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Dynamics of Multiple Matings in Monarch Butterflies

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Dynamics of Multiple Matings in Monarch Butterflies

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Bachelor of Science

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An abstract of

A thesis submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of Master of Science in Biology

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Abstract

Dynamics of Multiple Matings in Monarch Butterflies
By Andrew Mongue

While competition for sexual partners is often imagined in terms of dramatic visual displays and physical combat, there are just as many invisible forces driving the struggle for reproduction. Even when the process at play cannot be seen, the economics of benefits and costs to each party control the system. Insects make the perfect study organisms for such dynamics due to their short generation time and large offspring number. *Danaus plexippus*, the monarch butterfly, makes an intriguing study insect because of its apparent departure from butterfly mating norms. With that in mind, I compared the number and paternity of offspring of female monarch butterflies that mated with either one or two males to investigate benefits to both sexes. Females that remated lived longer and laid more eggs total compared to their singly mated counterparts. On the male side, there was a trend for last male sperm precedence, as detected with microsatellite paternity analysis, though significance was not achieved with the study sample size. With these results both sexes could potentially benefit from remating, but confounds in the mating design introduce uncertainty to these benefits. More concretely, this experiment demonstrates the usefulness of genetic markers developed for population differentiation studies on the pedigree scale.

Dynamics of Multiple Matings in Monarch Butterflies

By

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Bachelor of Science, Biology

Advisor: Jacobus de Roode, PhD

A thesis submitted to the Faculty of the
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Introduction

Sex has been identified as an important factor in evolution since Darwin's seminal work (1859) on natural selection where he pointed out that competition between two males of the same species over a mate is just as important as interspecific competition and the struggle against the environment to survive. While this male-male competition can often be easily observed, e.g. males killing the offspring of rival males (Hrdy 1974) or directly fighting each other (Le Boeuf 1974), just as often the conflict cannot be seen.

Sperm competition, the struggle of male gametes to fertilize an egg, occurs when more than one male has access to a receptive female. It has been demonstrated in everything from apes (Møller 1988) to zebra finches (Birkhead et al. 1988), as well as many invertebrates. Males employ a number of mechanisms to gain an advantage, including flushing out the previous male's ejaculate with their semen (Ono et al. 1989) or simply removing competing sperm with their penis (Waage 1979), but the end result is the same: the last male often fathers the most offspring. For instance, in some damselflies the last male to mate can father up to 95% of the offspring (Fincke 1984).

In some species however, the female has significant control over who mates with her last. Rutowski (1980) found that female *Pieris protodice*, checkered white butterflies, solicit males when they run low on sperm or, more likely, other spermatophore contents, thus giving the female a choice of second mate. The non-fertilizing portion of the spermatophore is often very valuable to females, as in grasshoppers where the nutrient content can enable a female to lay more eggs (Butlin et al. 1987). In other species, females select mates for less immediately quantitative reasons, like potential for their sons to father more offspring than the sons of other females, a dynamic often referred to as the sexy son hypothesis (see Pai and Yan 2002 for an example in flour beetles).

While females may get some quantitative (direct) or qualitative (indirect) benefit to remating, they also incur a cost; in butterflies for example, the act of copulation is often physically costly in the form of damage to females (Kawagoe et al. 2001), thus it pays for a female to be choosy with her mates. In many species, males must perform some form of courtship whether physical or chemical, to entice the female (see Wiklund and Forsberg 1986 for an example in orange tip butterflies). One of the few exceptions is *Danaus plexippus*, the monarch butterfly. Females monarchs have been observed in nature struggling to free themselves from males that are attempting to initiate copulation (Hill et al. 1976). Male monarchs have small, apparently vestigial hair-pencils (Pliske 1975) and do not make use of their androconia (Boppré 1993), suggesting that pheromone based courtship is not important in this species . With the apparent resistance of females and lack of enticement by males the inference can be made that mating in monarchs is a male-controlled behavior. This departure from the Lepidopteran norm makes the monarch an appealing choice for a study on sexual dynamics.

In general, the monarch butterfly is a charismatic and well-studied organism, both scientifically and publically. It has a robust citizen science program that tracks its yearly North American migration (Howard and Davis 2004), is one of a handful of butterflies to have a sequenced genome (Zhan et al. 2011), and is used in chemical ecology studies due to the toxic nature of its larval host plant (de Roode et al. 2011). In spite of all this attention, many questions remain surrounding this organism. It was not until recently that modern molecular techniques were applied to the question of monarch population structure in North America (Lyons et al. 2012) and such an update is still needed for the sexual dynamics of this butterfly.

This update was attempted by Solensky et al. (2009b) who used allozymes of phosphoglucose isomerase to assess paternity and establish the degree of sperm precedence in multiply mated female monarchs and found a second male advantage in paternity. But using a functional protein introduces the possible confound of fitness differences between phenotypes because a given male may father more offspring due to the efficiency of their allele of this particular enzyme rather than the order in which he mated.

Since the time of that study, neutral microsatellite markers have been developed by Lyons et al. (2012) specifically for use in monarchs. Pitting males with different microsatellite profiles against each other in sperm competition is less potentially biasing than using males with differing enzyme electrophoretic properties since the microsatellite loci are neutral. Thus the current study aims to use this advance to examine the mating dynamics of monarchs.

Multiple matings may exist for several reasons. It may be a female-controlled phenomenon which is driven by either direct or indirect benefits of remating to females. In both of these cases, there is no necessary pattern of first or last male success. For example if a female mates once to ensure she can produce offspring, but then mates again to have a choice in the sperm she uses, it is just as likely that she mates with the better male first (in terms of genetic traits) as second. On the other hand, remating may be driven by males competing to fertilize the most eggs, in which case I expect Solensky et al.'s results, i.e. last male dominance, to hold up to the scrutiny of a microsatellite paternity test.

