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Direct benefits from choosing a virgin male in the European grapevine moth, *Lobesia botrana*

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In Lepidoptera, male investment in the ejaculate usually declines over consecutive matings, a depletion that could have profound consequences for female reproductive output. Since successive matings can affect the ability of males to provide phenotypic benefits, there may be strong selection for females to discriminate between males with different mating experience. The aim of our study was to determine whether monandrous females of the European grapevine moth, *Lobesia botrana*, are able to discriminate between males of different quality based on their mating experience in order to maximize direct benefits (by receiving large spermatophores from virgin males) and minimize mating costs (by avoiding low-quality nonvirgin males). Nonvirgin males produced spermatophores five times smaller than those of virgin males; consequently, mating with nonvirgin males significantly reduced female fecundity and increased their motivation to remate. In a mate preference experiment, we found that females were more likely to mate with virgin males and more frequently rejected nonvirgin mates. Moreover, nonvirgin males required more time to achieve mating than virgin males. Our results suggest that females are able to discriminate between males with different mating experience, and prefer virgin males, thereby maximizing direct benefits associated with receiving large spermatophores.

Classical sexual selection theory predicts that males should maximize their reproductive success by mating with several females, whereas females boost their fitness by being selective and choosing the highest quality mate (Andersson & Simmons, 2006; Kokko, Jennions, & Brooks, 2006). Females may choose mates based on direct material benefits obtained in the present generation or on indirect, genetic benefits for their offspring obtained in the subsequent generation. Direct fitness benefits are commonly assumed to be of predominant importance because they have immediate effects and they do not require mechanisms that maintain genetic variance (Møller & Jennions, 2001). Male contribution to these direct benefits is limited, however, as reproduction imposes nontrivial costs on males, especially in gift-giving species (Edward & Chapman, 2011). Thus, when females vary in quality, males are also expected to be choosy because they have a finite quantity of resources to invest in reproduction (Bonduriansky, 2001).

In most insect species, one of the direct benefits that might drive female preference is access to nutritive resources, such as nuptial gifts offered by males before or during mating (Lewis & South, 2012; Vahed, 1998). These nuptial gifts include food items or male accessory gland secretions, and generally have a direct positive effect on female fitness through beneficial effects on a variety of fitness-related traits, including fecundity, fertility and longevity (South & Lewis, 2011). However, in some species, the nuptial gifts apparently provide no significant nutritional fitness benefits to females (for example, in the bushcricket, *Leptophyes laticauda*, Vahed & Gilbert, 1997, or in the decorated cricket, *Gryllodes sigillatus*, Will & Sakaluk, 1994). In Lepidoptera, nuptial gifts consist of a spermatophore containing sperm and accessory gland products rich in nutrients such as proteins (Marshall, 1982). Several studies using radiolabelled substances (e.g. amino acids, zinc, phosphorus, sodium) support the prediction that females allocate male-derived nutrients from spermatophores to egg production (Boggs & Gilbert, 1979; Rooney & Lewis, 1999), and further indicate that large spermatophores generally increase female fecundity (South & Lewis, 2011). Consequently, mated females that receive small spermatophores containing fewer resources have a reduced fitness

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compared with females that receive larger spermatophores (reviewed by South & Lewis, 2011).

Spermatophore quality depends on various factors, such as male mass, age at first mating (Oberhauser, 1989; Tigreros, 2013), male larval and adult nutrition (Cahenzli & Erhardt, 2013; Delisle & Bouchard, 1995; Muller, Thiéry, Moret, & Moreau, 2015) and male mating experience (Torres-Vila & Jennions, 2005). Because ejaculate production is costly (Dewsbury, 1982), males usually have to invest in either current or future reproduction (reviewed by Simmons, 2001). In many species, spermatophores become smaller with repeated mating and their nutritional and spermatic qualities decrease with the number of matings. Indeed, some studies have shown that lepidopteran females that copulate with a recently mated male receive a smaller spermatophore with fewer resources and sperm than those mating with a virgin male (Lauwers & Van Dyck, 2006; Marcotte, Delisle, & McNeil, 2005; Torres-Vila & Jennions, 2005). Moreover, male mating experience also affects male mating behaviour; for example, mating duration increases with increasing number of matings (Konopka & McNeil, 2015).

