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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, strong selection for females to discriminate among males based on the male mating experience may exist. The aim of our study was to determine if monandrous females of the European grapevine moth (*Lobesia botrana*) are able to discriminate among males of different qualities based on their mating experience in order to maximise direct benefits (by receiving large spermatophores from virgin males) and minimise mating costs (by avoiding low-quality non-virgin males). Non-virgin males produced spermatophores five times smaller than those of virgin males; consequently, mating with non-virgin males significantly reduced female fecundity (by ~25%) 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 non-virgin mates. Moreover, non-virgin males required more time to achieve mating than virgin males. Our results suggest that females are able to discriminate among males based on male mating experience, and demonstrate female preference for virgin males, thereby maximising direct benefits associated with receiving large spermatophores.

KEY WORDS: direct benefits, female mate choice, *Lobesia botrana*, male mating experience, monandry, spermatophore.

Classical sexual selection theory predicts that males should maximise 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 et al., 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 however limited, as reproduction imposed non-trivial costs to 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 significantly 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

68 further indicate that large spermatophores generally enhance female fecundity (South &
69 Lewis, 2011). Consequently, mated females that receive small spermatophores containing
70 fewer resources have a reduced fitness compared with females that receive larger
71 spermatophores (reviewed by South & Lewis, 2011).

72 Spermatophore quality depends on various factors, such as male mass, age at first mating
73 (Oberhauser, 1989; Tigreros, 2013), male larval and adult nutrition (Cahenzli & Erhardt,
74 2013; Delisle & Bouchard, 1995; Muller et al., 2015), and male mating experience (Torres-
75 Vila & Jennions, 2005). Because ejaculate production is costly (Dewsbury, 1982), males
76 usually have to invest in either current or future reproduction (reviewed by Simmons, 2001).
77 In many species, spermatophores become smaller with repeated mating and their nutritional
78 and spermatic qualities classically decrease with the number of matings. Indeed, some studies
79 have shown that lepidopteran females that copulate with a recently mated male receive a
80 smaller spermatophore with fewer resources and sperm compared to those mating with a
81 virgin male (Lauwers & Van Dyck, 2006; Marcotte et al., 2005; Torres-Vila & Jennions,
82 2005). Moreover, male mating experience also affects male mating behavior, for example,
83 mating duration increases with the increasing number of matings (Konopka & McNeil, 2015).

84 How male spermatophore size affects female reproductive output greatly depends on the
85 ecological context of the considered species, including feeding habits, timing of reproduction,
86 and mating system. On the one hand, the receipt of spermatophore-derived nutrients at mating
87 is particularly important for females, which do not have access to resources as adults. Indeed,
88 for capital breeding species in which resources necessary for somatic maintenance and
89 gametic investment are limited because they are accumulated only during larval development,
90 females need to receive larger ejaculates containing a considerable amount of nutrients to
91 maximise their fecundity (Boggs & Freeman, 2005). Since females must rely on reserves
92 stored as larvae to support their reproductive efforts, any additional input from large

93 spermatophores might represent an important resource that could be used for reproduction. On
94 the other hand, the effect of male-derived nutrients on female fitness depends on the species
95 mating system. In monandrous species, in which females restrict themselves to a single
96 opportunity to mate over their lifespan, females would be predicted to preferentially mate
97 with males that produce larger spermatophores in order to acquire sufficient sperm and
98 nutritional resources to fertilize all their eggs, as shown in butterflies (Jones, 2001; Velde et
99 al., 2011). Moreover, monandrous females receiving a small spermatophore from their first
100 mating tend to subsequently remate (Elzinga et al., 2011; Foster & Ayers, 1996; McNamara et
101 al., 2009). Thus, monandrous females should select large spermatophores, not only to receive
102 more nutrients, but also to reduce time and survival costs associated with remating, which
103 decreases the time available to females for egg-laying. Several studies have also reported that
104 each reproductive event is associated with increased predation risk and increased exposure to
105 parasites (Arnqvist & Nilsson, 2000; Harshman & Zera, 2007; Magnhagen, 1991). Since male
106 mating experience can affect the ability of males to provide phenotypic benefits, there may be
107 strong selection for females to discriminate among males based on mating experience and to
108 preferentially mate with males that give large spermatophores (i.e., virgin males) in order to
109 boost their reproductive potential.

110 However, evidence that females prefer virgin males in insects is equivocal. Whereas some
111 work has shown that females mate preferentially with virgin males in some species, including
112 the common fruit fly, *Drosophila melanogaster* (Markow et al., 1978), the tobacco budworm
113 *Heliothis virescens* (Klepetka & Gould, 1996), the black-lyre leafroller moth, *Cnephasia*
114 *jactatana* (Jimenez-Perez & Wang, 2004) and the cockroach *Schultesia nitor* (Monceau & van
115 Baaren, 2012), other studies have shown that females mate preferentially with experienced
116 males, e.g., in the stink bug, *Euschistus conspersus* (Krupke et al., 2008), the bella moth,
117 *Utetheisa ornatrix* (Iyengar, 2009), the almond moth, *Cadra cautella* (McNamara et al., 2009)