Materials and Methods

Mating Design

Initially 35 female and 61 male monarchs were taken from eight lineages of second generation offspring of wild monarchs from Cobb County, Georgia, raised in greenhouse conditions described below, with *Asclepias curassavica* as larval food. Prior to mating cage assignment individuals were visually checked for parasitic infection by examining abdomen scales under a light microscope. Uninfected individuals were transferred to mating cages in the greenhouse; thus the matings occurred under a natural light cycle and temperature range. As the experiment occurred in June and July in Atlanta, Georgia, temperatures climbed quite high (38+°C), which lead to the deaths of a number of individuals before the end of their natural lifespans. Consequently, fecundity analyses were carried out on only 33 females, though all of the starting butterflies were measured on at least one dimension of body size since corpses were kept post mortem.

For the first matings, each lineage was segregated by sex and paired with the opposite sex from another lineage so that there was no inbreeding. Males and females were placed into communal cages and copulations were noted. On completion, the female was moved to an individual cage stocked with *Asclepias curassavica* milkweed; the male was placed in a separate male-only cage to ensure males did not mate multiple times. Since production of apyrene sperm, an important but non-fertilizing component of a male's spermatophore, and egg maturation are continuous processes in adult males and females respectively (Friedlander 1997, O'Brien et al. 2004), it was important to provide a constant nutrient source to avoid the confound of resource depletion on any changes in fecundity. As such, in all cages butterflies had continuous access to food in the form of a honey water solution.

For second matings, females were chosen at random initially but some females assigned to the multiple mating treatment were recorded as single matings because they died before successfully remating; the final counts thus skewed in favor of single matings (n = 20) as opposed to multiple matings (n = 13). Females were introduced to one or two virgin males, depending on availability, after 6 consecutive days of egg laying. The use of virgin males prevented sperm precedence, i.e. the proportion of offspring fathered by the second (last) male to mate, bias based on time since last copulation. It is also a reasonable model of natural populations since sex ratios can become male skewed at overwintering sites, where breeding begins in the spring for migrating monarchs, such as those that pass through Georgia (Hill et al. 1976). Pairs were left together until successful mating occurred, after which both males were removed and milkweed was reintroduced to allow for continued egg laying. No individuals were killed and instead were allowed to continue laying until they died naturally, over 20 days later in some cases.

To obtain time series data on fecundity, each plant and cage with a fertile female was inspected and all eggs were collected and counted every 24 hours. The milkweed plant was changed daily after the count, thus no eggs were counted twice. Stored eggs were kept at least four days to allow for hatching to get a measure of egg viability. For caterpillars used in paternity analyses, individuals were reared to second or third instar so that they would have more tissue for DNA extraction. In either case, larvae were frozen until time of counting or extraction. Similarly deceased adults were saved for DNA extraction or body size measurements. Because monarch wings often rip and deteriorate over time, wing length could not be used as a measure of body size; instead thorax length and tibia length from the middle legs were recorded using digital calipers. Dry mass was also recorded.

Genetic Analyses

For paternity analyses up to, but no more than, ten caterpillars from the first and ten from last day of egg laying post remating were collected (n = 130 total) and used whole as a tissue source for DNA extraction using a MoBio kit (Carlsbad, CA, USA). Whole bodies were used as extraction tissue for caterpillars due to their small size. For parents a portion of abdomen was taken from both possible male parents. For females a portion of thorax was used instead to prevent potential extraction of DNA from stored sperm.

Post extraction, DNA was checked for concentration and quality on a NanoDrop (Wilmgton, DE, USA) before being used in PCRs. Paternal and maternal samples were run with primers developed specifically to amplify monarch microsatellites, using cycling parameters established by Lyons et al. (2012). PCR products were genotyped on an ABI 3100 genetic analyzer (Foster City, CA, USA) and interpreted with GeneMarker software (State College, PA, USA). After identifying a locus that could be used to distinguish paternity, DNA from all caterpillars for the trio was run using this primer and analyzed in the same way.

Statistics

Body characters were examined in relation to sex and lineage in a two way ANOVA to establish body size relations between the sexes after confirming that these characters were normally distributed. The relationship between tibia length and thorax length was examined with linear regression to allow the two measures to be interchanged in analyses due to availability (for example thoraxes were used for DNA extraction of females, so tibia length was used in those cases).

Relationships between age and egg output as well as egg viability were done using linear regression as well. Factors affecting egg output in females and offspring sired in males were examined using ANCOVAs. Because of the time delay between first and second matings, monarchs that died within the first 5 days after remating had to be assigned to the singly mated treatment. To minimize the confound this created, only singly mated monarchs that lived at least 6 days were used in comparative analyses (n = 12). Due to the repeated measures involved in daily egg laying and viability, a mixed effects linear model with each day's total for egg laying and viability was also used to examine these variables in relationship to mating treatment. However, daily egg output could not be modeled using standard R functions due to non-convergence so the number of larvae hatching on each day was used as a proxy given that the eggs laid per day is related to the larvae hatching by the viability (defined as number of larvae/number of eggs), and the number of larvae hatching is a more direct measure of fecundity than egg number. P2, the proportion of offspring sired by the second male to mate, was tested against chance using a general linear model with a quasibinomial error distribution.

Results

Body character

Body mass did not vary significantly between sexes or lineages ($F_{1,69}$ = 2.49; p = 0.119 & $F_{7,69}$ = 2.49, p = .097 respectively) but was affected by the interaction of the two ($F_{4,69}$ = 3.68; p = 0.009); males were larger than females in most, but not all lineages. Conversely, thorax length differed between sex ($F_{1,69}$ = 3.49; p = 0.006), with males averaging 0.6mm longer, and lineage ($F_{7,69}$ = 1.33, p = 0.007) but not with their interaction ($F_{4,69}$ = .038; p = 0.986), indicating that the sex differences held across lineages. Tibia length followed the same pattern: differing between sex and lineage ($F_{1,69}$ = 6.10; p = 0.016 & $F_{7,69}$ = 2.33, p = 0.034 respectively) but not the interaction ($F_{4,69}$ = 0.464; p = 0.762). Males had the longer tibia by 0.32mm (Figure 1). Furthermore tibia length and thorax length were positively related to other ($F_{1,80}$ = 33.61; r^2_{80} = 0.290; p<0.001, see Figure 2).