How male spermatophore size affects female reproductive output greatly depends on the ecological context of the species, including feeding habits, timing of reproduction and mating system. On the one hand, the receipt of spermatophore-derived nutrients at mating is particularly important for females, which do not have access to resources as adults. Indeed, for capital breeding species in which resources necessary for somatic maintenance and gametic investment are limited because they are accumulated only during larval development, females need to receive larger ejaculates containing a considerable amount of nutrients to maximize their fecundity (Boggs & Freeman, 2005). Since females must rely on reserves stored as larvae to support their reproductive efforts, any additional input from large spermatophores might represent an important resource that could be used for reproduction. On the other hand, the effect of male-derived nutrients on female fitness depends on the species' mating system. In monandrous species, in which females restrict themselves to a single opportunity to mate over their life span, females would be predicted to preferentially mate with males that produce larger spermatophores in order to acquire sufficient sperm and nutritional resources to fertilize all their eggs, as shown in butterflies (Jones, 2001; Velde, Damiens, & Van Dyck, 2011). Moreover, monandrous females receiving a small spermatophore from their first mating tend to subsequently remate (Elzinga, Chevasco, Grapputo, & Mappes, 2011; Foster & Ayers, 1996; McNamara, Elgar, & Jones, 2009). Thus, monandrous females should select large spermatophores, not only to receive more nutrients, but also to reduce time and survival costs associated with remating, which decreases the time available to females for egg laying. Several studies have also reported that each reproductive event is associated with increased predation risk and increased exposure to parasites (Arnqvist & Nilsson, 2000; Harshman & Zera, 2007; Magnhagen, 1991). Since male mating experience can affect the ability of males to provide phenotypic benefits, there may be strong selection for females to discriminate between males based on mating experience and to preferentially mate with males that give large spermatophores (i.e. virgin males) in order to boost their reproductive potential.

However, evidence that females prefer virgin males in insects is equivocal. Whereas some work has shown that females mate preferentially with virgin males in some species, including the common fruit fly, *Drosophila melanogaster* (Markow, Quaid, & Kerr, 1978), the tobacco budworm *Heliothis virescens* (Klepetka & Gould, 1996), the black-lyre leafroller moth, *Cnephasia jactatana* (Jiménez-Pérez & Wang, 2004) and the cockroach *Schultesia nitor* (Monceau & van Baaren, 2012), other studies have shown that females mate preferentially with experienced males, e.g. in the stink bug, *Euschistus conspersus* (Krupke, Brunner, & Jones, 2008), the bella moth, *Utetheisa ornatrix* (Iyengar, 2009), the almond moth, *Cadra cautella* (McNamara et al., 2009) and the European corn borer, *Ostrinia nubilalis* (Milonas, Farrell, & Andow, 2011). In some of these species, females prefer to mate with experienced males based on indirect benefits related to genetically superior partners or inherited male attractiveness (Iyengar, 2009; Krupke et al., 2008). Moreover, male mating history can have positive direct effects on female fitness by reducing manipulations by males, especially in species in which male ejaculates contain manipulative substances that are harmful to females (Edvardsson, Hunt, Moore, & Moore, 2008). The lack of overall consensus could reflect the fact that mating patterns greatly depend on the species under consideration (with or without nuptial gifts), feeding habits (capital versus income breeders), mating system (monandrous versus polyandrous females) and the effect of male mating experience on female reproductive output. To our knowledge, there has never been a conclusive study that explicitly tested predictions about female preferences driven by potential direct benefits in a capital breeding monandrous species in which nuptial gifts represent a crucial advantage for female reproductive output.

The European grapevine moth, *Lobesia botrana* (Lepidoptera: Tortricidae), an important pest of grapes worldwide, is an ideal candidate for assessing female preferences based on male mating experience. This moth is a capital breeder that acquires most of the resources necessary for its adult life during its larval stages in grapes. Adult females can also obtain resources during the adult stage, not by feeding but by receiving spermatophore-derived nutrients from males during mating (Muller et al., 2015; Torres-Vila, Stockel, & Rodriguez-Molina, 1997). This species is considered monandrous, with about 70% of wild females mating only once during their life span (Torres-Vila et al., 1997). Polyandry (involving less than 30% of the population) is a heritable trait ($h^2 = 0.40 \pm 0.12$, Torres-Vila, Gragera, Rodrıguez-Molina, & Stockel, 2002) and is strongly associated with physiological factors, such as larval food nutrition (Torres-Vila, Rodríguez-Molina, & Jennions, 2004) and the size of the spermatophore received by females (Torres-Vila et al., 1997). Thus, all the ecological conditions (a capital breeding monandrous species in which there is a strong effect of the spermatophore on female fitness) needed to detect female preference related to male mating experience are present in this species.

Here, we experimentally evaluated the influence of male mating experience on male spermatophore size, female reproductive output and motivation to remate in *L. botrana*. We first investigated the existence of direct benefits to females from mating with virgin males in terms of fecundity, fertility and longevity. Second, we quantified the motivation to remate in females mated with virgin or nonvirgin males. Finally, we assessed whether *L. botrana* females preferred to mate with virgin or recently mated males, with the assumption that female should optimize mate choice to obtain male-derived nutrients. We also monitored the premating behaviour of virgin and nonvirgin males to characterize their behavioural differences that might explain female mating preference. We predicted that females would be able to discriminate between a nonvirgin and a virgin male and, given the choice, would preferentially mate with the virgin.

