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Assortative mating based on heritable body size suggests potential ecological speciation in monarch butterflies

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
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Abstract

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Geographical isolation that separates a species into separate populations can cause the two populations on each side of the physical barrier to evolve independently, as they adapt to different environments and accumulate genetic and phenotypic differences. Consequently, adaptation to different ecological conditions can give rise to divergence in morphology and might result in reproductive isolation. In animals exhibiting courtship tactics, it is well-known that adaptive morphological changes can create reproductive barriers. However, it remains relatively unclear how morphology affects reproductive isolation in species adopting coercive mating strategies. Monarch butterflies employ sexual coercion, and their size varies among different populations. Monarchs originate in North America, where they are migratory, and have dispersed around the world to form non-migratory populations. In general, these non-migratory monarchs have smaller body size. Here, we examine the effects of body size on mating success in the tractable monarch butterfly system. Mating trials were conducted using wild type and size-manipulated monarchs, and the heritability of body size was measured. Our data show that monarchs mate assortatively based on size, and female choice plays a larger than expected role in driving the mating dynamics. In addition, we found that body size is a heritable trait in both migratory Eastern North American monarchs and non-migratory Puerto Rican monarchs. This study sheds light on how morphological adaptations can facilitate speciation in animals that use coercive mating strategies.

Key words: reproductive isolation, speciation, sexual coercion, heritability

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Introduction

Ecological speciation happens when new species form as adaptive divergence drives reproductive isolation (Matsubayashi et al., 2010; Rundle and Nosil, 2005). There are many circumstances under which ecological speciation can occur, one of which is allopatry. Allopatric speciation occurs when two populations of the same species are separated by a geographic barrier and are blocked from genetic exchange (Singh, 2021; Yamaguchi and Iwasa, 2013). Owing to the reduction in gene flow, the populations begin to accumulate genotypic and phenotypic differences as they adapt to different environments under distinct selective forces. As a result, morphological divergence might evolve and can in turn lead to reproductive isolation (Rundle and Nosil, 2005).

Allopatry has two possible causes, which are vicariance and dispersal (Futuyma and Kirkpatrick, 2018; Zink et al., 2000). Vicariance involves species being passively divided by the formation of a geographic barrier—such as a river or a mountain—that hinders movement and gene flow between populations. In contrast, dispersal is defined by the active movement of individuals from one geographic area to another and the ensuing colonization of new habitats (Futuyma and Kirkpatrick, 2018; Garcia and Trewick, 2015). Speciation by dispersal is researched in many different organisms. For example, Darwin’s finches dispersed after a common ancestor colonized the Galápagos archipelagos in South America about 1.5 million years ago, and the different finch populations were isolated from one another by the sea (Funk and Burns, 2017; Lamichhaney, 2015). The finches on different islands were exposed to different ecological circumstances with varying food sources. Over time, each finch population evolved a distinct beak type that is adapted for acquiring the corresponding food source (Reaney et al., 2020). Finches with short and blunt beaks are specialized in cracking seeds with hard shells; finches with long and pointed beaks forage by snatching insects and arthropods from the plants (Burns et al., 2002). Since gene flow is hampered by the ocean, the genetic differences can accumulate in the populations. Studies have shown that beak size is heritable and the variation of beak shape in Darwin's finches is associated with genetic variation in the *ALX1* gene (Lamichhaney, 2015), and thus the different beak shapes and sizes are passed down to their offspring. It was also shown that beaks play an important role in sound production, and vocal signal evolution is shaped by the diversification of beak morphology (Podos, 2001; Ryan, 2001). Males with larger beaks have a narrower frequency range, and males with smaller beaks can sing with a wider frequency range (Podos, 2001; Ryan, 2001). Since finch species rely on vocal signals to choose mates (Lamichhaney, 2018), the frequency of courtship songs is of great importance in mate recognition. Courtship songs can give rise to assortative mating based on acoustic cues (Podos, 2010), and the divergence in courtship songs can contribute to reproductive isolation (Podos, 2007). In organisms that exhibit courtship behavior, it is well-known that adaptive morphological changes can create reproductive isolation. However, how morphological adaptations affect speciation in coercive mating systems remains largely unknown.

Monarch butterflies (*Danaus plexippus*) display coercive mating behavior (Solensky, 2004) and the globally dispersed populations with different migratory behavior show divergence in wing morphology (Altizer and Davis, 2010). Thus, monarchs provide an excellent system for studying the effects of adaptive morphological changes on the formation of reproductive barriers.

Eastern North America monarch butterflies are famous for their annual migration up to 3000 miles from their northern breeding range in Canada and the USA to overwintering grounds in central Mexico (Brower, 1995; Reppert and de Roode, 2018). However, monarch butterflies are not only found in North America. They have also dispersed around the world from their North American origin (Freedman et al., 2020). The dispersal of monarch butterflies happened independently three times through the Pacific Ocean, through Central/South America, and through the Atlantic Ocean. As new populations formed to colonize new habitats, they became semi-migratory or non-migratory (Dockx et al., 2004; Freedman et al., 2020; Knight et al., 1999). As monarchs colonized new habitats and altered their migratory patterns, their morphology diverged from those of the North American population (Altizer and Davis, 2010). Based on previous studies, it is known that migratory monarchs are generally bigger, and migration distance is positively correlated with wing area (Flockhart et al., 2017). In contrast, non-migratory monarchs, such as those in Puerto Rico, are smaller in size (Altizer and Davis, 2010; Hanley et al., 2013; Flockhart et al., 2017). Although migratory and non-migratory monarchs differ in body size, most existing studies are observational, and it awaits to be answered how heritable body size is. It is important to unravel how much of the variation in monarch body size is due to genetics and how much is owing to the influence of the environment. The variation of a trait must be heritable and have a genetic component to be involved in the process of species evolution.

