

The high cardenolide concentrations of the bursae in early June may also indicate that many of the 1st generation females that arrive to Michigan have already been mated farther south by males reared on *A. viridis* or by *A. viridis*–derived males that also arrive in Michigan at the same time. This, along with the chorionated egg counts and the wing

wear data (Figures 1 and 2), provide evidence against the "oogenesis-flight syndrome" in migrating monarch females. Using wing wear as an indication of age we can show that older adult females have a large number of eggs compared to younger females (Figure 1). We also found that monarchs with a higher degree of wing wear were captured early in June suggesting that they are migrants from the south. Rather than entering reproductive diapause during the spring re-colonization, female monarchs are allocating energy to the production of eggs as well as to movement. We believe this is enabled by the abundance of larval resources available through the entire geographical range and so it is important to be able to lay eggs along the way. Unlike other migratory insects, the monarch butterfly may be able to compensate for reproductive trade-offs because the resources required for reproduction are distributed throughout their breeding range east of the Rocky Mountains, rather that spatially separated between discrete and aggregated entities.

Even though we sampled from four different sites on a regular basis, the Allegan site (Table 1) was the most productive with a total of 79 adult monarchs captured. We are unsure why this was the case, but we believe it was because it was the site closest to Lake Michigan. It is possible that the lake acts as a barrier so that the migrating population is forced to either travel up the west side through Wisconsin, or on the east side, directly over our sample site. Further research could be conducted to determine if this is the case. This site was also located in the goose management fields of the Allegan State Game Preserve in west Michigan and it has large populations of common milkweed, *A. syriaca.* The management regimes in the maize fields grown to attract geese, ducks and deer provide ideal habitat for the highly modular *A. syriaca* as well as abundant nectar resources for adult monarchs (Brown and Chippendale, 1974), such as *Aster*, *Cirsium* and *Trifolium.*

Due to the length of the annual monarch migration, research is still being conducted to test our hypotheses. Only 25% of the actual sample size has been processed, and we plan on continuing with processing and analyzing the data to build a more complete analysis of the summer population dynamics of the annual migration. We predict that female egg counts will reduce later in the season because of the onset of reproductive diapause in September (Herman, 1985). We plan on presenting our

complete data and analysis at the Midwest Ecology and Evolution conference in April 2012.

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Tables and Figures

Table 1: Number of wild-caught *D. plexippus* females at four sites in southwest Michigan, with mean and basic statistics for number of eggs per female.

Site ID	Female N	Mean No. eggs	SD	Minimum	Maximum
Allegan	20	24.3	37.3	0	143
Lawrence	1	20.0	-	20	20
PCCI	10	42.1	33.3	0	83
Kalamazoo	5	15.2	22.4	0	54
Summary	37	27.1	34.3	0	143

