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Author: Morton, Eugene S.

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IS MULTIPLE MATING BY FEMALE PROMETHEA MOTHS (*CALLOSAMIA PROMETHEA*) (DRURY)  
(LEPIDOPTERA: SATURNIIDAE) FERTILITY INSURANCE?

EUGENE S. MORTON

22318 Teepleville Flats Road, Cambridge Springs, PA 16403, USA; email: eugenemorton40@gmail.com

**ABSTRACT.** Multiple mating is apparently rare in female saturniids but male and female *Callosamia promethea* (Drury) (promethea moth) mate multiple times. Previous study showed that polyandrous females laid significantly more eggs than monandrous females, suggesting the hypothesis that yoke proteins from male ejaculates enhanced fecundity. However, multiple mating by females could arise if a single mating with a previously-mated male results in low fertility. To test this, I compared females mated once to either a virgin male or a male that had copulated the previous day. No differences were found in fecundity or fertility in females mated to virgin males vs. nonvirgin males, showing that multiple mating by female promethea moths is not related to lower fertility when mating with nonvirgin males. I discuss this finding with respect to known cases of polyandry in saturniids and suggest a hypothesis to explain the evolution of polyandry in this group.

**Additional key words:** fecundity, diurnal mating, seminal gifts, sexual conflict, polyandry

Multiple mating is common in insects and often illustrates sexual conflict (Arnqvist & Nilsson 2000, Härdling & Kaitala 2005) but is uncommon in saturniids. Thus I was surprised to discover that the mating system of the promethea moth (*Callosamia promethea*) includes both polyandry and polygyny (Morton 2009). All female *Promethea* (384) from northwestern Pennsylvania have called on multiple days following emergence since I began to study them there in 2004. Furthermore, five females that emerged from wild cocoons collected near Front Royal, Virginia, (430 km SSE of the Pennsylvania site) called multiple times. Females were observed to call daily for up to five days when allowed to do so; but I restricted the comparison to single vs. twice-mated females. These observations show polyandry is the normal mating system for *Promethea* females and probably throughout their range.

Comparing fertility and fecundity in females forced to mate monandrously with those allowed to mate twice showed that both achieved similar egg fertility but the polyandrous group laid 10% more eggs, a significant difference ( $P < 0.05$ ). This fecundity difference should be considered the minimal difference because it compares females mated once or twice, not with females mating several times, which is likely the norm. How many matings a female normally has needs study. But the fecundity difference between polyandrous and monandrous females in my study was not due to differences in body size, duration of copulations, size of eggs laid, or number of days in the laying period. This suggested the hypothesis that seminal gifts (LaMunyon 1997, Gwynne 2008), perhaps involving yolk proteins (Telfer & Rutberg 1960), boosted egg production in the polyandrous females (Morton 2009).

But this previous study did not eliminate fertility as a source of selection favoring polyandry. A virgin female

moth might mate with a male that has mated previously. Polyandry might evolve to compensate for low fertility in males that have already mated several times (Svärd & Wiklund 1986, Torres-Vila & Jennions 2005, Lauwers & Van Dyck 2006). Mating only with a depleted male could result in lower fertility and/or fecundity in monandrous compared to polyandrous females, and thus favor multiple mating in females. Here I test this “reduced fertility” hypothesis by comparing the fecundity and fertility of females forced to mate with a male that had mated the previous day to females mated with a virgin male. I then discuss the potential role of sexual conflict to influence the timing of mating in polyandrous saturniids.

## METHODS

To produce moths whose mating history was known, I raised *Promethea* larvae derived from 5 females that emerged from wild cocoons collected during the winter of 2013 in northwestern Pennsylvania (41°47'N, 79°57'W). The broods were raised separately within remay cloth sleeves (2.29m long and 1.70m in circumference) tied over branches of the same black cherry tree (*Prunus serotina*, Ehrhart 1784). The resulting cocoons were kept at ambient temperature in a screened porch (2.4m high × 2.8m wide × 6.6m long) attached to a house during the following winter.