Much of our current understanding of the morphological adaptations in monarchs is based on their function in migration, but little is known regarding how much these adaptations affect sexual selection, and the consequences of changes in body size for monarch reproduction remain unclear. Body size plays a critical role in mating for monarch butterflies because male monarchs display coercive mating behavior. Unlike most Lepidoptera, in which males attract females using pheromones or courtship dances, monarch males exclusively use forced copulation (Solensky 2004). A male monarch typically approaches a female mid-air and knocks her to the ground. Once on the ground, the male pins down the female and probes her abdomen with his claspers searching for her genitalia. Throughout this process, the female often struggles and curls her abdomen to resist copulation. These mating attempts can last up to 30 minutes and often end in failure (Solensky, 2004). The intense physical nature of this mating strategy likely favors larger, stronger males that are more likely to inseminate females (Shine & Mason, 2005). When copulations are obtained through physical force, body size can be crucial for monarch mating success. Since body size plays an important role in mating for butterflies, this morphological difference may affect mate choice among monarch populations and facilitate the formation of possible reproductive barriers in the long run.

This study aims to determine if morphological adaptations can create reproductive barriers between different populations in animals that mate coercively. Specifically, we analyzed the effects of body size on monarch butterfly mating success. Firstly, we developed a method to experimentally manipulate monarch size. Then, we conducted mating assays using wild-type and size-manipulated monarchs, tested their mate preference, and examined the dominating sex in shaping mating dynamics. Secondly, as a trait must be heritable for a reproductive barrier to have evolutionary significance, we measured the heritability of monarch body size using mid-parent-offspring regression. We hypothesized that body size dictates monarch mate choice. Since male monarchs obtain mates via force copulation, our second hypothesis was that mating dynamics are mostly driven by males. Our final hypothesis was that body size is a heritable trait in both migratory Eastern North American (EA) monarchs and non-migratory Puerto Rican (PR) monarchs, the two populations that form the focus of this study.

Methods

Mating Experiment

Experimental manipulation of body size

All monarchs used in the mating assay were descendants of wild-caught, eastern North American migratory monarchs from St. Marks, Florida. A total of 758 monarchs (16 lineages) were raised in this experiment. All monarchs were raised in a temperature-controlled greenhouse room (temp: 24°C, humidity: 17%) with natural light at Emory University. Caterpillars were raised on tropical milkweed (*Asclepias curassavica*). All milkweed plants were surrounded by a clear plastic tube (height: 21 in., diameter: 4 in.) stuck into the dirt of a milkweed pot (depth: 4.5 in.). In order to prevent caterpillars from escaping, the top of the tubes was covered by a white mesh net.

About two-thirds (n=504) of the larvae were provided food ad libitum throughout their development to pupae and served as our “Wild Type (WT)” group. The other one-third (n=253) of the larvae had their food supply restricted at the beginning of the 5th instar developmental stage. Food was restricted by removing all the leaves from their host plant, leaving only the stems behind as a minimum food source to keep the caterpillars alive. These diet-restricted small monarchs served as the “size-manipulated” group (Figure 1, 2). After the monarchs pupated, they were removed and glued by their cremaster to the lid of a plastic cup. These chrysalises were then placed in a climate controlled (26.5 °C) room with artificial light.

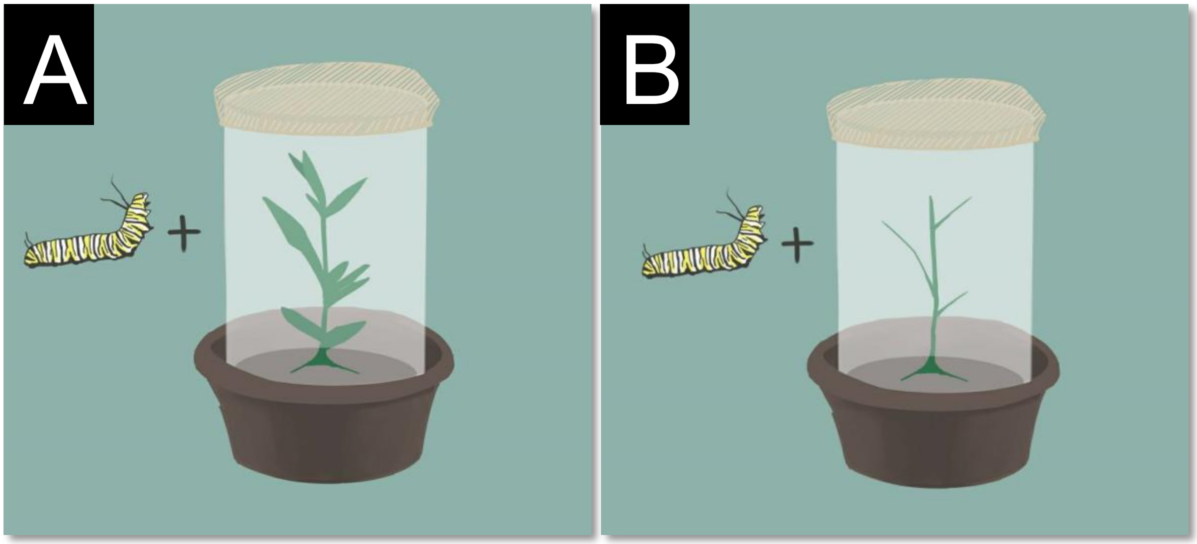


Figure 1. Experimental manipulation of monarch body size. A: Control monarchs that were provided food *ad libitum.* B: Size-manipulated monarchs that had their diet restricted by removing all the leaves from the milkweed plant at the fifth instar developmental stage.

Figure 2. Experimental variation in size of the lab-reared monarchs. The larger monarchs are control monarchs that were provided food *ad libitum.* The smaller monarchs had their diet restricted at the fifth instar. Males on the left; females on the right.