and the European corn borer, *Ostrinia nubilalis* (Milonas et al., 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 through the reduction of male manipulations, especially in species in in which male ejaculates contain manipulative substances that are harmful to females (Edvardsson et al., 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 vs. income breeders) mating system (monandrous vs. 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) (Denis and Schiffermüller), a very 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 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 et al., 1997). This species is considered monandrous, with about 70% of wild females mating only once during their lifespan (Torres-Vila et al., 1997). The remnant polyandry (involving less than 30% of the population) is an heritable trait ($h^2 = 0.40 \pm 0.12$, Torres-Vila et al., 2002) and is strongly associated with physiological factors, such as larval food nutrition (Torres-Vila et al., 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 spermatophore on female fitness) are present in this species to detect female preference related to male mating experience.

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. Secondly, we quantified the motivation to remate in females mated with virgin or non-virgin males. Finally, we assessed if whether *L. botrana* females preferred to mate with virgin males rather than with recently mated males, with the assumption that female should optimise mate choice to obtain male-derived nutrients. We also monitored the premating behaviour of virgin and non-virgin males to characterise behavioural differences of virgin and non-virgin males that might explain female mating preference. We predicted that female would be able to discriminate between a non-virgin and a virgin male and, given the choice, would preferentially mate with the virgin.

MATERIAL & 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 semi-artificial diet (1000 mL water, 15 g agar, 84.63 g maize flour, 41.25 g wheat germ, 45.48 g yeast, 6.03 g ascorbic acid, 3.35 g Wesson salts, 0.32 mL Scala, 5 mL ethanol [95%], 2.65 g benzoic acid, 2.76 g Nipagin), as described previously (Thiéry & Moreau, 2005), and maintained at 22 ± 1°C, 60 ± 10% relative humidity, under a 16:8 hour 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 semi-artificial 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 below 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. Females submitted to dissection were chilled 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 non-virgin 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. Couples were observed for 4 hours, corresponding to the period during which females called males by releasing their pheromone (Muller, personal observations), and couples that failed to mate within this period were excluded from the experiment.

Non-virgin males were obtained by first pairing 2-day-old virgin males with 2-day-old virgin females in a mating tube one 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% for virgin or non-virgin males), the male was excluded from the experiment. Virgin males were kept in their glass tubes during 3 days with no mating opportunity until they were used for 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 non-virgin males. The second experiment was conducted to investigate the effect of the male mating experience on the oviposition dynamics of females and their refractory period and motivation to remate.

Experiment 1a: Effect of mating experience on spermatophore volume

Immediately after the end of mating with a virgin (n = 76) or a non-virgin (n = 118) male, females were frozen at -25 °C for ten minutes 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 et al., 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 20X. 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: Effect of mating experience on both female oviposition and recalling dynamics and on female reproductive output

After the end of copulation with virgin (n = 34) or non-virgin (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 daily 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 (i) time spent ovipositing (number of days a female oviposited), (ii) daily fecundity (number of eggs laid per day), (iii) achieved fecundity (total number of eggs laid during a female's lifespan), (iv) female fertility (proportion of hatched eggs), and (v) 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 non-virgin male. In this species, a female that is ready to remate signals her readiness by releasing sex pheromone at dusk, an action that represents a fitness cost to her (Harari et al., 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 minutes during a 1-hour period at dusk (each day, females that did not recall at all were excluded from the analysis) – the longer the recalling 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 recalling behaviour. The female motivation to mate was measured by (i) the percentage of females that were motivated to remate during their remaining lifespan and (ii)

238 the proportion of time a female was observed in calling position during 1 hour. We also
239 measured the number of days a female exhibited the recalling behaviour.
240
241 *Experiment 2: Female choice trials and precopulatory mating behaviours of virgin and non-*
242 *virgin males*

243 To explore whether females preferred to mate with virgin males over recently mated males,
244 we performed a choice triad test in which two males (one 3-day-old-virgin and one 3-day-old-
245 non-virgin) were simultaneously presented to one virgin female in a mating tube, as described
246 above (n = 44 triad mating trials). Each triad was observed until copulation started. When
247 presenting simultaneous, males directly try to copulate with the female, and accidentally
248 encounter each other, meaning that there is virtually no direct interactions between males. To
249 control for the effect of male mass, males with equal pupal masses were selected and were
250 marked similarly by removing the scales on one of their wings. Marking did not influence
251 female choice ($\chi^2_1 = 0.41$, $p = 0.522$). Precopulatory behaviours of the three individuals were
252 videotaped (SONY HDR CX220E) until mating occurred. We recorded three behaviours
253 reflecting female and male sexual motivations (Muller et al., 2015). To evaluate the female
254 motivation to mate, we assessed the percentage of time in calling position, expressed as the
255 time spent calling divided by the onset time of mating × 100. To evaluate the male motivation
256 to mate, we recorded two different proxies: (i) the latency to the first male mating attempt,
257 corresponding to curving their abdomen and touching the female abdomen without successful
258 copulation, and (ii) the percentage of male activity, expressed by the time spent in movement
259 by the male divided by the onset time of mating × 100. In *L. botrana* species like in other
260 butterflies species, female have the opportunity to reject males after an intromission attempt
261 by kicking them or walking away (Jones, 2001; Jones & Elgar, 2004). *L. botrana* female
262 rejection behaviour is clearly observable and is mostly successful (when a female walks away