METHODS

Moth Rearing and General Procedures

All adults used were obtained from an inbred strain of *L. botrana* (INRA, UMR Save Bordeaux). The stock colony was maintained without diapause on a semiartificial diet (1000 ml of water, 15 g of agar, 84.63 g of maize flour, 41.25 g of wheat germ, 45.48 g of yeast, 6.03 g of ascorbic acid, 3.35 g of Wesson salts, 0.32 ml of Scala, 5 ml of ethanol (95%), 2.65 g of benzoic acid, 2.76 g of Nipagin), as described previously (Thiéry & Moreau, 2005), and maintained at 22 ± 1 °C, 60 ± 10% relative humidity, under a 16:8 h light:dark photoperiod. Males and females were placed in a large cage and bands of waxed paper (15 × 2 cm) were hung for oviposition support. Once the paper had received a sufficient number of eggs, it was placed in a plastic box containing the semiartificial larval diet. The larvae were maintained at a density of 100 individuals per 300 ml of diet. Larvae were checked daily until pupation, and then were weighed to the nearest 0.01 mg on a Precisa 262 SMA-FR microbalance and placed individually in glass tubes (70 × 9 mm diameter) stoppered with cotton plugs. Pupae were checked every morning, and newly emerging virgin adults were used for subsequent experiments under the same conditions as rearing.

Ethical Note

All experiments complied with French laws on animal experimentation. All individuals were reared under controlled laboratory conditions and fed regularly to maintain a healthy population (see above for rearing methods). Moths were treated carefully, and the abiotic conditions (temperature, humidity and photoperiod) they experienced corresponded to the natural conditions in their native habitat. Dissected females were frozen at −25 °C for 10 min in a freezer prior to decapitation.

Experiment 1: No-choice mating Trials

General procedure

At dusk, a randomly selected 2-day-old virgin female was mated with either a virgin or a nonvirgin 3-day-old male in a glass mating tube (100 × 15 mm diameter) and observed until the end of copulation. The onset time (time elapsed from the start of the session until genital coupling) and the duration of mating (time during which the pair was observed) were noted. Pairs were observed for 4 h, corresponding to the period during which females called males by releasing their pheromone (K. Muller, personal observations), and pairs that failed to mate within this period were excluded from the experiment.

Nonvirgin males were obtained by first pairing 2-day-old virgin males with 2-day-old virgin females in a mating tube 1 day before the experiment and then returning them to their glass tube after observing copulation. Females were dissected immediately after mating to check for spermatophore transfer. When copulation occurred but no spermatophore was found in the female's genital duct (in less than 5% of virgin and nonvirgin males), the male was excluded from the experiment. Virgin males were kept in their glass tubes for 3 days with no mating opportunity until they were used for the experiment.

Using this procedure, we performed two distinct experiments under the same conditions as moth maintenance (see above). The first experiment was conducted to assess mating success and the volume of the spermatophore transferred to the female during mating for virgin and nonvirgin males. The second experiment was conducted to investigate the effect of the male mating experience on the oviposition dynamics of females, their refractory period and their motivation to remate.

Experiment 1a: spermatophore volume

Immediately after the end of mating with a virgin ($N = 76$) or a nonvirgin ($N = 118$) male, females were frozen (see above) and then dissected on a glass slide. The bursa copulatrix containing the male spermatophore was removed in order to estimate its size. Estimating spermatophore size by extrapolating its volume is the method classically used in moths, including *L. botrana* (Milonas et al., 2011; Muller et al., 2015; Torres-Vila, Rodriguez-Molina, Roehrich, & Stockel, 1999). To assess spermatophore size, we measured its dimensions (length [l], width [w] and thickness [t]) under a stereomicroscope (NIKON SMZ1500) with a magnification of 20×. The volume of the spermatophore was estimated as an ellipsoid balloon [$V = \pi/6\ (l \times w \times t)$], as described previously (Torres-Vila et al., 1999).

Experiment 1b: female remating and reproductive output

After the end of copulation with virgin ($N = 34$) or nonvirgin ($N = 36$) males, males were removed and females were maintained in their mating tube until death to assess reproductive output and motivation to remate. These females were allowed to oviposit freely on the inside surface of the glass tub until their death. Every day, newly laid eggs were counted through the tube. Female survival was also checked daily; after death, the eggs were incubated for 7 days under the same conditions as used for moth maintenance. With this procedure, we recorded (1) time spent ovipositing (number of days a female oviposited), (2) daily fecundity (number of eggs laid per day), (3) achieved fecundity (total number of eggs laid during a female's life span), (4) female fertility (proportion of hatched eggs) and (5) longevity.

To assess motivation to remate, each day until their death we simultaneously monitored the behaviour of females that had previously copulated with either a virgin or nonvirgin male. In this species, a female that is ready to remate signals her readiness by releasing sex pheromone at dusk, which represents a fitness cost to her (Harari, Zahavi, & Thiéry, 2011). The female does this by assuming a calling position with wings raised and pheromone gland exposed. To obtain an index of female motivation, we recorded the occurrence of this calling behaviour every 3 min during a 1 h period at dusk (each day, females that did not call at all were excluded from the analysis); the longer the calling period, the higher the motivation to mate (Torres-Vila et al., 2002). With this procedure, we also obtained the refractory period of females, corresponding to the number of days elapsed between the mating and the first day we observed the calling behaviour. The female motivation to mate was measured by (1) the percentage of females that were motivated to remate during their remaining life span and (2) the proportion of time a female was observed in the calling position during 1 h. We also measured the number of days a female exhibited the recalling behaviour.