Mating assay

Once monarchs emerged, they were each checked for *Ophryocystis elektroscirrha*, a common protozoan parasite. This was done by using a clear sticker to tape the scales off of the abdomen and the scales were checked under the microscope for parasite spores. All the monarchs used in this experiment were parasite-free. The monarchs were weighed and used to conduct two types of mating trials, which are choice trials and no-choice trials. The no-choice 1x1 trials were divided into two different setups—(a) the WT male and WT female combination and (b) the small male and WT female combination. The choice trials were also divided into two different setups— (c) the 2x1 combination using one WT male, one small male, and one WT female, and (d) the 2x2 combination using one WT male, one WT female, one small male, and one small female (Figure 3). Males and females in each cage were from different genetic lineages to avoid potential effects of inbreeding.

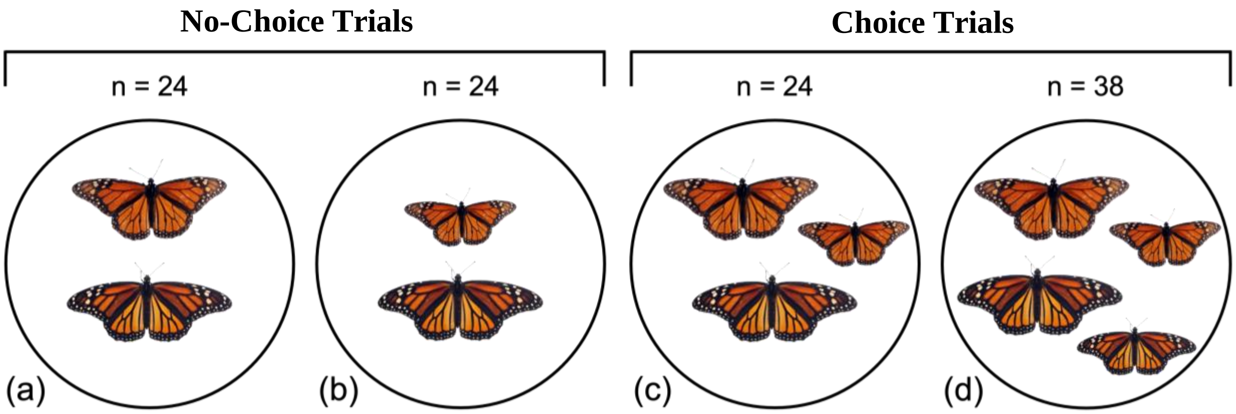


Figure 3. Summary of mating assays testing the effects of wing size on reproductive success. (a). WT male and female. (b). Size-manipulated male and WT female. (c). One WT and one size-manipulated male with a WT female. (d). WT male and female with size-manipulated male and female. Males on top; females on bottom.

Monarch size was approximated by using body mass as a frame of reference, since there is a positive correlation between monarch forewing size and body mass (de Roode, unpublished data) (Figure 4). The butterflies for each trial were placed in a small Carolina Biologicals cylindrical popup mesh cage (height: 12 in., diameter: 11 in.) with 10% honey water *ad libitum* for food and allowed to mate as many times as they could for five consecutive days in an incubator (temp: 25°C, humidity: 58%). Monarchs that started mating on the fifth day were given an extra day to complete copulation. Matings were recorded by checking the pairs in copula after dusk (~18:00-21:30h). In each setup of both trial types, 10 mating cages were filmed continuously for the entire experiment using high–definition Owl AHD10-171 841-B cameras, and the remaining cages were spot-checked daily. Cameras were equipped with infrared bulbs to film in complete darkness. All cameras were hung approximately 12 inches above a cage and provided a clear recording 24 hours per day. These filmed cages allowed us to quantify finer-scale mating behavior beyond the evening checks, which quantified the individuals that were in copula each day.