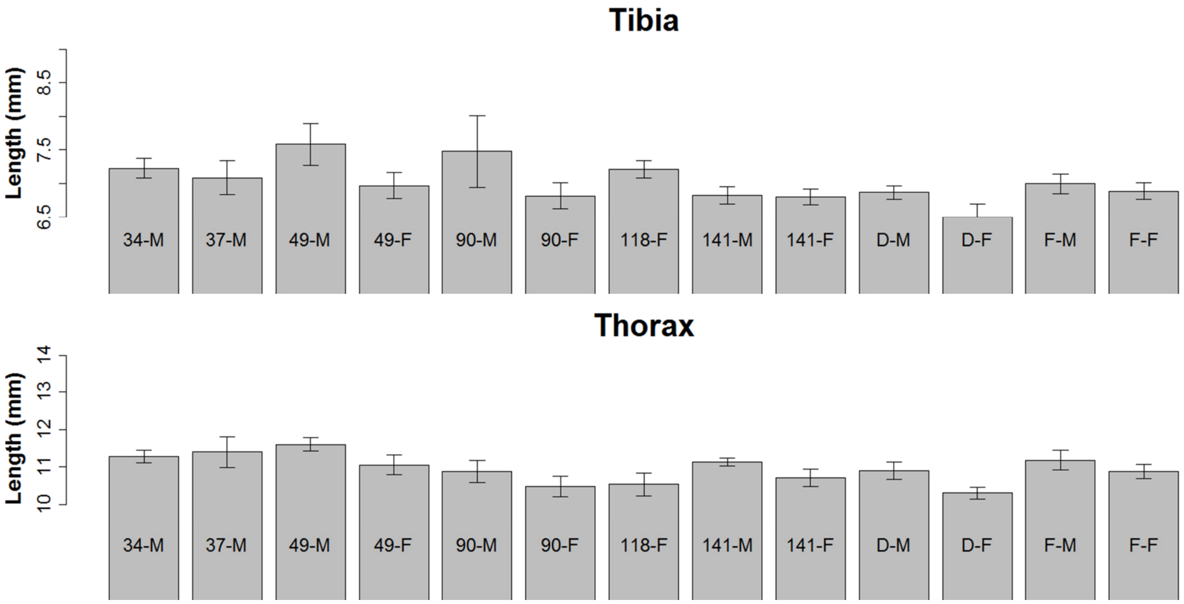

Figure 1. Top: middle leg tibia lengths by sex and lineage. Bottom: thorax lengths by sex and lineage. M denotes male and F denotes female. As above, for lineages 118, 37, and 34 all individuals were of one gender.

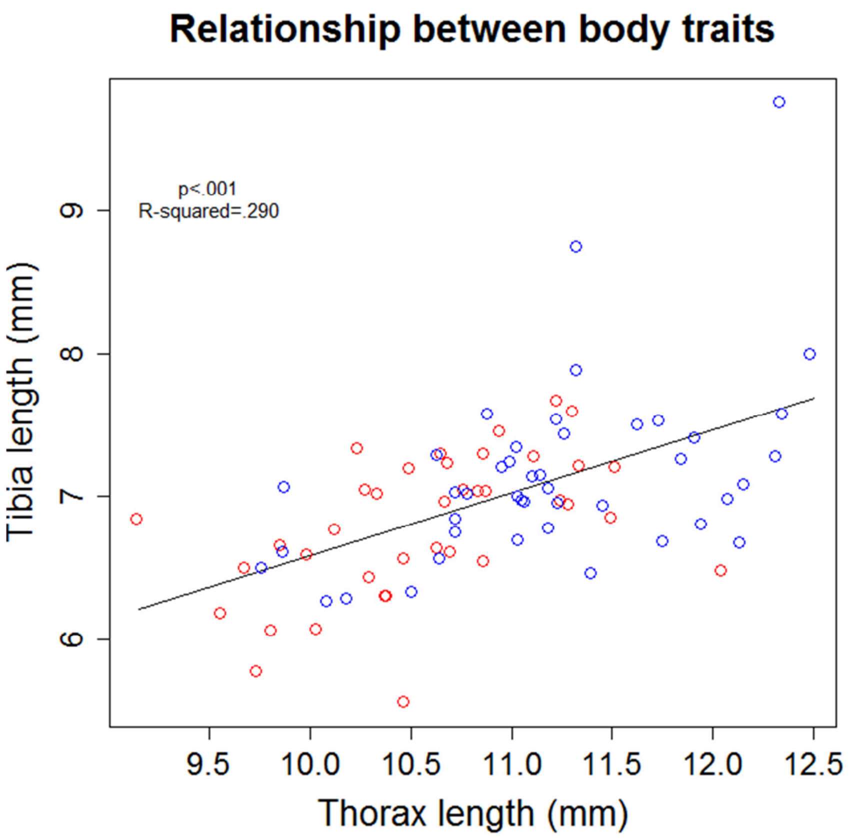

Figure 2. Relationship between tibia length and thorax length for both males (blue) and females (red).

Fecundity

Table 1. Results of ANCOVA for factors affecting total lifetime egg output, daily egg output, and daily egg viability as well as lifespan.

Test	total lifetime egg output			daily egg output			daily egg viability		
Factor	F	df	p	F	df	p	F	df	p
mating treatment	18.16	1,21	<0.001*	0.957	1,21	0.339	0.129	1,21	0.723
lineage	1.94	5,21	0.131	1.17	5,21	0.359	0.013	5,21	0.882
tibia length	0.127	1,21	0.725	0.04	1,21	0.851	0.112	1,21	0.741
lifespan	0.195	1,21	0.663	1.25	1,21	0.276	0.782	1,21	0.387
Test	total lifetime egg output			Test			total lifetime egg output		
Factor	F	df	p	Factor			F	df	p
mating treatment	34.89	1,27	0.013*	mating treatment			6.20	1,25	0.019*
lineage	2.68	5,27	0.043*	lifespan			11.87	1,25	0.002*

Under the full model, lifetime egg output was only significantly affected by mating treatment (Table 1, Figure 3). By simplifying the model, effects of lineage and lifespan on total egg output emerged as well. Tibia length was not a good predictor of total egg production in any model. Finally, none of these factors significantly impacted daily egg output or viability (Table 1).