In May, 2014, I attached the cocoons individually with duct tape and a staple to the tips of 35 cm twigs held by gravel in open topped quart jars and set the jars on a sill inside the screened porch. Females and males emerged in June–July. After emergence, females remained on their cocoons and called from 1500–1830 h EDT. Calling is a conspicuous behavior involving the protrusion of a yellow scent gland (see Fig. 1 in Tuskes et al. 1996). On days when a male(s) also emerged, I hand paired (after

Peigler 1977) him to a virgin female calling within the screened porch. After the pair separated, I placed the male in a paper bag overnight. If, fortuitously, a female emerged on the following day, I hand paired this virgin female with the male who had mated the previous day. This was accomplished after she began calling in the afternoon of her emergence day. I allowed the mating pair to copulate until separation and include only hand pairings that lasted a normal length of time for a first copulation, generally within  $271.2 \pm 10.2$  min (Morton 2009). The female was then placed in a paper bag and allowed to lay her full clutch of eggs over the following days. After she died, I cut the eggs from the paper bag, counted them, and placed the pieces of paper bag containing the eggs in a plastic container kept at ambient temperature in the screened porch. After the eggs hatched, I considered hatched eggs fertile and dissected those that had not hatched under a compound microscope for the presence of an embryo. Eggs with no development were considered infertile, following the protocol of the earlier study (Morton 2009) (see also Fig. 1 in Collins & Rawlins 2013 for electronmicrograph of this technique).

I obtained data from 9 matings between nonvirgin males and virgin females and compared these females' fecundity and fertility to that of 17 captive-raised females that had copulated once with virgin males. Data were analyzed with the JMP 13 SAS statistical package (Sall et al. 2005) using one-way ANOVA tests. Significance was set at  $P < 0.05$  and two-tailed tests were used throughout. Standard error of the mean (SE) was used as a measure of dispersion.

## RESULTS

Females mated to nonvirgin males ( $N = 9$ ) laid an average of  $247 \pm 10.94$  eggs (range = 181–309) compared to an average of  $235 \pm 7.96$  eggs (range = 190–282) for females mated to virgin males ( $N = 17$ ). There was no significant difference in number of eggs laid by the two groups of females (ANOVA, d.f. = 25,  $F = 0.8175$ ,  $P < 0.38$ ).

Comparing the percent of fertile eggs between the two mating types, females mated to nonvirgin males averaged  $243.8 \pm 10.856$  fertile eggs (99%) to  $230.7 \pm 7.899$  fertile eggs (98%) for females mated to virgin males; no significant difference (ANOVA, d.f. = 25,  $F = 0.9565$ ,  $P < 0.34$ ). For both fecundity and fertility rate, the females mated to nonvirgin males had nonsignificant, but higher, values than for females mated to virgin males. Females mated with virgin males averaged 4.12 infertile eggs per clutch whereas females mated to nonvirgin males averaged 3.22 infertile eggs per clutch, an insignificant difference statistically.

## DISCUSSION

My reduced fertility hypothesis suggested that females mated to nonvirgin males would have lower fertility. Instead, mating history of males had no effect on females in fecundity or fertility. There was no statistical difference between the two groups. In fact, females mated to nonvirgin males laid more eggs than females mated to virgin males, so the effect found was in the opposite direction of the prediction. A power test showed that a mean difference of 28 eggs (sensitivity) or a sample size of 125 would be needed to show a significant difference between the two groups in fecundity, but it would confirm the null hypothesis not the prediction. I therefore accept the null hypothesis that a female mated to a male that had copulated the previous day does not differ in fertility or fecundity from a female mated to a virgin male.

Therefore, multiple mating is not due to fertility enhancement in *Promethea*. This was found earlier in an arctiine moth (LaMunyon 1997) who found that fertility was not affected by male mating status in *Utetheisa ornatrix* (Linnaeus) but multiple mating resulted in increased fecundity, as I found for *Promethea* (Morton 2009). LaMunyon suggested that fecundity was limited by resources needed for egg construction and that male spermatophores contributed these resources. Indeed, nutrients from spermatophores are thought to be the most common benefit of multiple mating in insects (Arnqvist & Nilsson 2000). Thus the question becomes: Why don't more saturniid species, all of which are non-feeding as adults, exhibit multiple mating, given that nutrition from spermatophores is the only way they can obtain more nutrients for egg production than those gained through larval feeding?