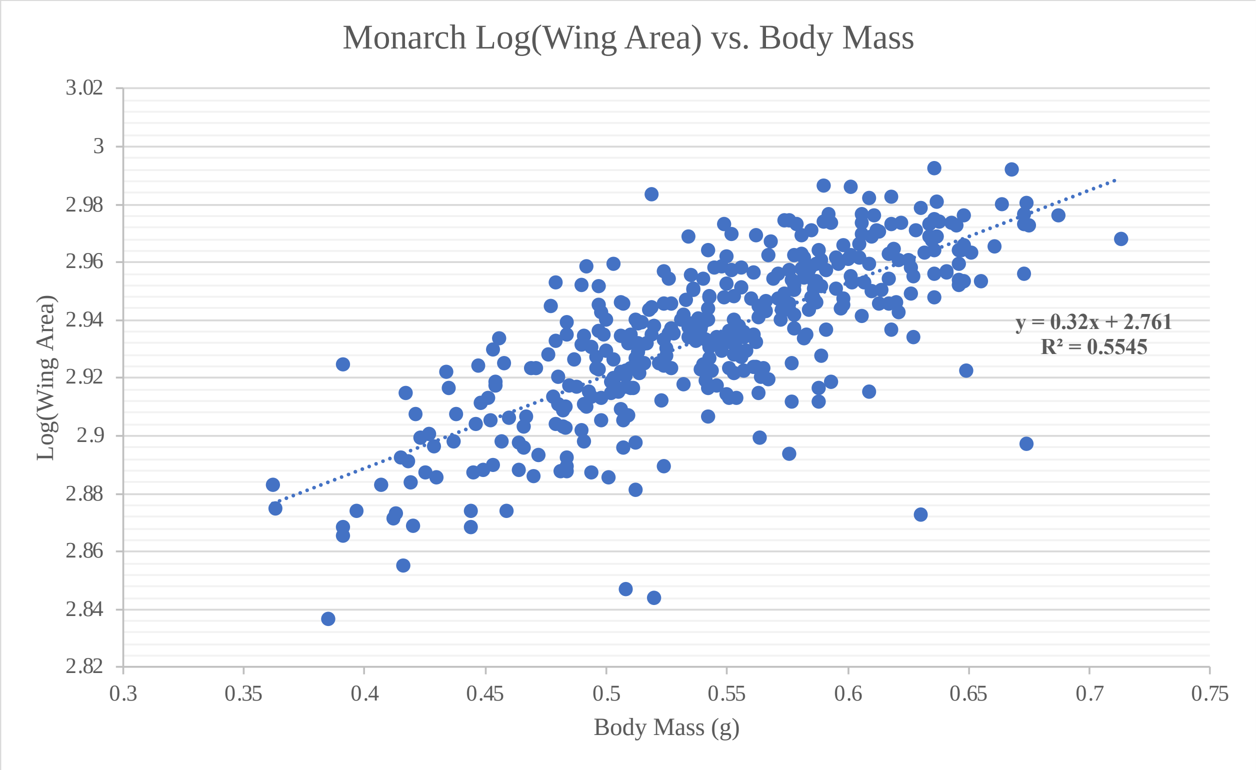


Figure 4. Positive correlation between the logarithm of monarch forewing area and body mass.

Behavioral analysis for recorded cages

Monarch mating behavior was divided into two stages: the precopulatory attempt stage and the copulatory stage. The attempt stage begins when the male grabs the female and probes the female with his abdomen and tries to coerce the female into copulation (Solensky, 2004). Mating attempts can be easily distinguished from when males randomly come into contact with females when they fly around in the cages, because the latter does not involve male probing or female struggling. Successful mating attempts end with copulation, and unsuccessful attempts end with either the male giving up or the female escaping from the male. The copulatory stage begins when the male and female are in a back-to-back posture, which is stereotypic in the Lepidoptera family, with the male's genital claspers locked in the female's reproductive tract in the abdomen (Cannon, 2020). When the mating pair separates, the copulation ends.

Behavior in recorded cages was analyzed by watching footages of the mating assays. The attempt number, attempt starting time, attempt stopping time, whether the attempt was successful, the copulation starting time, and the copulation stopping time were all recorded manually. A detailed description of the mating attempts was also recorded.

Quantification of copulations in spot-checked cages

All the cages were spot-checked daily between 6:00 PM and 9:30 PM in the evening to record which monarchs mated successfully and were in copulation. Copulations in the 10 recorded cages in each type of trial were assessed by both spot-checking and video recordings; the remaining cages relied solely on spot-checking for the number of copulations. Monarchs are diurnal, and their mating activities happen before dusk. Thus, the successful mating pairs are in copula by the time when lights are turned off (around 8:00 PM), and the butterflies remain mostly static at night and do not initiate matings (Oberhauser, 1988). Since monarchs copulate at maximum once per day and all copulations end before the next photophase (Svärd and Wiklund, 1988), it is sufficient to confirm the number of successful mating pairs by doing one spot-checking in the evening.

Statistics

All data were analyzed using JMPv15.0. The tests performed included Chi-squared test and Fisher's exact test. First, we used Chi-squared test to determine if the pattern for the number of matings is random or there is significant bias toward certain combinations of size in the choice trials. Then, we asked if attempt success rate significantly differ among different combinations of size in the choice trials. We used Fisher's exact test to compare the difference in success rate for the 2x1 trial, and for each combination in the 2x2 trials to the two WT combination.

Heritability Experiment

Raising Eastern North American monarch butterflies

Monarch parents (n=112) and offspring (n=756) from 56 families were raised in a temperature-controlled greenhouse room (temp: 24°C, humidity: 17%) with natural light at Emory University. Caterpillars were raised on swamp milkweed (*Asclepias incarnata*), and were provided food *ad libitum* throughout their development to pupae. All the milkweed plants were surrounded by a clear plastic tube (height: 21 in., diameter: 4 in.) stuck into the soil of the milkweed pot. In order to prevent caterpillars from escaping, the top of the tubes was covered by a white mesh net. After the monarchs pupated, they were removed and glued by their cremaster to the lid of a plastic cup. These chrysalises were then placed in a climate controlled (26.5 °C) room with artificial light. Once monarchs emerged, they were frozen for scanning.

Raising Puerto Rican and Eastern North American monarch butterflies for the common garden experiment

Wild Puerto Rican monarchs were captured in the Puerto Rico archipelago in Summer 2021. The Eastern North American monarchs were descendants of wild-caught migratory ENA monarchs from St. Marks, Florida. The offspring of Puerto Rican (n=36) and ENA (n=36) monarchs were raised in a temperature-controlled greenhouse room (temp: 24°C, humidity: 17%) with natural light at Emory University for the common garden experiment. All monarchs were provided food *ad libitum* throughout their development to pupae. All the milkweed plants were surrounded by a clear plastic tube (height: 21 in., diameter: 4 in.) stuck into the soil of the milkweed pot. In order to prevent caterpillars from escaping, the top of the tubes was covered by a white mesh net. After the monarchs pupated, they were removed and glued by their cremaster to the lid of a plastic cup. These chrysalises were then placed in a climate controlled (26.5 °C) room with artificial light. Once monarchs emerged, they were frozen for scanning.

Scanning butterflies

The wings were taken off from the base of the monarch body by turning the wings 90° to the side using forceps. Care was taken to avoid damaging the surface of the wings. Marking stickers were attached to the sides of the scanner as a reference for where the wing was placed. All the wings were placed approximately at the same location. The dorsal side of the right forewing was scanned along with a Tiffen color control patch as a frame of reference for wing color. All monarchs were scanned using the CanoScan 9000F cannon scanner and the VueScan app.