Experiment 2: Female Choice Trials and Male Mating Behaviours

To explore whether females preferred to mate with virgin males over recently mated males, we performed a choice triad test in which two males (one 3-day-old-virgin and one 3-day-old-nonvirgin) were simultaneously presented to one virgin female in a mating tube, as described above ($N = 44$ triad mating trials). Each triad was observed until copulation started. In this situation, males accidentally encounter each other while trying to copulate with the female, but otherwise direct interactions between males are rare. To control for the effect of male mass, males with equal pupal masses were selected and were marked similarly by removing the scales on one of their wings. Marking did not influence female choice ($\chi^2_1 = 0.41$, $P = 0.522$). Precopulatory behaviours of the three individuals were videotaped (SONY HDR CX220E) until mating occurred. We recorded three behaviours reflecting female and male sexual motivation (Muller et al., 2015). To evaluate the female motivation to mate, we assessed the percentage of time spent in the calling position, expressed as the time spent calling divided by the onset time of mating × 100. To evaluate the male motivation to mate, we recorded two different proxies: (1) the latency to the first male mating attempt, corresponding to curving their abdomen and touching the female abdomen without successful copulation, and (2) the percentage of male activity, expressed by the time spent in movement by the male divided by the onset time of mating × 100. In *L. botrana*, as in other butterfly species, female have the opportunity to reject males after an intromission attempt by kicking them or walking away (Jones, 2001; Jones & Elgar, 2004). In *L. botrana*, female rejection behaviour is clearly observable and is mostly successful (when a female walks away from a male during courtship, the male cannot mate with her), suggesting that mating is to a large degree under female control. Preliminary tests suggested that *L. botrana* females rejected their mate several times before copulation (K. Muller, personal observations); thus, we scored these female rejection behaviours against virgin and nonvirgin males.

Statistical Analysis

All statistical tests were performed using R Software version 3.2.0 (R Core Team, 2015). Each model includes all the main effects, deleting nonsignificant interactions. For each analysis, we first tested the full model with the main effects and their interactions and then we deleted the interactions when they were not significant. However, all main effects were always included in the model, following the recommendations of Forstmeier and Schielzeth (2011). To assess the mating success of males (percentage of successful mating) according to male mating experience (virgin versus nonvirgin) in no-choice mating trials, we used Pearson chi-square tests. The effects of mating experience on spermatophore volume and mating duration were analysed with ANCOVAs, with the pupal masses of males and females as covariates.

We used generalized linear modelling to estimate the sources of variation in fecundity. Because lifetime fecundity was best approximated by an overdispersed Poisson distribution, we fitted the model with a negative binomial error structure (Bolker et al., 2009). We also improved the model by specifying that the data were zero inflated. This analysis was performed using the glmmADMB library (Skaug, Nielsen, Magnusson, & Bolker, 2013), including time, male mating experience and their interactions as fixed effects, male and female mass as covariates, and female identity as a random factor. A generalized linear model (GLM) using a quasibinomial error structure and a logit link function was used to analyse the proportion of eggs hatched by females mated with virgin and nonvirgin males. Finally, a Cox regression analysis was applied to assess the influence of male mating experience, female pupal mass and fecundity on female longevity.

Because female percentage calling could not be normalized, a nonparametric factorial repeated measures analysis of longitudinal data was performed using the package nparLD in R (Noguchi et al., 2012) to determine how time, male mating experience and their interaction affected the intensity of female motivation to remate. Repeated measures were analysed using the F1-LD-F1 model of the nparLD function to calculate an ANOVA-type statistic. Owing to heteroscedasticity, female motivation to remate (percentage of time spent in the calling position) and male activity (percentage of time spent in movement) were arcsine square root-transformed prior to analysis. The effects of male mating experience on male and female precopulatory behaviours were analysed with ANCOVAs, with the pupal masses of males and females as covariates, respectively. Because data on male mating attempts were counted and were overdispersed, a GLM with a negative binomial distribution (NBGLM) was used to assess the effect of male mating experience on mating attempts.

RESULTS

Experiment 1a: Spermatophore Volume

In no-choice mating trials, no effect of male mating experience (virgin versus nonvirgin) was detected on mating success, with approximately 85% of males successfully copulating ($\chi^2_1 = 0.001$, $P = 0.973$; Table 1). The onset time of mating did not vary with male mating experience ($W = 0.3$, $P = 0.586$; Table 1) or male mass ($W = 1.4$, $P = 0.237$). Mating duration was affected by male mating experience: copulation with a nonvirgin male lasted 33% longer than that with a virgin male ($F_{1,192} = 128.1$, $P < 0.0001$, corrected for male mass; Table 1). Spermatophores transferred by virgin males were on average more than five times larger than those transferred by nonvirgin males (Table 1). In addition, heavier males produced larger spermatophores when they were virgin, but male mass did not affect spermatophore volume of nonvirgin males (main model: $F_{3,190} = 338.4$, $P < 0.0001$; interaction male mating experience $*$ male mass: $P = 0.010$).