from a male during courtship, the male could not 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 (Muller, unpublished results); thus, we scored these female rejection behaviours against virgin and non-virgin 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 non-significant 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 & Schielzeth (2011). To assess the mating success of males (percentage of successful mating) according to male mating experience (virgin vs. non-virgin) in no-choice mating trials, we used Pearson's χ^2 tests. The effects of mating experience on spermatophore volume and mating duration were analysed with ANCOVAs, with the pupal mass of males and females as covariates.
We used generalised linear modeling to estimate the sources of variation in fecundity. Because lifetime fecundity was best approximated by an over-dispersed 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 et al., 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 generalised linear model (GLM) using a quasi-binomial error structure and a logit link function was used to analyse the proportion of eggs hatched by females mated with virgin and non-virgin 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 percent calling could not be normalised, 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. Due to heteroscedasticity, female motivation to remate (percent of time spent in recalling position) and male activity (percent of time spent in movement) were arcsin square root-transformed prior to analysis. The effect of male mating experience on male and female precopulatory behaviours were analysed with ANCOVAs, with the pupal mass of males and females as covariates, respectively. Because data on male mating attempts were counted and were over-dispersed, a GLM with a negative binomial distribution (NBGLM) was used to assess the effect of male mating experience on mating attempts.

RESULTS

Experiment 1a: No choice trials – Effect of mating experience on spermatophore volume

In no-choice mating trials, no effect of male mating experience (virgin vs. non-virgin) 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 non-virgin 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 non-virgin males (Table 1). In addition, heavier males produced larger

spermatophores when they were virgin, but male mass did not affect spermatophore volume of non-virgin males (main model: $F_{3,190} = 338.4$, $P < 0.0001$; interaction male mating experience × male mass: $P = 0.010$).

Experiment 1b: No choice trials – Effect of mating experience on female oviposition dynamics, on female reproductive output and on female recalling dynamics

Females that mated with virgin males had higher achieved fecundity (corresponding to the total number of eggs laid during a female's lifespan) 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 Figure 1, daily fecundity, corresponding to the number of eggs laid by females per day, greatly depended on time elapsed since mating ($F = 191.56$, $df = 14$, $P < 0.0001$), male mating experience ($F = 8.78$, $df = 1$, $P = 0.003$) and their interaction ($F = 49.94$, $df = 14$, $P < 0.0001$), and was positively correlated with female mass ($F = 11.52$, $df = 1$, $p = 0.0007$). 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 non-virgin 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 non-virgin 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 affected

neither female fertility (corresponding to the proportion of hatched eggs) ($F_{1,67}$ = 9.67, P = 0.447, corrected for male and female mass) nor female longevity (Cox regression, W = 2.46, P = 0.29, Table 2). 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 proportion of females were motivated to remate during their lifetime after mating with a non-virgin male (n = 32/36, 88.9%) compared to females mated with a virgin male (n = 22/34, 64.7%) (χ^2_1 = 4.51, P = 0.034). The mean length of the refractory period was longer in females mated with virgin males (4.0 ± 0.3 days) than for those mated with non-virgin males (2.0 ± 0.3 days) (Cox regression: W = 12.42, P = 0.0004). Females mated with non-virgin males resumed calling longer (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 non-virgin males were more motivated to remate than females mated with virgin males and their motivation increased over time (time effect: F = 3.29, df = 4.92, P = 0.006; male mating experience effect: F = 48.4, df = 1, P < 0.0001; Fig. 3).

Experiment 2: Female choice trials – Mating success and precopulatory mating behaviours of virgin and non-virgin males

When females could choose between two males, they were more likely to mate with a virgin than a non-virgin male (respectively 68.18% of virgin vs. 31.82% of non-virgin males mated with females, χ^2_1 = 10.23, P = 0.001; Table 2). Non-virgin 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 and was 50% longer for non-virgin 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

362 experience did not affect the latency to the first mating attempt (W = 1.86, P = 0.394,
363 corrected for male mass) or the percentage of time spent in activity ($F_{1,86}$ = 0.98, P = 0.326,
364 corrected for male mass; Table 2). Females did not mate preferentially with a more active
365 male, whether virgin (χ^2_1 = 0.83, P = 0.361) or non-virgin (χ^2_1 = 0.07, P = 0.789). Non-virgin
366 males attempted copulation significantly more frequently than virgin males, suggesting that
367 females were more likely to reject a non-virgin male (number of female rejections, $F_{1,86}$ =
368 8.45, P = 0.003, corrected for male mass; Table 2).