Statistical analysis of monarch size

Wing scans were processed using ImageJ. Data were analyzed using JMPv15.0. Heritability was measured using the linear regression model: mid-parent offspring regression. The average phenotype of two parents was calculated, and the average phenotype of their offspring was also calculated. The points were graphed across sets of parents and offspring, and the slope of the best-fit-line (least-squares linear regression) describes the strength of the “heritability” of the trait. The slope of the best-fit-line, demonstrated by h^2, has a range of 0 to 1. An h^2 value of 0 means the trait is not heritable, and the closer the h^2 value is to 1, the more heritable the trait is. For the plasticity of Puerto Rican and Eastern North American monarchs, a linear model was run to compare the size of monarchs from two populations, with monarch population, rearing environment and their interaction as the explanatory factors.

Results

Mating Experiment

Experimental manipulation of body size

We raised a total of 758 monarch butterflies. Restricting the larval diet at the 5th instar stage resulted in smaller-bodied adults. Diet-restricted male butterflies were on average 41% smaller than the control butterflies that were provided food ad libitum. Diet-restricted female butterflies were 39% smaller than the control butterflies that were provided food ad libitum (Figure 5). Lab-rearing of both groups was highly successful. The artificially small monarchs lived just as long and appeared to behave similarly to the control monarchs.

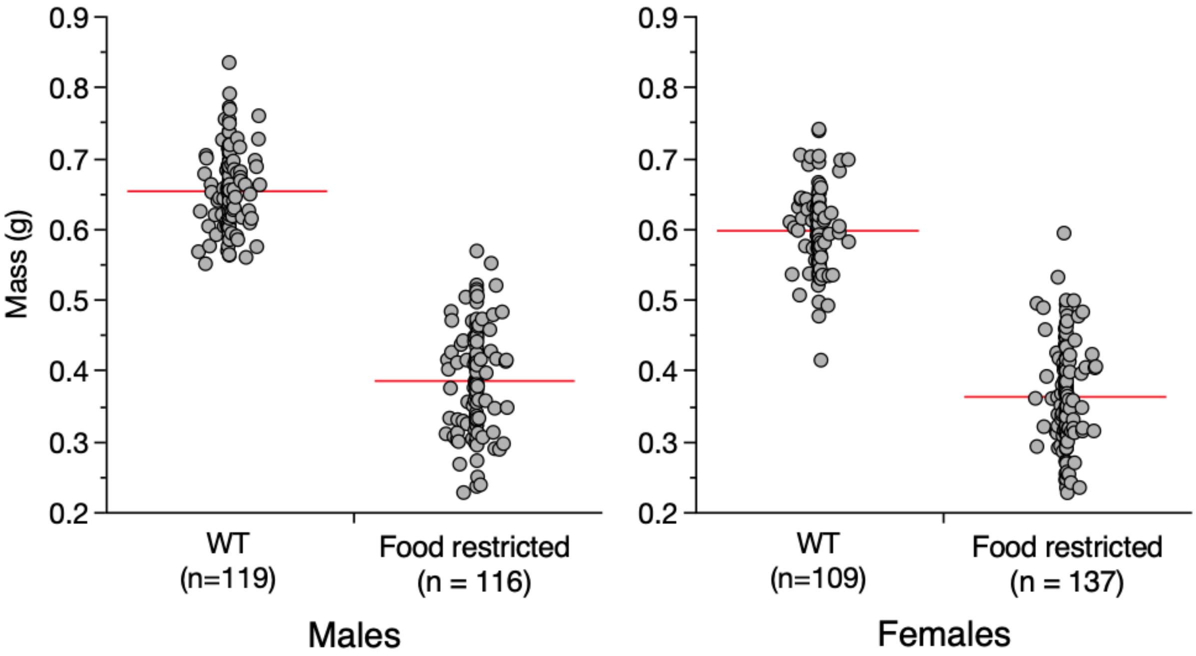


Figure 5. Experimental reduction of monarch body size. Compared to wild type males, size-manipulated males were reduced to 41%; compared to wild-type females, size-manipulated females were reduced to 39%. The red line indicates the mean value of mass.

Mating assay

Spot-checked cages

For no-choice trials, size-manipulated males had a comparable number of successful matings as the wild type males. Size-manipulation did not appear to impair small males' ability to mate. In type (c) choice trials, size-manipulated males were outcompeted by wild type males in terms of the number of matings. In type (d) choice trials, the number of matings in the two wild types and two size-manipulated combinations were more than twice as high as that of the WT and size-manipulated combinations. Size-matched pairs were more likely to mate. In other words, monarchs mate assortatively based on body size (Figure 6).