Experiment 1b: Female Remating and Reproductive Output

Females that mated with virgin males had higher achieved fecundity (corresponding to the total number of eggs laid during a female's life span) than those mated with males that had previously mated ($F_{1,67} = 10.03$, $P = 0.002$, corrected for male and female mass; Table 1). As shown in Fig. 1, daily fecundity, corresponding to the number of eggs laid by females per day, greatly depended on time elapsed since mating ($F_{14} = 191.56$, $P < 0.0001$), male mating experience ($F_1 = 8.78$, $P = 0.003$) and their interaction ($F_{14} = 49.94$, $P < 0.0001$), and was positively correlated with female mass ($F_1 = 11.52$, $P = 0.001$). The number of eggs laid was the highest 2 days after mating and decreased daily until the death of the female, regardless of the experience of males (Fig. 1). However, females mated with virgin males laid more eggs at the end of their life than females mated with nonvirgin males (Figs. 1 and 2). This increase in eggs laid by females mated with virgin males at the end of their life was confirmed by dividing oviposition period into two sequences: eggs laid in the first half of the oviposition period versus eggs laid in the second half of the oviposition period for a given female (Fig. 2). Females mated with virgin males laid more eggs than females mated with nonvirgin males in the second half of the oviposition sequence, whereas there was no difference during the first half of the oviposition sequence (first sequence: $F_{1,67} = 2.0$, $P = 0.163$; second sequence: $F_{1,67} = 23.7$, $P < 0.0001$, corrected for male and female mass; Fig. 2). In addition, females mated with virgin males spent more time ovipositing ($F_{1,67} = 14.41$, $P = 0.0003$, corrected for male and female mass; Table 1). Male mating experience did not affect female fertility (corresponding to the proportion of hatched eggs; $F_{1,67} = 9.67$, $P = 0.447$, corrected for male and female mass) or female longevity (Cox regression: $W = 2.46$, $P = 0.29$; Table 1). However, we found a trade-off between fecundity and longevity: females with higher fecundity had diminished longevity regardless of the experience of their mate (Cox regression: $W = 8.91$, $P = 0.012$).

A higher percentage of females were motivated to remate during their lifetime after mating with a nonvirgin male ($N = 32/36$, 88.9%) than those mated with a virgin male ($N = 22/34$, 64.7%; $\chi^2_1 = 4.51$, $P = 0.034$). The mean refractory period was longer in females mated with virgin males (4.0 ± 0.3 days) than for those mated with nonvirgin males (2.0 ± 0.3 days; Cox regression: $W = 12.42$, $P = 0.0004$). Females mated with nonvirgin males called for a longer period of time (5.7 ± 0.6 days) than females mated with virgin males (3.5 ± 0.4 days; $F_{1,52} = 7.54$, $P = 0.008$, corrected for female mass). In addition, females mated with

Table 1

Summary results from no-choice trials showing mating success, onset time, duration of mating and spermatophore volume (experiment 1) and reproductive traits and longevity of *L. botrana* females (experiment 2) according to the mating experience of males (virgin versus nonvirgin)

Male mating experience	Experiment 1				Experiment 2			
	Mating success[a] (%)	Onset time of mating[b] (min)	Duration of mating[c] (min)	Spermatophore volume ($\times 10^{-6}$ mm^3)[c]	Fecundity (eggs/female)[d]	Time spent ovipositing (days)[c]	Fertility (% of eggs hatched)[e]	Longevity (days)[b]
Virgin	84.89	4.6±0.3	62.8±1.3 (a)	133.3±2.8 (a)	103.2±7.9 (a)	7.1±0.4 (a)	85.8±2.6	11.5±0.4
Nonvirgin	87.36	5.0±0.6	83.7±1.2 (b)	26.7±1.1 (b)	77.9±7.7 (b)	5.3±0.3 (b)	87.9±2.9	11.7±0.5

Values are expressed as a percentage (mating success) or as means ± SEM (all other parameters). Values in each column denoted by different lowercase letters in parentheses are significantly different ($P < 0.05$).

[a] Pearson chi-square.

[b] Cox regression.

[c] ANCOVA.

[d] GLMMADMB.

[e] GLM with quasibinomial error.

nonvirgin males were more motivated to remate than females mated with virgin males and their motivation increased over time (time effect: $F_{4,92} = 3.29$, $P = 0.006$; male mating experience effect: $F_1 = 48.4$, $P < 0.0001$; Fig. 3).

Experiment 2: Female Choice Trials and Male Mating Behaviours

When females could choose between two males, they were more likely to mate with a virgin than a nonvirgin male (68.18% of virgin versus 31.82% of nonvirgin males mated with females; $\chi^2_1 = 10.23$, $P = 0.001$; Table 2). Nonvirgin males required more time to successfully mate than virgin males (Cox regression: $W = 5.31$, $P = 0.021$; Table 2). As in no-choice tests, mating duration was affected by male mating experience, being 50% longer for nonvirgin than for virgin males ($F_{1,42} = 14.43$, $P = 0.0004$, corrected for male mass; Table 2). Female motivation to mate was comparable among all females regardless of the mating status of the male with which they mated ($F_{1,42} = 0.75$, $P = 0.392$; Table 2). In addition, male mating experience did not affect the latency to the first mating attempt ($W = 1.86$, $P = 0.394$, corrected for male mass) or the percentage of time spent in activity ($F_{1,86} = 0.98$, $P = 0.326$, corrected for male mass; Table 2). Females did not mate preferentially with a more active male, whether virgin ($\chi^2_1 = 0.83$, $P = 0.361$) or nonvirgin ($\chi^2_1 = 0.07$, $P = 0.789$). Nonvirgin males attempted copulation significantly more frequently than virgin males, suggesting that females were more likely to reject a nonvirgin male (number of female rejections: $F_{1,86} = 8.45$, $P = 0.003$, corrected for male mass; Table 2).