The mixed effect models adjusted for repeated measures agreed with the ANCOVAs. Remating had no effect on offspring viability ($t_{203} = 1.18$; $p = 0.240$) though the difference in number of larvae produced by a female on a given day was nearly significant ($t_{203} = 1.76$; $p = 0.079$). Furthermore, both the daily number of eggs and proportion of hatching caterpillars decreased over time ($r^2_{236} = 0.122$; $p<0.001$ & $r^2_{236} = 0.093$; $p<0.001$, see Figures 4 & 5).

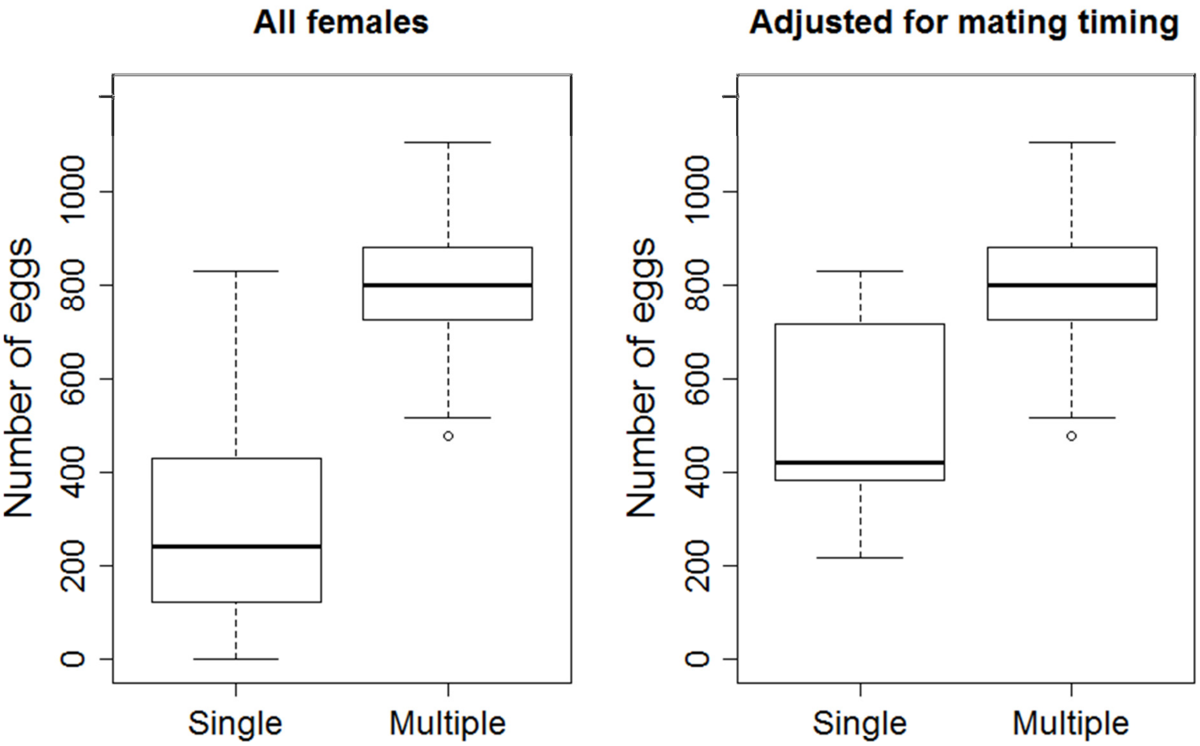

Figure 3. Total egg output by mating treatment. On the left, for all females with egg data, on the right only singly mated females that lived long enough to be eligible to remate. The adjusted data were used for comparisions.

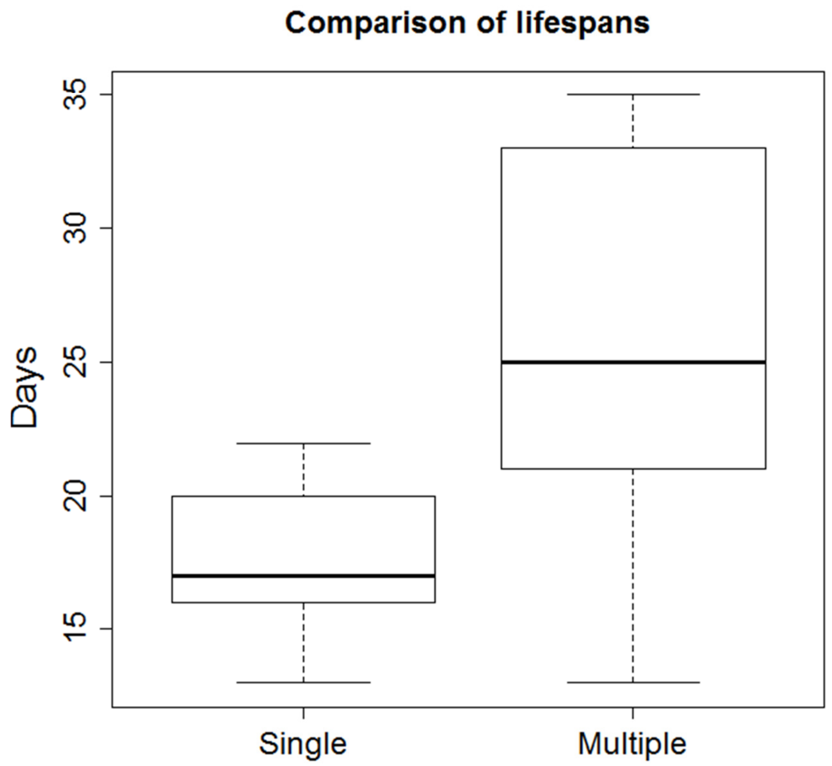

Figure 4. Multiply mated females lived longer than singly mated females, even controlling for the confound of mating timing.