369 **DISCUSSION**

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

385 As in several other studies, we found that spermatophore size decreased and copulation
386 duration increased if the male had recently mated before (Konopka & McNeil 2015; Torres-

387 Vila & Jennions, 2005). Mating with a non-virgin male increased mating costs by increasing
388 the time in copula (33% longer with non-virgin males). This prolonged copulation could be a
389 non-virgin male strategy to reduce sperm competition with future ejaculates if the male itself
390 acts as a mating plug that prevents the female from remating before oviposition ("in copula"
391 guarding hypothesis, reviewed by Alcock, 1994). Indeed, this post-copulatory strategy could
392 be particularly effective because in *L. botrana* species, the mating period occurs each day at
393 dusk during a few hours and we observed that females receiving small spermatophores tended
394 to recall sooner after mating, sometimes only 1 hour after copulation (Muller, personal
395 observations). Remaining in copula for more than 1 hour may be sufficient to prevent female
396 remating on the same day, ensuring sperm transfer from the non-virgin male. Indeed, in
397 several butterfly and moth species, sperm generally reaches the spermatheca between 2 and 5
398 hours after mating (Marcotte et al., 2003, Seth et al., 2002). Spermatophore size was
399 positively correlated with pupal mass for virgin males, but not for non-virgin males, as
400 observed in several other lepidopteran species (Watanabe et al., 1998; Wedell & Cook, 1999).
401 In our study, spermatophores delivered by non-virgin males were about five times smaller
402 than those transferred by virgin males, and probably contained low amounts of accessory
403 gland secretions, as observed in a wide range of species (Blanco et al., 2009; Marcotte et al.,
404 2005; Wedell & Cook, 1999). In capital breeders, the production of spermatophores is limited
405 by reserves stored as larvae. Therefore, the first mating of a capital breeder male is certainly
406 the most quantitatively important because a large proportion of his stored reserves is directly
407 used in the production of its first spermatophores. Further work is needed to determine how
408 larval nutrition affects energy reserves of adult males and how these stored reserves are
409 reallocated into reproductive traits of males (reproductive capacity, spermatophore quality).

410 Females mated to non-virgin males suffered a 25% reduction in lifetime fecundity, a finding
411 in agreement with reports on the majority of Lepidoptera species (reviewed by Torres-Vila &

412 Jennions, 2005). At the beginning of the oviposition period, females mated with non-virgin
413 males laid as many eggs as females mated with virgin males. These eggs probably correspond
414 to the initial egg load of the female (i.e., the number of mature eggs a female has available to
415 lay at emergence). However, in the second half of the oviposition period, females that had
416 mated with non-virgin males almost stopped laying compared with females that had received
417 a large spermatophore from virgin males. Thus, females mated with virgin males produced
418 more eggs at the end of their oviposition period, corresponding to the time the female needs to
419 incorporate male-derived nutrients into their eggs; these laid eggs probably correspond to the
420 male contribution. Several studies have demonstrated that the time required to incorporate
421 spermatophore-derived nutrients into maturing oocytes is highly variable and depends on the
422 species and its reproductive effort throughout life, for example, occurring within 2 days after
423 mating for a lampyrid species (*Photinus ignitus*, Rooney & Lewis, 1999) and not before 15–
424 20 days for a lepidopteran species (*Heliconius charitonius*, Boggs, 1990). Despite these
425 benefits in fecundity, females mated with non-virgin males that had received a small
426 spermatophore did not exhibit reduced fertility (85.8% of hatched eggs vs. 87.9% for females
427 mated with virgin males), suggesting that a large spermatophore is not a condition for
428 functional sperm transfer. This absence of effect on female fertility is certainly attributable to
429 the fact that the male usually transfers considerably more sperm than is necessary to fertilize
430 all female eggs, even if it has recently mated (Bezzerides et al., 2008; Curril & LaMunyon,
431 2006). A further experiment should be performed to evaluate the number of fertilizing
432 eupyrene sperm contained in spermatophores transferred by virgin and non-virgin males.
433 Females that mate with previously mated males may not receive enough male-derived
434 nutrients to boost their reproductive potential and should compensate by being more likely to
435 remate or by remating sooner than females mated to virgin males (Foster & Ayers, 1996;
436 Marcotte et al., 2007). We found that, after mating with a non-virgin male, 88.9% of females

recalled at dusk and were motivated to remate, whereas only 64.7% of those mated with virgin males did. Moreover, females mated with non-virgin males recalled males sooner (2 days) than females mated with non-virgin males (4 days), a finding consistent with the literature on Lepidoptera species (Marcotte et al., 2005; Torres-Vila et al., 1997; 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 activate stretch receptors in the female reproductive tract (Sugarawa, 1979), inducing a long refractory period. In addition to this proximate mechanism for post-mating non-receptivity, 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 et al., 1991; Karube & Kobayashi, 1999). However, female receptivity in butterflies is not only affected by the presence of sperm; some males transfer anti-aphrodisiacs at mating that temporarily reduce female attractiveness (Andersson et al., 2003). Further investigations on the composition of the male spermatophore (sperm and anti-aphrodisiac 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 non-virgin males and would prefer virgin males so as to acquire more direct benefits (Wedell et al., 2002). Consistent with these predictions, our study revealed that virgin males were twice as likely to mate with females compared to non-virgin 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 et al., 2013), and non-virgin males