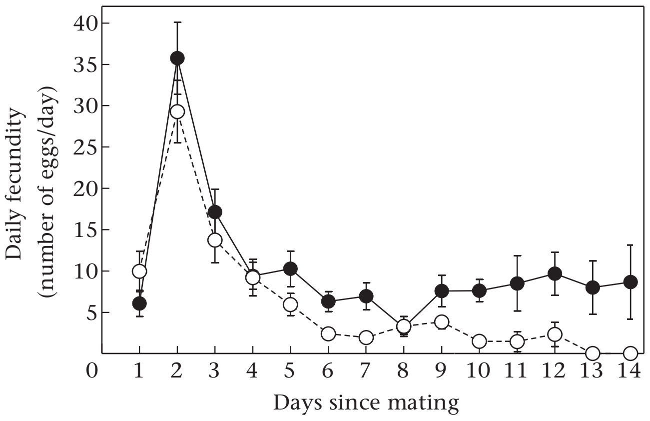

Figure 1. Number of eggs laid daily by females mated with virgin males (black circles and line) or nonvirgin males (white circles and dashed line). Results are presented as means ± SEM. Day 0 corresponds to the day of copulation.

DISCUSSION

The aim of our study was to determine whether monandrous *L. botrana* females are able to discriminate between males of different quality based on their mating experience so as to maximize direct benefits (by receiving large spermatophores from virgin males) and minimize mating costs (by avoiding low-quality nonvirgin males). Our results revealed that, in a no-choice test, females mated equally with a virgin or a nonvirgin male, despite a fitness cost in terms of reduced fecundity when mating with a nonvirgin male. However, when virgin and nonvirgin males were presented simultaneously, the virgin male was accepted twice as often by the female. This difference in mating success has apparently arisen as a result of female preference for virgin males, as there were no observable behavioural differences between virgin and nonvirgin males in their motivation to mate (latency to first mating attempt and general activity levels). Moreover, females more frequently rejected nonvirgin over virgin males, strengthening the existence of female preference for virgin males. Females mated with virgin males were less motivated to remate, suggesting that only one mating with a virgin male is sufficient to boost their reproductive output. These results suggest that females derive immediate fitness benefits through increased fecundity from their choice.

As in several other studies, we found that spermatophore size decreased and copulation duration increased if the male had

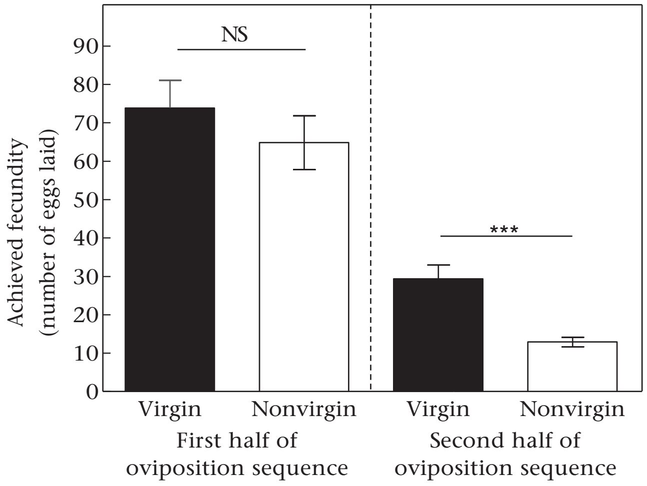

Figure 2. Number of eggs laid by females mated with virgin (black bars) or nonvirgin (white bars) males in the first and the second half of their oviposition period (oviposition period was separated into two sequences by the median for a given female). Results are presented as means ± SEM. ***$P < 0.0001$.

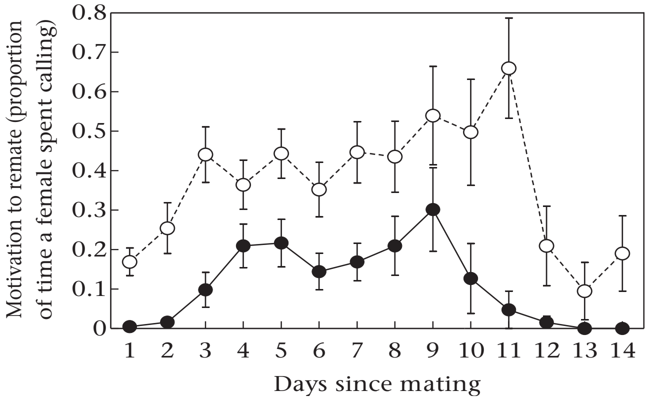

Figure 3. Daily motivation to remate in females mated with virgin males (black circles and line) or nonvirgin males (white circles and dashed line), expressed as the proportion of time observed in the calling position during 1 h. Results are presented as means ± SEM, excluding females that did not call at all.