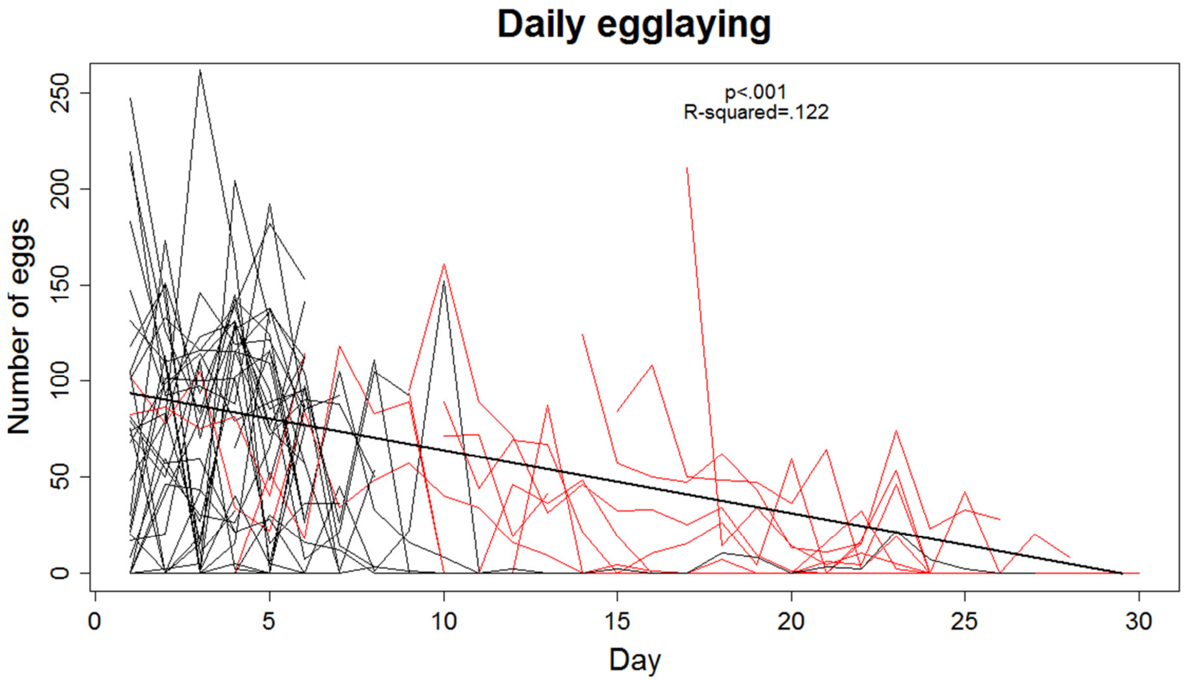

Figure 5. Daily egg laying decreases over time. Daily egg laying for both singly mated (black) and doubly mated (red) females. Lines connect the daily values of individuals over time, gaps in lines represent days where no eggs were laid, e.g. during remating or general inactivity.

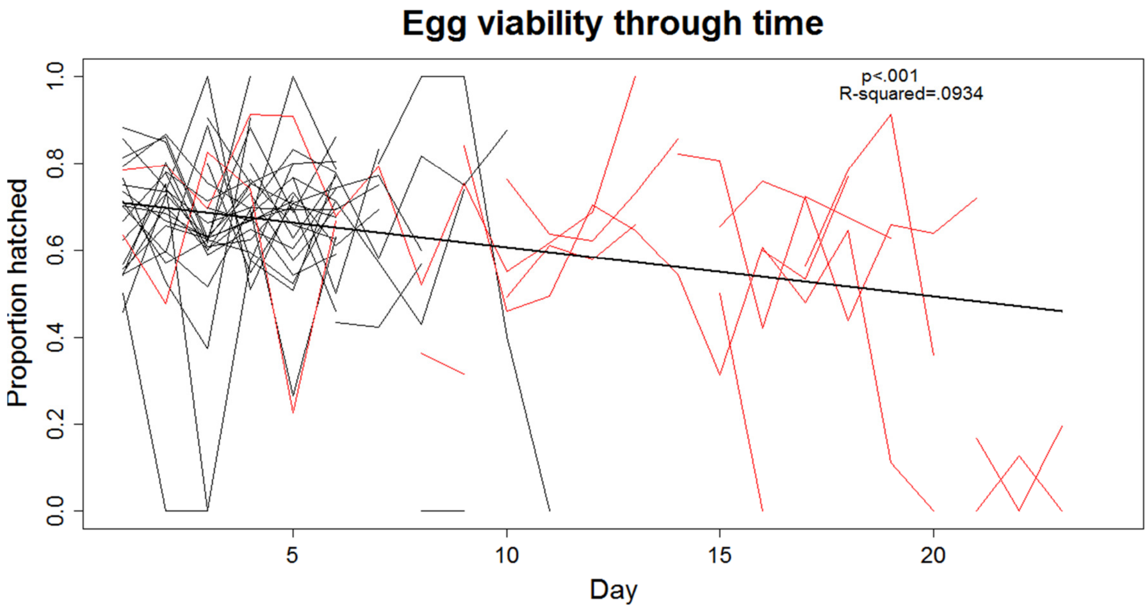

Figure 6. Daily egg viability decreases over time. Black represents single mating treatment and red indicates double mating treatment. Lines connect values from specific individuals over time, as above interruptions in the lines represent days where no eggs were laid.

Sperm precedence

All possible parent trios were successfully genotyped for at least one locus, and some for two (for details see Supplementary Table 1). In all but one mating trio, P2, the proportion of offspring fathered by the last male to mate, was greater than 0.5 for at least one of the two days and at least 0.5 on both days for five of the seven (Figure 7). These results were only marginally significant on the first day ($t_7 = 2.02$; p = 0. 0837) and not quite significant for the last day ($t_6 = 1.78$; p = 0.126); considering the two together, i.e. pooling the P2 for both days for each male, there was still no significant overall last male precedence ($t_7 = 1.75$; p = 0.111) even though P2 did not differ significantly between days ($t_{14} = .242$; p = 0.812).

Relative thorax length was the only good predictor of success on the first day after remating ($F_{1,4} = 12.91$; p = .023), as defined as the proportion of offspring fathered, with the larger male being more successful. Relative tibia length and even order of mating were not good predictors ($F_{1,4} = 2.89$; p = 0.164 & $F_{1,4} = .134$; p = 0.733 respectively). None of these variables: relative thorax length, tibia length, or mating order significantly affected success on the last day of egg laying post remating ($F_{1,4} = .772$; p = 0.444 , $F_{1,4} = 4.97$; p = 0.112 & $F_{1,4} = .1.21$; p = 0.351 respectively). Simpler models yielded the same results.