I suggest the answer involves tradeoffs that include time and female control of mating. Time is important because copulations take several hours in *Promethea*. Multiple mating would constrain time for oviposition and dispersal if mating overlapped in time with oviposition. Given that females control mating via calling (a non-calling female is invisible to males) they are in control of the timing of mating (e.g., Allison & Cardé 2016). It is unlikely that females would opt to oviposit in the daytime because of the threat of predation by birds. Instead, I suggest saturniid species known to have multiple mating call during the day time and have nocturnal hours reserved for egg laying and dispersal (Table 1).

For example, *Callosamia* species differ in mating time, with *C. securifera* (Maaassen) and *C. promethea* mating diurnally and *C. angulifera* (Walker) mating nocturnally (Tuskes et al. 1996). D. Bayer (pers. com.)

TABLE 1. Multiple mating in saturniids may be predicted by a separation of diurnal mating and nocturnal egg laying. Multiple mating species are listed together with unstudied species showing the diurnal/nocturnal mating/egg laying association.

Species	Multiple mating?	Mating time	Egg laying time	Reference
<i>Callosamia promethea</i>	yes	diurnal	nocturnal	Morton 2009
<i>Callosamia angulifera</i>	no	nocturnal	nocturnal	Morton 2009
<i>Callosamia securifera</i>	yes	diurnal	nocturnal	D. Bayer, pers. comm., Brown 1972
<i>Saturnia pavonia</i>	yes?	diurnal	nocturnal	Tutt 1902
<i>Eupackardia calleta</i>	yes	diurnal	nocturnal	Louwagie & Peigler 2016
<i>Hyalophora cecropia</i>	no	nocturnal	nocturnal	Tuskes et al. 1996
<i>Rothschildia lebeau</i>	no	nocturnal (20h)	nocturnal	Agosta 2008
<i>Automeris phrynon</i>	no data	diurnal	nocturnal	Marquis, R. J. 1984
<i>Saturnia albofasciata</i>	no data	diurnal	nocturnal	Tuskes & Collins 1981
<i>Hemileuca burnsi</i>	no data	diurnal	nocturnal	Tuskes 1984
<i>Anisota</i> ssp.	no data	diurnal	nocturnal	Tuskes et al. 1996

captured female *C. securifera* at lights at night and obtained fertile eggs from them over that same night. He then noticed the caged females called and attracted males the next day, even though they had already been fertilized, and allowed two of these females, several weeks apart, to copulate again. I had predicted, a priori, that *C. securifera*, as well as *Promethea*, would mate multiple times because of their shared characteristic of diurnal mating, while the nocturnal *angulifera* is monandrous (Morton 2009). The day mating *Eupackardia calleta* (Westwood) also mates multiple times (Louwagie & Peigler 2016) as possibly does *Saturnia pavonia* (Linnaeus), also day mating (Tutt 1902, as quoted in Louwagie & Peigler 2016). In all these cases, the females are not dayflying, only the males, suggesting females control whether or not multiple mating occurs and they control the time of mating through their pheromone emission to obtain what is optimum for their reproductive success. I hypothesize that the optimum is to temporally separate mating and oviposition, and the finding here that fertility assurance is not an issue in these cases of polyandry in saturniids, enhances this interpretation.

Female control (Eberhard 1996) is hypothesized to lead to diurnal mating. As a result of female control, females force males to fly in the daytime but can remain nocturnal themselves to avoid predation. Males, on the other hand, are faced with diurnal predators. In response, they traded one form of communication, iconic (crypsis), for indexical (Morton 2017) by mimicking distasteful diurnal butterflies (Jeffords et al. 1979, Louwagie & Peigler 2016).

An alternative neutral hypothesis might be that calling by nonvirgin females is an incidental byproduct of

physiological processes affected by circadian rhythms. Females call again due to a non-adaptive activation of calling behavior due to these physiological processes (e.g. Riddiford & Williams 1971). This is not likely because of the proven increase in fecundity due to polyandry that provides a source of selection that favors females that call many times.

We need more information on the timing of mating and oviposition to strengthen the relation between polyandry, diurnal mating, nocturnal ovipositing, and female control. There are suggestions polyandry may be more frequent in saturniids. For example, Tuskes (1984) stated that "certain females" of the dayflying *Hemileuca maia* "mate again" after laying a first clutch of eggs. He also stated that all members of the *Hemileuca* mate once. He describes *H. burnsi* (J. H. Watson) males as flying during the day but females ovipositing at night. If my prediction that mating diurnally and egg laying/dispersing nocturnally are adaptations for multiple mating this species may be polyandrous.

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