recently mated (Konopka & McNeil, 2015; Torres-Vila & Jennions, 2005). Mating with a nonvirgin male increased mating costs by increasing the time in copula (33% longer with nonvirgin males). This prolonged copulation could be a nonvirgin male strategy to reduce sperm competition with future ejaculates if the male himself acts as a mating plug that prevents the female from remating before oviposition (‘in copula’ guarding hypothesis, reviewed by Alcock, 1994). Indeed, this postcopulatory strategy could be particularly effective because, in *L. botrana*, the mating period occurs each day at dusk during a few hours and we observed that females receiving small spermatophores tended to call sooner after mating, sometimes only 1 h after copulation (K. Muller, personal observations). Remaining in copula for more than 1 h may be sufficient to prevent female remating on the same day, ensuring sperm transfer from the nonvirgin male. Indeed, in several butterfly and moth species, sperm generally reaches the spermatheca between 2 and 5 h after mating (Marcotte, Delisle, & McNeil, 2003; Seth, Kaur, Rao, & Reynolds, 2002). Spermatophore size was positively correlated with pupal mass for virgin males, but not for nonvirgin males, as observed in several other lepidopteran species (Watanabe, Wiklund, & Bon'no, 1998; Wedell & Cook, 1999). In our study, spermatophores delivered by nonvirgin males were about five times smaller than those transferred by virgin males, and probably contained low amounts of accessory gland secretions, as observed in a wide range of species (Blanco, Rojas, Groot, Morales-Ramos, & Abel, 2009; Marcotte et al., 2005; Wedell & Cook, 1999). In capital breeders, the production of spermatophores is limited by reserves stored as larvae. Therefore, the first mating of a capital breeder male is certainly the most quantitatively important because a large proportion of his stored reserves is directly used in the production of his first spermatophores. Further work is needed to determine how larval nutrition affects energy reserves of adult males and how these stored reserves are reallocated into reproductive traits of males (reproductive capacity, spermatophore quality).

Females mated to nonvirgin males suffered a 25% reduction in lifetime fecundity, a finding in agreement with reports on the majority of Lepidoptera species (reviewed by Torres-Vila & Jennions, 2005). At the beginning of the oviposition period, females mated with nonvirgin males laid as many eggs as females mated with virgin males. These eggs probably correspond to the initial egg load of the female (i.e. the number of mature eggs a female has available to lay at emergence). However, in the second half of the oviposition period, females that had mated with nonvirgin males almost stopped laying compared with females that had received a large spermatophore from virgin males. Thus, females mated with virgin males produced more eggs at the end of their oviposition period, corresponding to the time the female needs to incorporate male-derived nutrients into her eggs; these laid eggs probably correspond to the male contribution. Several studies have demonstrated that the time required to incorporate spermatophore-derived nutrients into maturing oocytes is highly variable and depends on the species and its reproductive effort throughout life, for example occurring within 2 days after mating for a lampyrid species (*Photinus ignitus*, Rooney & Lewis, 1999) and not before 15–20 days for a lepidopteran species (*Heliconius charitonia*, Boggs, 1990). Despite these benefits in fecundity, females mated with nonvirgin males that had received a small spermatophore did not exhibit reduced fertility (85.8% of hatched eggs versus 87.9% for females mated with virgin males), suggesting that a large spermatophore is not a condition for functional sperm transfer. This absence of effect on female fertility is certainly attributable to the fact that the male usually transfers considerably more sperm than is necessary to fertilize all female eggs, even if he has recently mated (Bezzerides, Iyengar, & Eisner, 2008; Curril & LaMunyon, 2006). A further experiment should be performed to evaluate the number of fertilizing eupyrene sperm contained in spermatophores transferred by virgin and nonvirgin males. Females that mate with previously mated males may not receive enough male-derived nutrients to boost their reproductive potential and should compensate by being more likely to remate or by remating sooner than females mated to virgin males (Foster & Ayers, 1996; Marcotte, Delisle, & McNeil, 2007). We found that, after mating with a nonvirgin male, 88.9% of females called at dusk and were motivated to remate, whereas only 64.7% of those mated with virgin males did. Moreover, females mated with nonvirgin males called males sooner (2 days) than females mated with nonvirgin males (4 days), a finding consistent with the literature on Lepidoptera species (Marcotte et al., 2005; Torres-Vila et al., 1997;

Table 2

Summary results from choice trials ($N = 44$) of *L. botrana* females successfully mated with virgin ($N = 30$) or nonvirgin ($N = 14$) males showing mating parameters and precopulatory behaviours

Male mating experience	Mating parameters			Precopulatory behaviours of males and females			
	Number of successful matings[a]	Onset time of mating[b] (s)	Duration of mating[c] (min)	Percentage of time spent in calling position[c]	Latency to the first mating attempt (s)[b]	Percentage of male activity[c]	Number of female rejections[d]
Virgin	30/44 (a)	182.6±22.6 (a)	60.9±2.1 (a)	27.2±3.6	72.4±17.1	52.0±4.2	4.4±0.5 (a)
Nonvirgin	14/44 (b)	309.0±57.1 (b)	91.9±9.7 (b)	32.9±5.3	90.6±17.1	47.5±3.6	7.5±0.9 (b)

Values are expressed as a ratio (successful matings) or as means ± SEM (all other parameters). Values in each column denoted by different lowercase letters in parentheses are significantly different ($P < 0.05$).