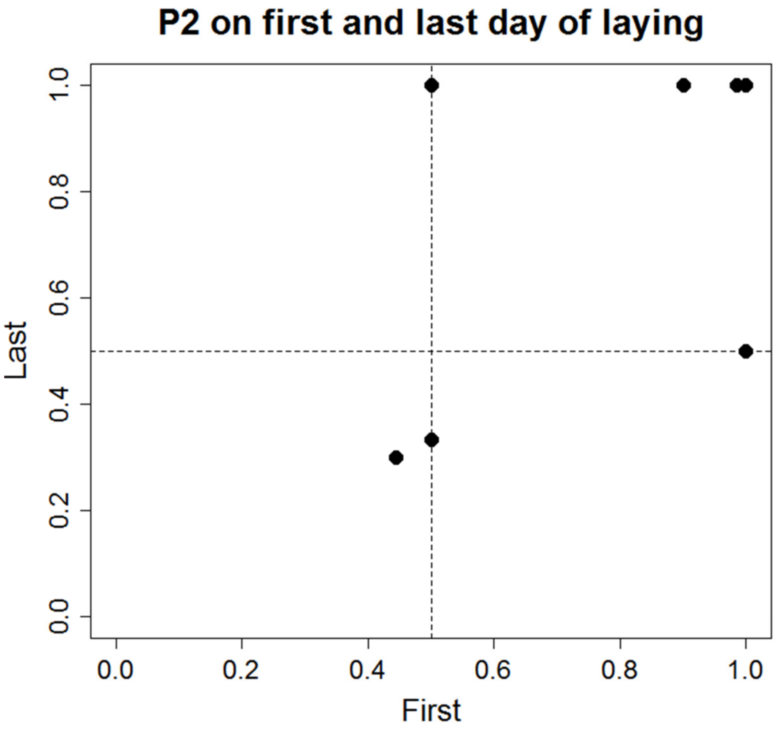

Figure 7. Proportion of offspring fathered by the second male to mate with a given female. Each point represents data for one male on both the first and last day after remating.

Discussion

Males were larger than females from the perspective of thorax and middle tibia length (Figure 1). As noted by Wiklund et al. (1988), in most insect species females tend to be larger than males. An appealing explanation for this reversal of insect norms is the male controlled mating dynamics of the species. Male to female size ratio has been shown to increase with the level of polygamy in Lepidoptera species (Wiklund and Forsberg 1991). It should be noted that Wiklund (and many others) use forewing length as a measure of size. Wing length was not measured in this instance because body measurements were done post mortem and many individuals had wing damage preventing an accurate measure of length. In future experiments, this can be avoided by measuring traits directly after emergence from pupae, which would also decrease the uncertainty in the mass measurement discussed below.

Body mass also had no apparent effect on egg production, a result at odds with much of insect sexual dynamic literature (Honěk 1993). The easiest explanation is that in this experiment, individuals were not massed until well after death. There was observable variation in the body condition between individuals, as to be expected since mating is a large cause of body damage in monarchs (Leong et al. 1993). Additionally some of the heaviest females were those that, on autopsy, had mature eggs in their abdomens but did not lay them. These eggs obviously were not counted as part of fecundity and thus increased the effective mass of some of the less fecund females.

Treatment and lifespan are somewhat confounded in this experiment since only monarchs that lived six or more days after initially mating were eligible to be remated. For analyses of female benefits, this problem was solved by removing females that did not live at least 6 days after their first mating. This did not eliminate the problem for male benefits though; even for females that survived to remating only eight individuals lived for more than one day of egg laying and only seven had viable eggs on more than one day, shrinking the number of usable individuals for the examination of sperm precedence over multiple days. This raises the immediate potential for a follow up experiment where multiply mated females would be mated again immediately after their first mating, rather than six days later. Furthermore, beginning matings earlier in the spring would decrease the likelihood of extreme temperatures prematurely killing off butterflies. Increased survival would create a larger sample size for the multiple mating treatment and allow clarification if some of the effects approaching marginal significance, e.g. the second male advantage, are truly significant.

There was an observed increase in total lifetime egg output for multiply mated females compared to their singly mated counterparts (Table 1); the lack of a similar effect on daily egg output combined with the difference in lifespans between treatments (Figure 4) suggests that remated females laid more eggs because they lived longer, not because they laid more eggs per day. Similarly, remating had no effect on egg viability (Figure 6); also of note from Figure 6 is the fact that viability decreases dramatically just prior to the end of an individual's life. Combined with the decrease in daily egg laying over time (Figure 5), the beginning of a female's laying period is disproportionately important compared to the rest of her adult life.

The decreases in egg output and viability over time raise questions about fecundity in natural settings. Some North American monarch populations are known to undertake a yearly migration to central Mexico where they overwinter. During the trip and the stay in Mexican forests, individuals enter reproductive diapause, i.e. do not mate or lay any eggs. Herman et al. (1981) states uncertainly that diapause likely has no effect on female fecundity, but this assertion has yet to be tested. Likely the decrease in egg output is due to the females ovipositing faster than their eggs mature so monarchs in diapause would not experience decreased egg output after diapause. The reason behind the decreasing viability is less clear and thus may be relevant to this migrating population. Further comparisons of the fecundity of migrating and year-round breeding populations are warranted given evidence in mosquitos (Ellers and Van Alphen 2002) and spider mites (Kroon and Veenendaal 1998) that diapause subsequently decreases egg production.

The presence of P2 variation by thorax size is not surprising given that male size is a known factor affecting the outcome of sperm competition in other butterflies (Bissoondath and Wiklund 1997). The lack of a similar relationship with tibia length is probably a sample size effect since there was greater variance in tibia length compared to thorax length despite the two being related (Figure 2). Sample size is again an issue on the results of the ANCOVA and the quasibinomial test for sperm precedence as only seven male-male-female trios laid viable eggs for more than one day of remating. As discussed above modifying the timing of the second mating would potentially increase the number of females living for multiple days of egg laying after remating. With that in mind the lack of significance for factors affecting paternity of larvae may be an artifact of the small sample size, so a modified follow up experiment is warranted.