462 could be physically unable to produce the same level of courtship as sexually vigorous virgin
463 males (Janowitz & Fischer, 2010). However, in our study, both virgin and non-virgin males
464 were equally active during courtship, and virgin males did not attempt to copulate earlier with
465 females than non-virgin males, suggesting that virgin males have the same motivation to mate
466 as non-virgin males. Thus, we did not find that males changed their mating behaviour as they
467 gained experience, unlike others have reported (Kaitala & Wiklund, 1994). These behavioural
468 observations allow us to rule out the possibility that virgin males are superior competitors
469 because female preference for virgin males are not likely driven by differences in male
470 courtship. However, we observed female behaviour consistent with discrimination against
471 non-virgin males. The greater reproductive success of virgin males certainly reflects an active
472 female preference for these males, because females more frequently actively rejected non-
473 virgin males (7.5 times) than virgin males (4.4 times) before accepting mating, and non-virgin
474 males required more than twice as much time to successfully mate with females than virgin
475 males. These results strongly suggest that *L. botrana* females can evaluate male mating
476 experience using different visual and/or olfactory cues (Costanzo & Monteiro, 2007; Harris &
477 Moore, 2005). Even if our study was not designed to establish the proximal causes of female
478 discrimination, our results allow us to formulate some hypotheses. Among these cues,
479 chemical cues are important in mate choice in a variety of species and may be used by one or
480 both sexes to acquire information about potential mates (Carazo et al., 2004). In insect
481 species, pheromone or cuticular hydrocarbon substances produced by females and acquired by
482 males during copulation may advertise their previous mating experience (Harris & Moore,
483 2005; Scott et al., 1988). It is possible that non-virgin males are imbued with female
484 substances from their first mating. Females may use chemical cues left behind during
485 previous male-female interactions to gain information about the mating experiences of males.

Such “perfumed” non-virgin males could be recognised and avoided by virgin females in order to minimise 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 maximise benefits by preferentially 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 qualities, 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 considered species may not be as high as that for *L. botrana* species. 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.

REFERENCES

Alcock, J. (1994). Postinsemination associations between males and females in insects: the mate-guarding hypothesis. *Annual Review of Entomology, 39,* 1-21.

Andersson, J., Borg-Karlson, A. K., & Wiklund, C. (2003). Antiaphrodisiacs in pierid butterflies: a theme with variation!. *Journal of Chemical Ecology, 29,* 1489-1499.

Andersson, M., & Simmons, L. W. (2006). Sexual selection and mate choice. *Trends in Ecology & Evolution, 21,* 296-302.

Arnqvist, G., & Nilsson, T. (2000). The evolution of polyandry: multiple mating and female fitness in insects. *Animal Behaviour, 60,* 145-164.

Bezzerides, A. L., Iyengar, V. K., & Eisner, T. (2008). Female promiscuity does not lead to increased fertility or fecundity in an arctiid moth (*Utetheisa ornatrix*). *Journal of Insect Behavior, 21,* 213-221.

Blanco, C. A., Rojas, M. G., Groot, A. T., Morales-Ramos, J., & Abel, C. A. (2009). Size and chemical composition of *Heliothis virescens* (Lepidoptera: Noctuidae) spermatophores. *Annals of the Entomological Society of America, 102,* 629-637.

Boggs, C. L. (1990). A general model of the role of male-donated nutrients in female insects' reproduction. *American Naturalist, 136,* 598-617.

Boggs, C. L., & Freeman, K. D. (2005). Larval food limitation in butterflies: effects on adult resource allocation and fitness. *Oecologia. 144,* 353-361.

Boggs, C. L., & Gilbert, L. E. (1979). Male contribution to egg production in butterflies: evidence for transfer of nutrients at mating. *Science, 206,* 83-84.

Bolker, B. M., Brooks, M. E., Clark, C. J., Geange, S. W., Poulsen, J. R., Stevens, M. H. H., & White, J. S. S. (2009). Generalized linear mixed models: a practical guide for ecology and evolution. *Trends in Ecology & Evolution, 24,* 127-135.

528 Bonduriansky, R. (2001). The evolution of male mate choice in insects: a synthesis of ideas
529 and evidence. *Biological Reviews, 76,* 305-339.
530 Cahenzli, F., & Erhardt, A. (2013). Nectar amino acids enhance reproduction in male
531 butterflies. *Oecologia. 171,* 197-205.
532 Carazo, P., Sanchez, E., Font, E., & Desfilis, E. (2004). Chemosensory cues allow male
533 *Tenebrio molitor* beetles to assess the reproductive status of potential mates. *Animal*
534 *Behaviour, 68,* 123-129.
535 Costanzo, K., & Monteiro, A. (2007). The use of chemical and visual cues in female choice in
536 the butterfly *Bicyclus anynana*. *Proceedings of the Royal Society B: Biological Sciences, 274,*
537 845-851.
538 Curril, I. M., & LaMunyon, C. W. (2006). Sperm storage and arrangement within females of
539 the arctiid moth *Utetheisa ornatrix*. *Journal of Insect Physiology, 52,* 1182-1188.
540 Delisle, J., & Bouchard, A. (1995). Male larval nutrition in *Choristoneura rosaceana*
541 (Lepidoptera: Tortricidae): an important factor in reproductive success. *Oecologia, 104,* 508-
542 517.
543 Dewsbury, D. A. (1982). Ejaculate cost and male choice. *American Naturalist, 119,* 601-610.
544 Edvardsson, M., Hunt, J., Moore, P. J., Moore, A. J. (2008). Female agreement over male
545 attractiveness is not affected by cost of mating with experienced males. *Behavioral Ecology,*
546 *19,* 854-859.
547 Edward, D. A., & Chapman, T. (2011). The evolution and significance of male mate choice.
548 *Trends in Ecology & Evolution, 26,* 647-654.
549 Elzinga, J. A., Chevasco, V., Grapputo, A., & Mappes, J. (2011). Influence of male mating
550 history on female reproductive success among monandrous Naryciinae (Lepidoptera:
551 Psychidae). *Ecological Entomology, 36,* 170-180.