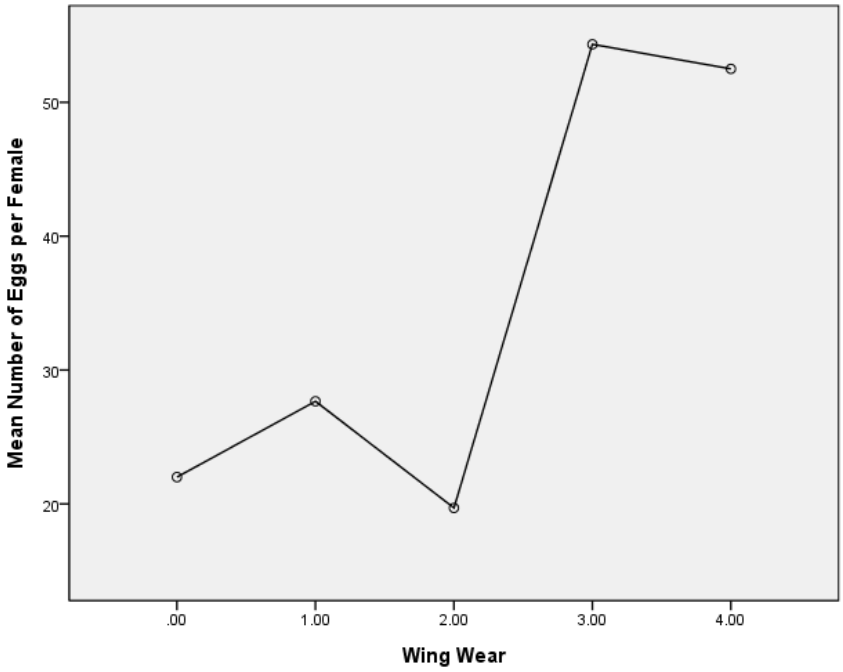

Figure 1. Mean number of eggs per dissected *D. plexippus* female plotted against wing wear of the female. Wing wear was estimated on a scale of 0-5 where 0 is perfect condition and 5 is extremely worn.

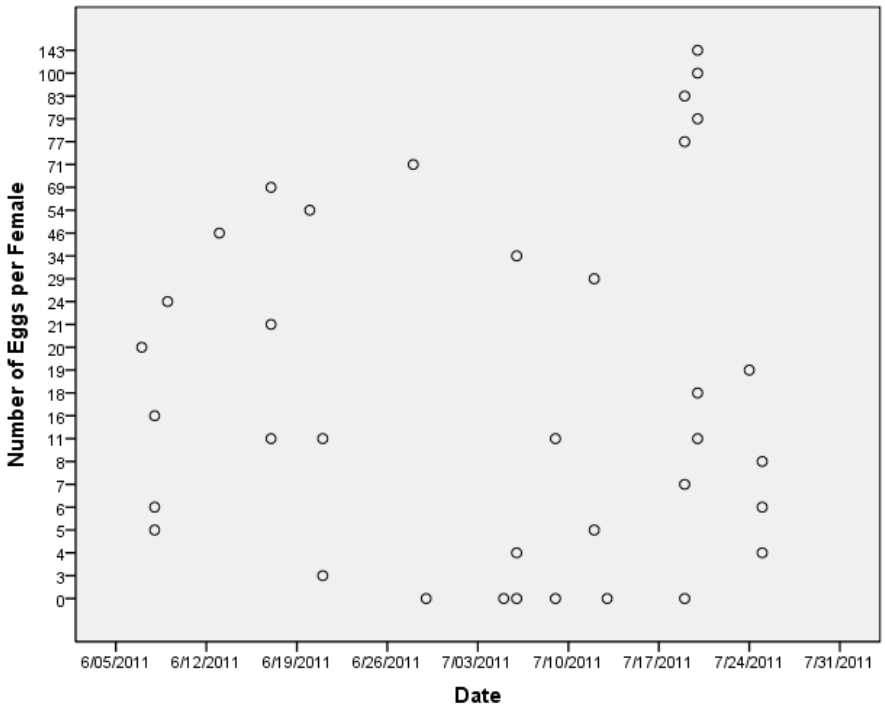

Figure 2. The number of chorionated eggs per dissected *D. plexippus* female plotted against the date that the female was caught.