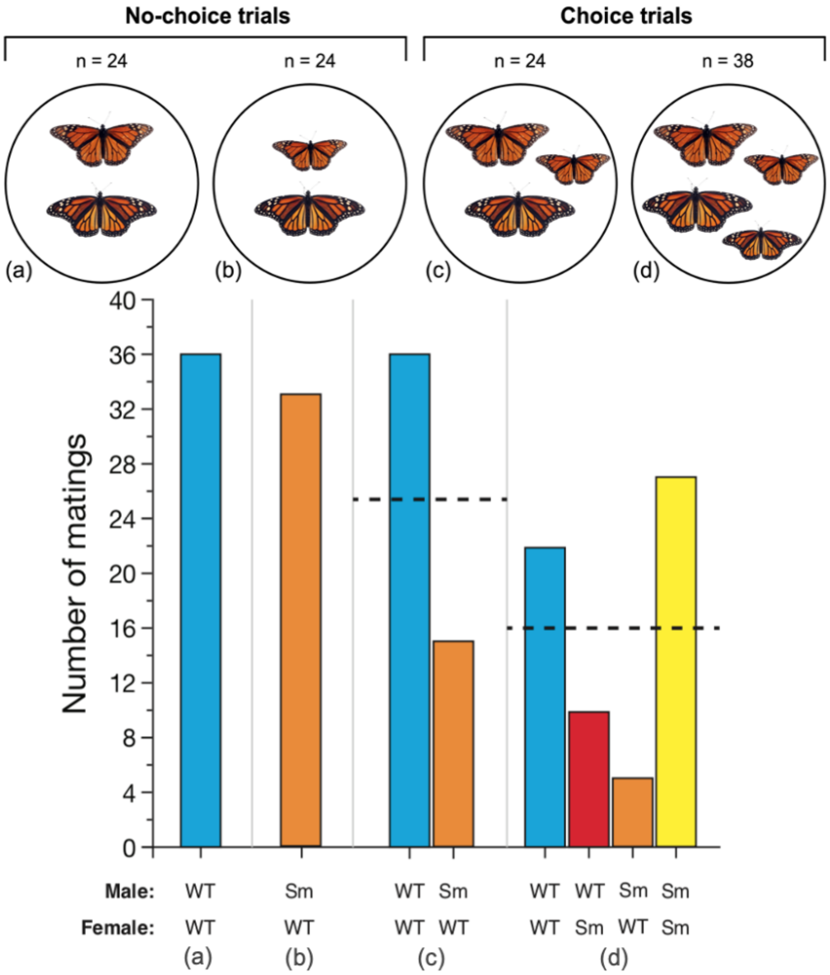


Figure 6. Summary of all mating trials testing the influence of monarch body size on the number of successful matings. All cages were spot-checked every day to quantify copulations. (a). WT male and female; (b). Sm male and WT female; (c). Significant mating bias towards 2WT combination (Chi-squared test; n = 51, df = 1, Chi-square = 8.647, P = 0.0033); (d). Significant mating bias towards 2WT and 2Sm combination (Chi-squared test; n = 64, df = 3, Chi-square = 19.625, P = 0.0002). Dotted line represents threshold of matings by random choice. Males on top; females on bottom.

Recorded cages

For no-choice trials, size-manipulated males had a lower mating success rate than the wild-type male. However, the success rate of size-manipulated male was still relatively high with half of mating attempts ending in copulation. In type (c) choice trials, size-manipulated males were outcompeted by wild type males, and their success rate plummeted to less than one-third. In type (d) choice trials, the mating success rate in the two wild types and two size-manipulated combinations were more than twice as much as that of the WT and size-manipulated combinations. Size-matched pairs had higher success rate in mating attempts (Figure 7).

The comparison among (a), (b), and (c) suggests that female choice might be driving the mating dynamics. Although the attempt success rate is more than or equal to 50% for both WT and size-manipulated males in the 1x1 trials, when females are given a choice between WT and size-manipulated males in the 2x1 trials, WT males seem to be favored over size-manipulated males because the success rate of WT male is still above 50%, but the success rate of size-manipulated male dropped to only 13.3%. In (c), despite making more attempts in total, small males seem more likely to be rejected by females (Figure 7).