552 Forstmeier, W., & Schielzeth, H. (2011). Cryptic multiple hypotheses testing in linear models:
553 overestimated effect sizes and the winner's curse. *Behavioral Ecology and Sociobiology, 65,*
554 47-55.
555 Foster, S. P., & Ayers, R.H. (1996). Multiple mating and its effects in the lightbrown apple
556 moth, *Epiphyas postvittana* (Walker). *Journal of Insect Physiology, 42,* 657-667.
557 Friberg, U. (2006). Male perception of female mating status: its effect on copulation duration,
558 sperm defence and female fitness. *Animal Behaviour, 72,* 1259-1268.
559 Giebultowicz, J. M., Raina, A. K., Uebel, E. C., & Ridgway, R. L. (1991). Two-step
560 regulation of sex-pheromone decline in mated gypsy moth females. *Archives of Insect*
561 *Biochemistry and Physiology, 16,* 95-105.
562 Harari, A. R., Zahavi, T., & Thiéry, D. (2011). Fitness cost of pheromone production in
563 signaling female moths. *Evolution, 65,* 1572-1582.
564 Harris, W. E., & Moore, P. J. (2005). Female mate preference and sexual conflict: females
565 prefer males that have had fewer consorts. *American Naturalist, 165,* 64-71.
566 Harshman, L. G., & Zera, A. J. (2007). The cost of reproduction: the devil in the details.
567 *Trends in Ecology & Evolution, 22,* 80-86.
568 Iyengar, V. K. (2009). Experience counts: females favor multiply mated males over
569 chemically endowed virgins in a moth (*Utetheisa ornatrix*). *Behavioral Ecology and*
570 *Sociobiology, 63,* 847-855.
571 Janowitz, S. A., & Fischer, K. (2010). Costing reproduction: effects of mating opportunity on
572 mating success in male *Bicyclus anynana* butterflies. *Behavioral Ecology and Sociobiology,*
573 *64,* 1999-2006.
574 Jiménez-Pérez, A., & Wang, Q. (2004). Male remating behavior and its effect on female
575 reproductive fitness in *Cnephasia jactatana* Walker (Lepidoptera: Tortricidae). *Journal of*
576 *Insect Behavior, 17,* 685-694.

577 Jones, T. M. (2001). A potential cost of monandry in the lekking sandfly *Lutzomyia*
578 *longipalpis*. *Journal of Insect Behavior, 14*, 385-399.
579 Jones, T. M., & Elgar, M. A. (2004). The role of male age, sperm age and mating history on
580 fecundity and fertilization success in the hide beetle. *Proceedings of the Royal Society B:*
581 *Biological Sciences, 271*, 1311-1318.
582 Kaitala, A., & Wiklund, C. (1994). Female mate choice and mating costs in the polyandrous
583 butterfly *Pieris napi* (Lepidoptera: Pieridae). *Journal of Insect Behavior, 8*, 355-363.
584 Karube, F., & Kobayashi, M. (1999). Presence of eupyrene spermatozoa in vestibulum
585 accelerates oviposition in the silkworm moth, *Bombyx mori*. *Journal of Insect Phsyiology, 45*,
586 947-957.
587 Klepetka, B., & Gould, F. (1996). Effects of age and size on mating in *Heliothis virescens*
588 (Lepidoptera: Noctuidae): implications for resistance management. *Environmental*
589 *Entomology, 25*, 993-1001.
590 Kokko, H., Jennions, M. D., & Brooks, R. (2006). Unifying and testing models of sexual
591 selection. *Annual Review of Ecology, Evolution and Systematics, 37*, 43-66.
592 Konopka, J. K., & McNeil, J. N. (2015). Mating status regulates post-mating refractory period
593 in *Striacosta albicosta* females. *Entomologia Experimental et Applicata, 155*, 80-85.
594 Kotiaho, J. S. (2002). Sexual selection and condition dependence of courtship display in three
595 species of horned dung beetles. *Behavioral Ecology, 13*, 791-799.
596 Krupke, C. H., Brunner, J. F., & Jones, V. P. (2008). Factors influencing mate choice in
597 *Euschistus conspersus* Uhler (Heteroptera: Pentatomidae). *Environmental Entomology, 37*,
598 192-197.
599 Lauwers, K., & Van Dyck, H. (2006). The cost of mating with a non-virgin male in a
600 monandrous butterfly: experimental evidence from the speckled wood, *Pararge aegeria*.
601 *Behavioral Ecology and Sociobiology, 60*, 69-76.