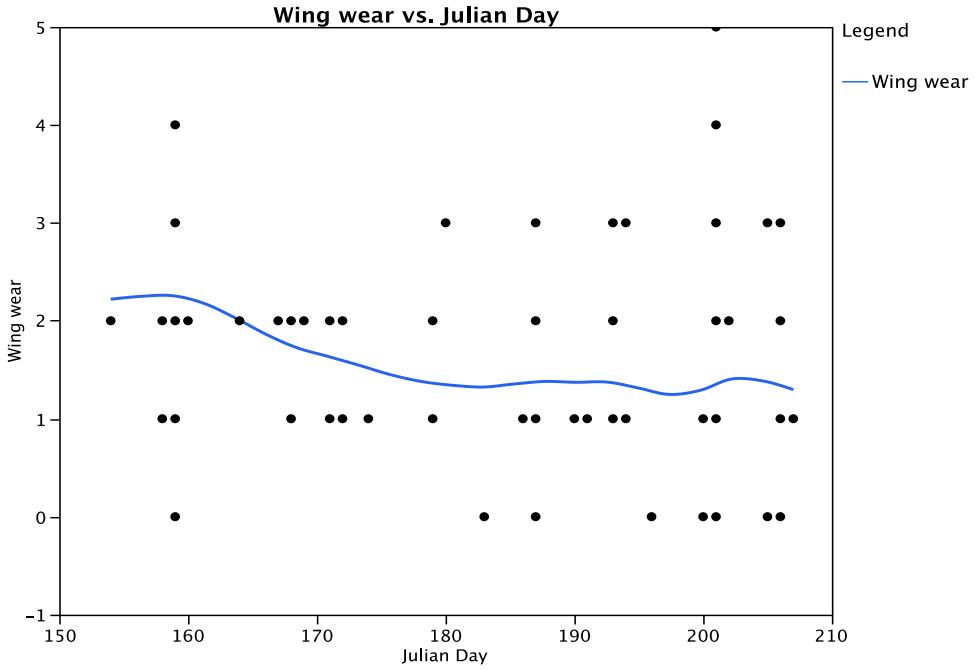

Figure 3. Monarch wing wear against time (Julian day). Wing wear was estimated on a scale of 0-5 where 0 is perfect condition and 5 is extremely worn. The line is fitted by JMP8 as a best-fit polynomial to indicate trends in the data. Julian day 150 = May 30, 160 = June 9, 170 = June 19, 180 = June 29, 190 = July 9, 200 = July 19, 210 = July 29.

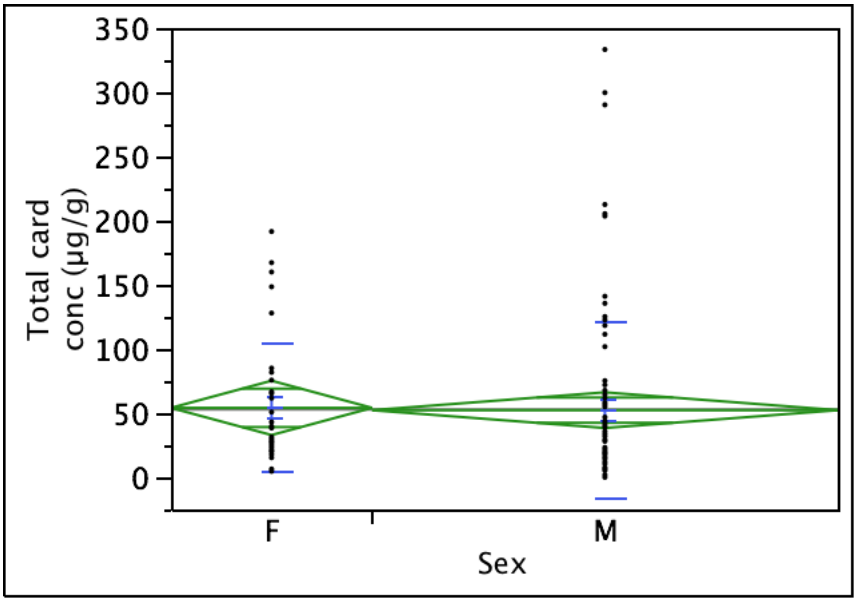

Figure 4. Mean and standard deviation of cardenolide concentrations (µg/g DW) estimated by HPLC in *D. plexippus* males (M) and females (F).