As illustrated by Figure 7, second male precedence was greater, although not significantly so, on the first day post remating than on the last day after remating. If P2 truly is greater on the first day remating, it would follow much of established theory. While females have many reasons to be choosy with the paternity of their offspring, as discussed below, this selectivity may fall apart near the end of an individual's lifespan. Close to death a female has no time to select another mate and thus can only increase her fecundity by using whatever sperm is available to her at the time. Choosiness has already been shown to decrease over time in both crickets (Gray 1999) and guppies (Kodric ‐ Brown and Nicoletto 2001), and the disappearance of the larger male advantage over time may be another example, if females are using male size as a criterion for choice.

Even if a larger sample in a follow up experiment revealed a significant P2 advantage, Birkhead (1998) would be quick to point out that this observation alone is not enough to distinguish a male-driven phenomenon like sperm competition from female-driven cryptic choice. To truly show sperm competition, a follow up experiment would have to show that P2 for the same two males does not vary between females; conversely variation in the success of the same males between females would imply female choice. Regardless, given the incomplete sperm precedence shown, a male has the potential to increase his fecundity by mating with more females, even if those females have mated before or mate afterward, giving males an incentive to mate as many times as possible. On top of that, females have no practical way to prevent copulation. This form of male-driven forced copulation is common across phyla (e.g. , Dukas and Jongsma 2012) but rare in butterflies, which often have courtship rituals. As mentioned above however, monarchs lack some aphrodisiac compounds and thus do not conform to the standard sexual dynamic of butterflies. But while males have motive and means to control the copulation dynamic it should be noted that this situation does not preclude a female benefit to multiple matings.

Like many insects, male monarchs transfer much more than just sperm to females. It has been shown in other organisms that the non-sperm portion of the spermatophore can be used by the female as a source of nutrients (Bowen et al. 1984). In this way a female monarch that mates twice could gain more nutrients than a female that only mates once. As discussed above, remated females did not lay more eggs per day or receive an increase in egg viability. Thus it seems unlikely that the spermatophore contents are used directly to increase daily egg number or quality. Multiply mated females did however live longer than their singly mated counterparts (Table 1, Figure 4), and thus ultimately laid more eggs. With the insight that the nutrient benefit from spermatophores is much greater for females have limited food access (Butlin et al. 1987) it follows that the honey water diet prevented females from starving, but possibly lacked some nutrients found in spermatophores. Essentially remated females may have lived longer from a healthier diet, for lack of a better word.

There is another possible benefit to receiving spermatophore contents was not tested in this experiment. It has been noted that cardiac glycoside compounds can be transferred paternally to offspring (Hunter 2012). These compounds, sequestered during the larval stages, are dependent on the host plant eaten and have demonstrated protective properties. It has long been known that cardiac glycosides make monarchs unpalatable to predators like birds (Brower and Fink 1985). Subsequent research has shown that these compounds also have anti-parasitic effects (de Roode et al. 2008). Given that there is variation in cardiac glycoside content between individual monarchs, there must be variation in the glycosides transferred in the spermatophore. Currently it is unclear what portion of the spermatophore, e.g. the sperm cells or the extra cellular nutrients, contain the these compounds, but in either case, with such strong benefits, it would be in the interest of a female monarch to remate in order to give her offspring a higher cardiac glycoside content. Even if remating gave her no additional offspring, it could give the female more protective chemicals and thus increase her fecundity by increasing the survival of those offspring in early instars before they have sequestered a significant concentration of cardiac glycosides from feeding. This line of thinking is only speculative at this point, but it is supported by the fact that other insects have been shown to paternally transfer protection to their offspring, e.g. immune priming in flour beetles (Jokela 2010). Furthermore, there is already evidence of transgenerational medication in monarchs; parasite infected females have been shown to preferentially lay eggs on the milkweed species with the higher cardiac glycoside content when given a choice (Thierry Lefe`vre and Roode 2012). If these compounds are sequestered in the sperm cells, it would not be surprising if male monarchs transferred protective compounds as a way to incentivize females to use their sperm, especially given that in other species nuptial gifts are often given more for the males' benefit, e.g. making the female less likely to remate (Wedell 1993) or more likely to mate with the male giver (Stålhandske 2001), than for the females' benefit. This hypothesized benefit could easily be tested using a similar experiment but having all females remate and instead partitioning the males into groups raised on cardiac glycoside rich and cardiac glycoside poor milkweed species and then examining mating success between groups.

From a molecular ecology perspective, this study has demonstrated the viability of existing microsatellite markers on a pedigree scale where previously they had only been used on a population level. With this increased resolution many new doors are opened. One has been described above; for another, sperm precedence patterns that hold for two males can break down for three or more males mating with one female in some species (Zeh and Zeh 1994). An investigation of this phenomenon in monarchs would be easily accomplished with our current genetic markers.

Moreover, as Lepidoptera, monarch butterflies produce both nucleated, eupyrene, and anucleated, apyrene, sperm (Friedlander 1997). This life history trait has been theorized as a strategy in sperm competition, namely, anucleated sperm are an energetically cheaper way (in terms of cost of production) to fill a female's spermatheca and ensure other males have less room for their sperm (Silberglied et al. 1984). Since sperm precedence is incomplete in monarchs, as shown above, it would normally be advantageous for a male to mate with as many females as possible over the course of his life (Oberhauser 1988). However infections, such as those with the protozoan parasite *Ophryocystis elektroscirrha* can dramatically decrease an individual's adult lifespan (de Roode et al. 2007) and may make this strategy unrealistic for diseased males. Previous studies have shown that the relative ratio of eupyrene to apyrene sperm is a plastic trait (Solensky and Oberhauser 2009a). Thus an infected male, which might not live long enough to remate, might choose to invest more eupyrene sperm in his spermatophore to better compete with other males in a single female. On the hand, chronic infection may tax an individual so much that he cannot devote as many resources to reproduction. Either way, these and other hypotheses can be tested with the tools and analyses demonstrated in this study.