Lewis, S. M., & South, A. (2012). The evolution of animal nuptial gifts. *Advances in the Study of Behavior, 44,* 53-97.

Magnhagen, C. (1991). Predation as a cost of reproduction. *Trends in Ecology & Evolution, 6,* 183-186.

Marcotte, M., Delisle, J., & McNeil, J. N. (2003). Pheromonostasis is not directly associated with post-mating sperm dynamics in *Choristoneura fumiferana* and *C. rosaceana* females. *Journal of Insect Physiology, 49,* 81-90.

Marcotte, M., Delisle, J., & McNeil, J. N. (2005). Impact of male mating history on the temporal sperm dynamics of *Choristoneura rosaceana* and *C. fumiferana* females. *Journal of Insect Physiology, 51,* 537-544.

Marcotte, M., Delisle, J., & McNeil, J. N. (2007). Effects of different male remating intervals on the reproductive success of *Choristoneura rosaceana* males and females. *Journal of Insect Physiology, 53,* 139-145.

Markow, T. A., Quaid, M., & Kerr, S. (1978). Male mating experience and competitive courtship success in *Drosophila melanogaster*. *Nature, 276,* 821-822.

Marshall, L. D. (1982). Male nutrient investment in the Lepidoptera: what nutrients should males invest? *American Naturalist, 120,* 273-279.

McNamara, K. B., Elgar, M. A., & Jones, T. M. (2009). Large spermatophores reduce female receptivity and increase male paternity success in the almond moth, *Cadra cautella*. *Animal Behaviour, 77,* 931-936.

Milonas, P. G., Farrell, S. L., & Andow, D. A. (2011). Experienced males have higher mating success than virgin males despite fitness costs to females. *Behavioral Ecology and Sociobiology, 65,* 1249-1256.

Møller, A., & Jennions, M. (2001). How important are direct fitness benefits of sexual selection? *Naturwissenschaften, 88,* 401-415.

627 Monceau, K., & van Baaren, J. 2012. Female teneral mating in a monandrous species.
628 *Ecology and Evolution. 2,* 1426-1436.
629 Muller, K., Thiéry, D., Moret, Y., & Moreau, J. (2015). Male larval nutrition affects adult
630 reproductive success in wild European grapevine moth (*Lobesia botrana*). *Behavioral*
631 *Ecology and Sociobiology, 69,* 39-47.
632 Noguchi, K., Latif, M., Thangavelu, K., Konietschke, F., Gel Y. R., & Brunner, E. (2012).
633 Package nparLD: Nonparametric Analysis of Longitudinal Data in Factorial Experiments,
634 2012. R package version 2.0. http://CRAN.R-project.org/package=nparLD.
635 Oberhauser, K. S. (1989). Effects of spermatophores on male and female monarch butterfly
636 reproductive success. *Behavioral Ecology and Sociobiology, 25,* 237-246.
637 R Development Core Team. (2015). R: *A language and environment for statistical computing.*
638 Vienna, Austria: R Foundation for Statistical Computing. (http://www.R-project.org/).
639 Rooney, J., & Lewis, S. M. (1999). Differential allocation of male-derived nutrients in two
640 lampyrid beetles with contrasting life-history characteristics. *Behavioral Ecology, 10,* 97-104.
641 Scharf, I., Peter, F., & Martin, O. Y. (2013). Reproductive trade-offs and direct costs for
642 males in arthropods. *Evolutionary Biology, 40,* 169-184.
643 Scott, D., Richmond, R. C., & Carlson, D. A. (1988). Pheromones exchanged during mating:
644 a mechanism for mate assessment in Drosophila. *Animal Behaviour, 36,* 1164-1173.
645 Seth, R. K., Kaur, J. J., Rao, D. K., & Reynolds, S. E. (2002). Sperm transfer during mating,
646 movement of sperm in the female reproductive tract, and sperm precedence in the common
647 cutworm *Spodoptera litura*. *Physiological Entomology, 27,* 1-14.
648 Simmons, L. W. (2001). Sperm competition and its evolutionary consequences in the insects.
649 Princeton, New Jersey: Princeton University Press.
650 Skaug, H. F. D., Nielsen, A., Magnusson, A., & Bolker, B. (2013). GlmmADMB package.
651 (http://glmmadmb.r-forge.r-project.org/)

South, A., & Lewis, S. M. (2011). The influence of male ejaculate quantity on female fitness: a meta-analysis. *Biological Reviews, 86,* 299-309.

Sugawara, T. (1979). Stretch reception in the bursa copulatrix of the butterfly, *Pieris rapae crucivora*, and its role in behaviour. *Journal of Comparative Physiology, 130,* 191-199.

Thiéry, D., & Moreau, J. (2005). Relative performance of European grapevine moth (*Lobesia botrana*) on grapes and other hosts. *Oecologia, 143,* 548-557.