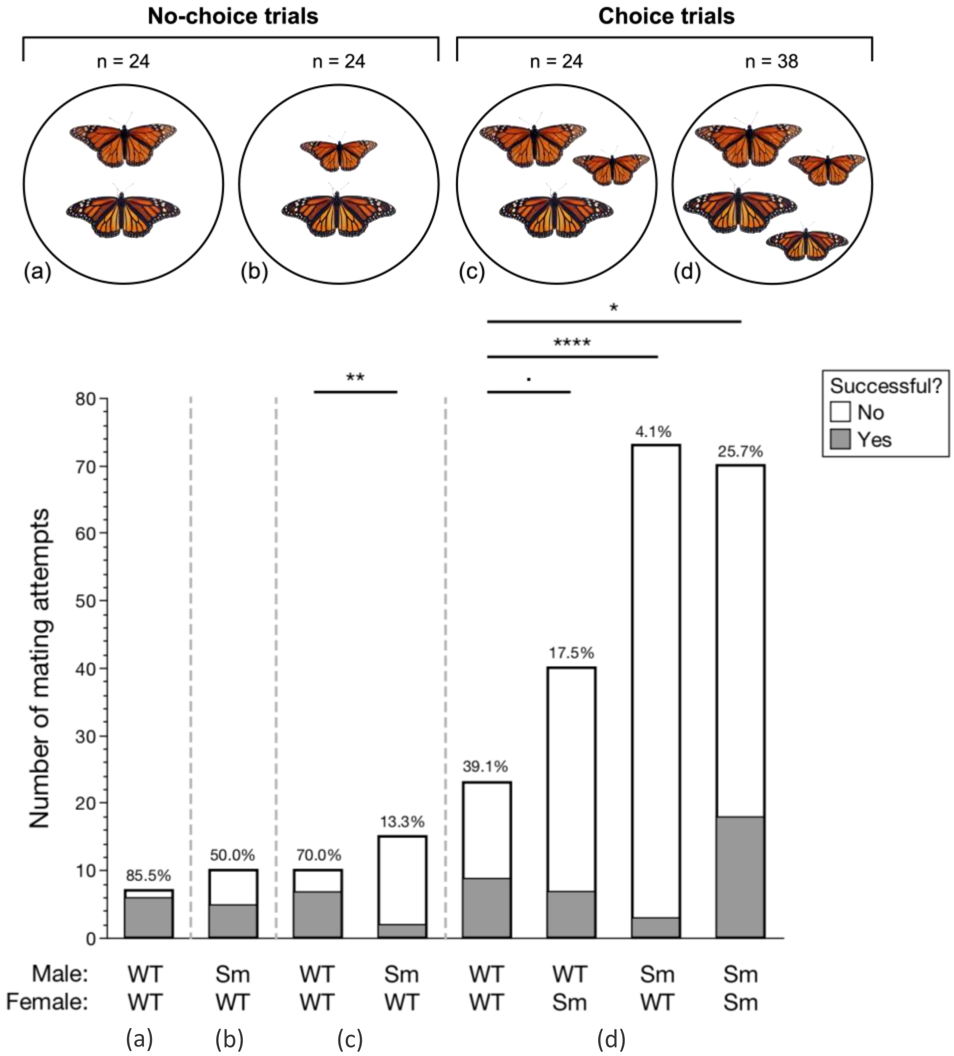


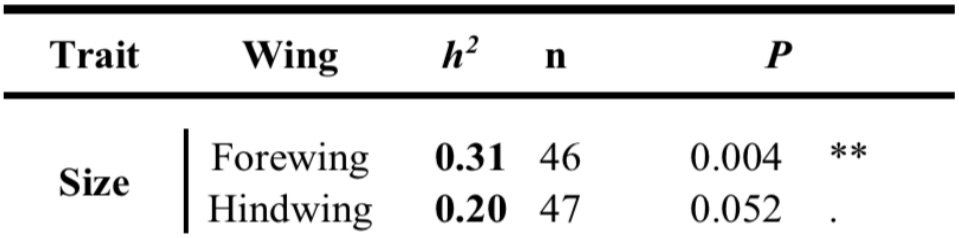
Figure 7. Summary of recorded mating trials testing the influence of monarch body size on mating attempts and the attempt success rate. Only ten cages were recorded in each type of trial for behavioral analysis. The percentage of attempts that ended in copulation is shown on top of each bar. (a). WT male and female; (b). Sm male and WT female; (c). Significant difference between success rate of WT and Sm male with WT female (Fisher's exact test; n =25, P = 0.009); (d). Trend between 2WT and WT x Sm combination (Fisher's exact test; n =63, P = 0.075), significant difference between 2WT and Sm xWT combination (Fisher's exact test; n =96, P = 0.00001), significant difference between 2WT and 2Sm combination (Fisher's exact test; n =83, P = 0.015).

Heritability Experiment

Wing size heritability of Eastern North American monarchs

Results showed that 31% of the forewing size variation in Eastern North American monarchs is explained by genetics. Forewing size appears to be highly heritable (p=0.004).

Hindwing size also appears to be heritable with a h^2 value of 20% ($p \approx 0.05$) (Table 1). The 95% confidence interval for forewing size is 0.14-0.63, and for hindwing size is 0.00-0.53. Since there is an overlap in confidence interval, forewing and hindwing size are not statistically different.



Trait	**Wing**	h^2	**n**	***P***	
Size	Forewing	**0.31**	46	0.004	**
	Hindwing	**0.20**	47	0.052	.

Table 1. Results of linear models investigating the heritability of forewing and hindwing size for Eastern North American monarchs raised on milkweed species *Asclepias incarnata*.

Body size plasticity of Puerto Rican and Eastern North American monarchs

Results demonstrate that in the common garden experiment, where wild caught Puerto Rican monarchs were raised under the same environmental conditions as Eastern North American monarchs, the offspring of the wild-caught Puerto Rican monarchs maintained smaller body sizes, like their parents (Figure 8). Additionally, results suggest Eastern North American monarchs are larger than Puerto Rican monarchs.

Linear comparisons show that Puerto Rican and Eastern North American monarchs are statistically different in size ($p<0.00001$), (Table 2). Population effects have a significant influence on monarch body size ($p=0.001$). Environmental effects or the interaction of population and environmental effects do not have a significant influence on body size ($p>0.05$). Data indicate that size does not have a plastic component. Differences in the body size of Puerto Rican and Eastern North American monarchs are mostly due to genetic differences, instead of environmental differences or the GxE interactions.

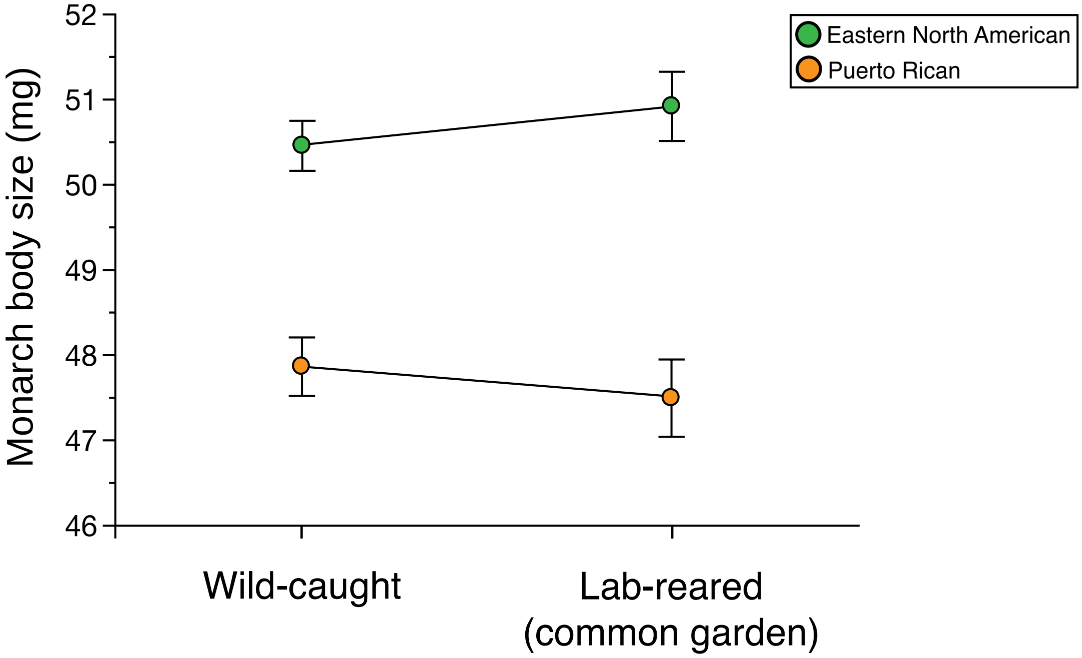
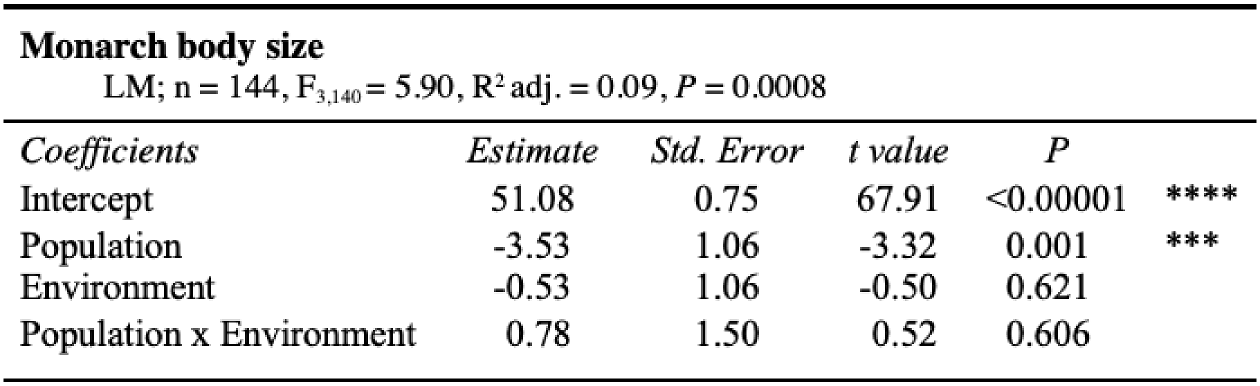