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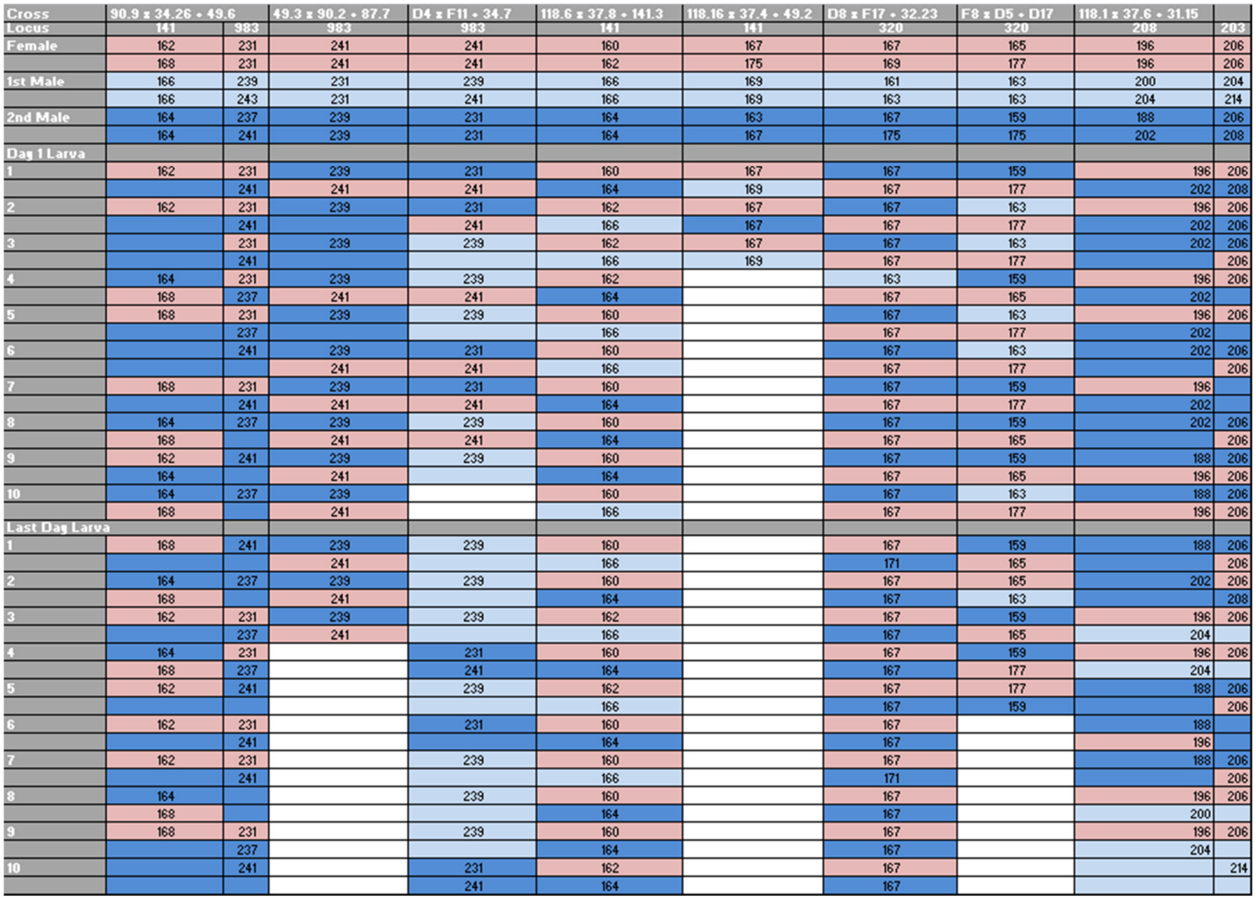

Cross	90.9 x 34.26 • 49.6		49.3 x 90.2 • 87.7	D4 x F11 • 34.7	118.6 x 37.8 • 141.3	118.16 x 37.4 • 49.2	D8 x F17 • 32.23	F8 x D5 • D17	118.1 x 37.6 • 31.15	
Locus	141	983	983	983	141	141	320	320	208	203
Female	162	231	241	241	160	167	167	165	196	206
	168	231	241	241	162	175	169	177	196	206
1st Male	166	239	231	239	166	169	161	163	200	204
	166	243	231	241	166	169	163	163	204	214
2nd Male	164	237	239	231	164	163	167	159	188	206
	164	241	239	231	164	167	175	175	202	208
Day 1 Larva										
1	162	231	239	231	160	167	167	159	196	206
		241	241	241	164	169	167	177	202	208
2	162	231	239	231	162	167	167	163	196	206
		241		241	166	167	167	177	202	206
3		231	239	239	162	167	167	163	202	206
		241			166	169	167	177		206
4	164	231	239	239	162		163	159	196	206
	168	237	241	241	164		167	165	202	
5	168	231	239	239	160		167	163	196	206
		237			166		167	177	202	
6		241	239	231	160		167	163	202	206
			241	241	166		167	177		206
7	168	231	239	231	160		167	159	196	
		241	241	241	164		167	177	202	
8	164	237	239	239	160		167	159	202	206
	168		241	241	164		167	165		206
9	162	241	239	239	160		167	159	188	206
	164		241		164		167	165	196	206
10	164	237	239		160		167	163	188	206
	168		241		166		167	177	196	206
Last Day Larva										
1	168	241	239	239	160		167	159	188	206
			241		166		171	165		206
2	164	237	239	239	160		167	165	202	206
	168		241		164		167	163		208
3	162	231	239	239	162		167	159	196	206
		237	241		166		167	165	204	
4	164	231		231	160		167	159	196	206
	168	237		241	164		167	177	204	
5	162	241		239	162		167	177	188	206
					166		167	159		206
6	162	231		231	160		167		188	
		241			164		167		196	
7	162	231		239	160		167		188	206
		241			166		171			206
8	164			239	160		167		196	206
	168				164		167		200	
9	168	231		239	160		167		196	206
		237			164		167		204	
10		241		231	162		167			214
				241	164		167			

Supplementary Table 1. Complete list of larvae analyzed for paternity. Colors correspond to origin of allele with pink representing the female, light blue the first male to mate, and dark blue the second male to mate.