[a] Pearson chi-square.

[b] Cox regression.

[c] ANCOVA.

[d] NBGLM.

Wedell & Cook, 1999). Moreover, the length of the refractory period in moths can be affected by several factors, including spermatophore size (McNamara et al., 2009) and the quality and quantity of viable sperm and secretions of the male accessory gland transferred to the female during mating (Simmons, 2001; Wedell, 2005). In our experiment, it is possible that larger spermatophores produced by virgin males activated stretch receptors in the female reproductive tract (Sugawara, 1979), inducing a long refractory period. In addition to this proximate mechanism for postmating nonreceptivity, the presence of sperm in the spermatheca may be required to elicit pheromonostasis in *L. botrana*, similar to other Lepidoptera species in which females only become unreceptive if their spermatheca is full of sperm (Giebultowicz, Raina, Uebel, & Ridgway, 1991; Karube & Kobayashi, 1999). However, female receptivity in butterflies is not only affected by the presence of sperm; some males transfer antiaphrodisiacs at mating that temporarily reduce female attractiveness (Andersson, Borg-Karlson, & Wiklund, 2003). Further investigations on the composition of the male spermatophore (sperm and antiaphrodisiac substances) in *L. botrana* are needed to better understand the effect of male mating experience on female motivation to remate.

Because of the beneficial effect of mating with a virgin male, one might expect that virgin females would avoid mating with nonvirgin males and would prefer virgin males so as to acquire more direct benefits (Wedell, Gage, & Parker, 2002). Consistent with these predictions, our study revealed that virgin males were twice as likely to mate with females than nonvirgin males in choice trials. Some studies have shown that the level of activity of males may play a role in their probability of mating (Kotiaho, 2002; Scharf, Peter, & Martin, 2013), and nonvirgin males could be physically unable to produce the same level of courtship as sexually vigorous virgin males (Janowitz & Fischer, 2010). However, in our study, both virgin and nonvirgin males were equally active during courtship, and virgin males did not attempt to copulate earlier with females than nonvirgin males, suggesting that virgin males have the same motivation to mate as nonvirgin males. Thus, we did not find that males changed their mating behaviour as they gained experience, unlike others have reported (Kaitala & Wiklund, 1994). These behavioural observations allow us to rule out the possibility that virgin males are superior competitors because female preference for virgin males are not likely to be driven by differences in male courtship. However, we observed female behaviour consistent with discrimination against nonvirgin males. The greater reproductive success of virgin males certainly reflects an active female preference for these males, because females more frequently actively rejected nonvirgin males (7.5 times) than virgin males (4.4 times) before accepting mating, and nonvirgin males required more than twice as much time to successfully mate with females than virgin males. These results strongly suggest that *L. botrana* females can evaluate male mating experience using different visual and/or olfactory cues (Costanzo & Monteiro, 2007; Harris & Moore, 2005). Although our study was not designed to establish the proximal causes of female discrimination, our results allow us to formulate some hypotheses. Among these cues, chemical cues are important in mate choice in a variety of species and may be used by one or both sexes to acquire information about potential mates (Carazo, Sanchez, Font, & Desfilis, 2004). In insect species, pheromone or cuticular hydrocarbon substances produced by females and acquired by males during copulation may advertise their previous mating experience (Harris & Moore, 2005; Scott, Richmond, & Carlson, 1988). It is possible that nonvirgin males are imbued with female substances from their first mating. Females may use chemical cues left behind during previous male–female interactions to gain information about the mating experiences of males. Such 'perfumed' nonvirgin males could be recognized and avoided by virgin females in order to minimize the mating costs associated with sperm-depleted males.

In conclusion, the European grapevine moth provides evidence that, in a usually monandrous capital breeder species (see Torres-Vila, 2014 for more details), male mating experience greatly influences female fitness, and that female mate choice may evolve to maximize benefits by preferential mating with virgin males. These results were expected since all the required conditions for the evolution of these female preferences for virgin males have been fulfilled in this capital breeding monandrous species. In such a system, males should also discriminate between females of different quality, such as body mass or mating experience (Bonduriansky, 2001; Friberg, 2006), because nutritious ejaculates are costly to produce and should be invested prudently. In studies that did not confirm these findings or even demonstrated the reverse (Iyengar, 2009; Krupke et al., 2008; McNamara et al., 2009; Milonas et al., 2011), it is likely that the effect of male mating experience on female reproductive output on the species studied may not be as high as that for *L. botrana*. Thus, some caution should be exercised in exploring the reproductive strategies of a species, because its mating patterns greatly depend on the combined effects of its mating system, its feeding habits and the importance of male-donated nuptial gifts.

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