Figure 8. Common garden experiment showing the plasticity of Puerto Rican and Eastern North American monarch body size.



Monarch body size

LM; n = 144, $F_{3,140} = 5.90$, R^2 adj. = 0.09, $P = 0.0008$

Coefficients	*Estimate*	*Std. Error*	*t value*	*P*	
Intercept	51.08	0.75	67.91	<0.00001	****
Population	-3.53	1.06	-3.32	0.001	***
Environment	-0.53	1.06	-0.50	0.621	
Population x Environment	0.78	1.50	0.52	0.606	

Table 2. Summary of linear model results comparing the body size of monarchs from two populations (Eastern North American or Puerto Rican) reared in two different environments (wild-caught or lab-reared). The intercept is set to lab-reared Eastern North American monarchs.

Discussion

Our study illustrates that monarch butterflies mate assortatively based on size: large males mate more often than expected by chance with large females, and small males mate more often than expected than chance with small females (Figure 6, 7). Assortative mating is common in nature with great evolutionary significance, and it contributes to species diversification (Taborsky, 2014). For instance, a recent study shows that the Big Bird lineage of Darwin's finches evolved a different beak morphology and consequently a unique song type than its coexisting competitors (Lamichhaney et al., 2018). Since Darwin's finches mate assortatively based on vocal cues (Podos, 2010), a prezygotic barrier is established by the difference in courtship songs determined by their beak morphology. As a result, the Big Bird lineage was reproductively isolated from other finch species. Since assortative mating can assist the formation of reproductive barriers, it is possible that the large migratory Eastern North American monarchs and the small non-migratory Puerto Rican monarchs will become reproductively isolated from each other and diverge into different species in the future, given that the two populations are already geographically isolated from each other by the ocean and diverged morphologically. Although monarchs display forced copulatory behavior (Solensky, 2004), sexual coercion does not seem to counteract the pattern of assortative mating. Additionally, female choice seems to influence attempt success rate in choice trials. The phenomenon of female choice driving mating dynamics in coercive mating systems is also detected in other species, such as eastern mosquitofish (Bisazza et al., 2001). This could be because females exert some control over mating through evasive behavior or struggling even though males use physical force to mate. This point is supported by the many observations in mating videos that females curl their abdomen towards their thorax with legs to avoid mating with unwanted males.

Our results indicate that variation in body size between Eastern North America and Puerto Rican monarch populations is due to genetic differences between the populations. In addition, variation in body size within the North American population was significantly heritable (Table 1). In addition, our finding that Eastern North American monarchs are larger in size than Puerto Rican monarchs (Figure 8, Table 2) is consistent with previous research on the divergence of wing size among migratory and non-migratory monarch populations (Altizer and Davis, 2010). One possible explanation for the heritable size difference between these two populations is adaptation to their respective ecological conditions. Monarchs belong to the tropical Danaini butterfly group, and similar to their common ancestor, monarchs are unable to survive in winter with freezing temperatures (Agrawal, 2017). Thus, every autumn, Eastern North American monarchs migrate up to 4000 km from Canada and the U.S. to overwintering sites in Mexico (Brower, 1995; Reppert and de Roode, 2018). Multiple studies have shown that migratory monarchs from long-distance migratory populations have larger forewings than their non-migratory counterparts (Altizer and Davis, 2010; Flockhart et al., 2017). The larger wing sizes in the Eastern North American monarch population could be an adaptation for migratory behavior since monarchs with larger forewing areas migrate greater distances (Flockhart et al., 2017). In contrast to Eastern North American Monarchs that inhabit the North American continent, Puerto Rican monarchs are confined to a small archipelago. Puerto Rico is in the tropics with no changing of seasons and a constant availability of milkweed plants, enabling monarchs to breed year-round. Hence, without the influence of seasonality, the Puerto Rican population might be under relaxed selection for body size because they no longer need to migrate. It makes sense that environmental noise or GxE interactions do not have a significant influence on monarch body size because fitness-related traits are generally canalized and less affected by the environment (Table 2).

In summary, these results demonstrate that assortative mating based on heritable wing size could lead to potential ecological speciation in monarch butterflies. Our study provides insights into how morphological adaptions can facilitate reproductive isolation in organisms that use coercive mating strategies.

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