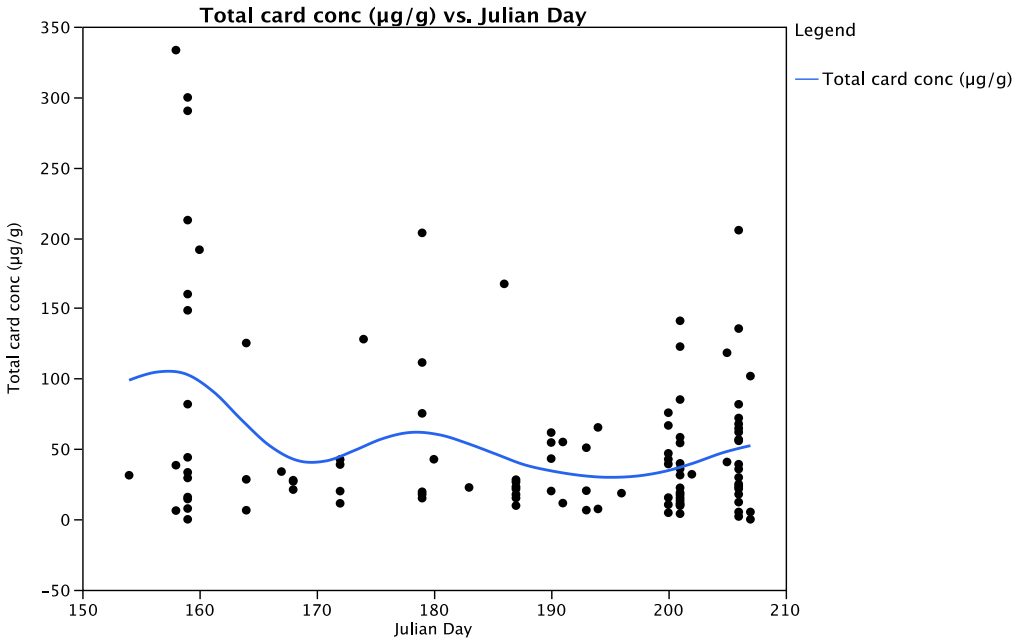

Figure 5. Cardenolide concentrations (µg/g DW) of *D. plexippus* adults against time (Julian day). The line is fitted by JMP8 as a best-fit polynomial to indicate trends in the data. Julian day 150 = May 30, 160 = June 9, 170 = June 19, 180 = June 29, 190 = July 9, 200 = July 19, 210 = July 29.

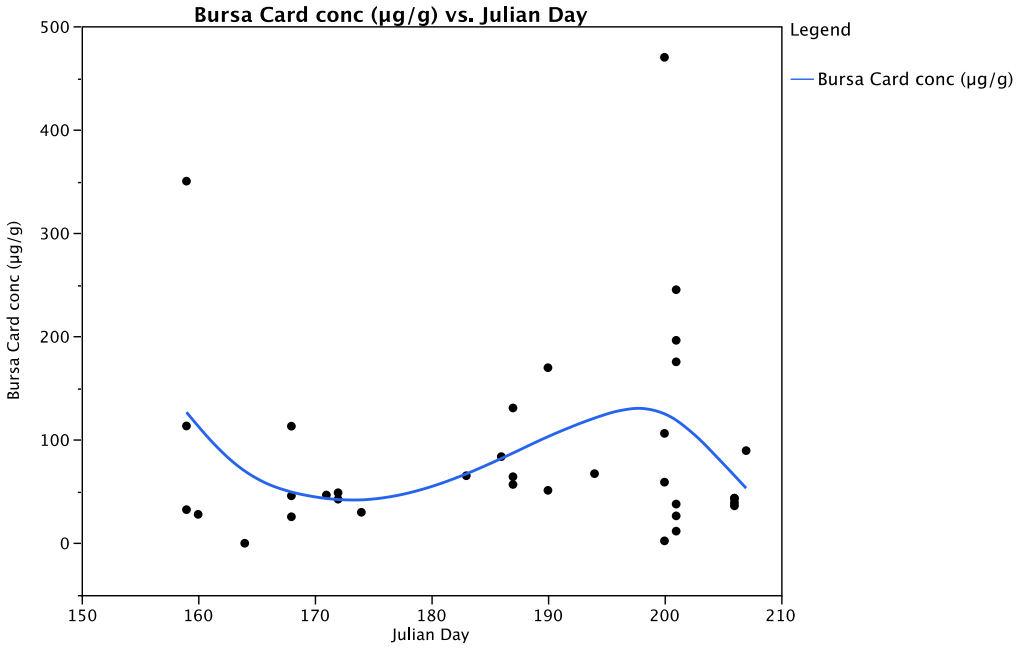

Figure 6. Bursa cardenolide concentrations (µg/g DW) of *D. plexippus* females against time (Julian day). The line is fitted by JMP8 as a best-fit polynomial to indicate trends in the data. Julian day 150 = May 30, 160 = June 9, 170 = June 19, 180 = June 29, 190 = July 9, 200 = July 19, 210 = July 29.