Tigreros, N. (2013). Linking nutrition and sexual selection across life stages in a model butterfly system. *Functional Ecology, 27,* 145-154.

Torres-Vila, L. M. (2014). Polyandry-fecundity relationship in insects: methodological and conceptual problems. *Journal of Evolutionary Biology, 26,* 325-334.

Torres-Vila, L. M., Gragera, J., Rodríguez-Molina, M. C., & Stockel, J. (2002). Heritable variation for female remating in *Lobesia botrana*, a usually monandrous moth. *Animal Behaviour, 64,* 899-907.

Torres-Vila, L. M., & Jennions, M. D. (2005). Male mating history and female fecundity in the Lepidoptera: do male virgins make better partners? *Behavioral Ecology and Sociobiology, 57,* 318-326.

Torres-Vila, L. M., Rodríguez-Molina, M. C., & Jennions, M. D. (2004). Polyandry and fecundity in the Lepidoptera: can methodological and conceptual approaches bias outcomes? *Behavioral Ecology and Sociobiology, 55,* 315-324.

Torres-Vila, L. M., Rodriguez-Molina, M. C., Roehrich, R., & Stockel, J. (1999). Vine phenological stage during larval feeding affects male and female reproductive output of *Lobesia botrana* (Lepidoptera: Tortricidae). *Bulletin of Entomological Research, 89,* 549-556.

Torres-Vila, L. M., Stockel, J., & Rodriguez-Molina, M. C. (1997). Physiological factors regulating polyandry in *Lobesia botrana* (Lepidoptera: Tortricidae). *Physiological Entomology, 22,* 387-393.

677 Vahed, K., & Gilbert, F. (1997). No effect of nuptial gift consumption on female reproductive
678 output in the bushcricket *Leptophyes laticauda* Friv. *Ecological Entomology, 22,* 479-482.
679 Vahed, K. (1998). The function of nuptial feeding in insects: a review of empirical studies.
680 *Biological Reviews, 73,* 43-78.
681 Velde, L. V., Damiens, D., & Van Dyck, H. (2011). Spermatophore and sperm allocation in
682 males of the monandrous butterfly *Pararge aegeria*: the female's perspective. *Ethology, 117,*
683 645-654.
684 Watanabe, M., Wiklund, C., & Bon'no, M. (1998). The effect of repeated matings on sperm
685 numbers in successive ejaculates of the cabbage white butterfly *Pieris rapae* (Lepidoptera:
686 Pieridae). *Journal of Insect Behavior, 11,* 559-570.
687 Wedell, N. (2005). Female receptivity in butterflies and moths. *Journal of Experimental*
688 *Biology, 208,* 3433-3440.
689 Wedell, N., & Cook, P. A. (1999). Butterflies tailor their ejaculate in response to sperm
690 competition risk and intensity. *Proceedings of the Royal Society B: Biological Sciences, 266,*
691 1033-1039.
692 Wedell, N., Gage, M. J., & Parker, G. A. (2002). Sperm competition, male prudence and
693 sperm-limited females. *Trends in Ecology & Evolution, 17,* 313-320.
694 Will, M. W., & Sakaluk, S. K. (1994). Courtship feeding in decorated crickets: is the
695 spermatophylax a sham?. *Animal Behaviour, 48,* 1309-1315.
696

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

Figure 2. Number of eggs laid by females mated with virgin (black bars) or non-virgin (white bars) males in the first and the second half of their oviposition period (oviposition period was separated in two sequences by the median for a given female). Results are presented as means ± SEM (*** $P < 0.0001$; NS, non-significant difference [$P > 0.05$]).

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

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

No-choice trials	**Experiment 1**				**Experiment 2**			
Male mating experience	Mating success[1] (%)	Onset time of mating[2] (min)	Duration of mating[3] (min)	Spermatophore volume ($\times 10^{-6}$ mm^3)[3]	Fecundity (eggs/female)[4]	Time spent ovipositing (days)[3]	Fertility (% of eggs hatched)[5]	Longevity (days)[2]
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
Non-virgin	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

[1]Pearson χ^2
[2]Cox regression
[3]ANCOVA
[4]GLMMADMB
[5]GLM with quasi-binomial error

726 Table 2. Summary results from choice trials (n= 44) of *L. botrana* females successfully mated with virgin (n = 30) or non-virgin (n=14) males
727 showing mating parameters and precopulatory behaviours. Values are expressed as a ratio (successful matings) or as means ± SEM (all other
728 parameters). Values in each column denoted by different lowercase letters are significantly different ($P < 0.05$).

Choice trials	Mating parameters			Precopulatory behaviours of males and females			
Male mating experience	Number of successful matings[1]	Onset time of mating[2] (s)	Duration of mating[3] (min)	Percentage of time spent in calling position[3]	Latency to the first mating attempt (s)[2]	Percentage of male activity[3]	Number of female rejections[4]
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)
Non-virgin	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)

729 [1]Pearson χ^2
730 [2]Cox regression
731 [3]ANCOVA
732 [4]NBGLM
733

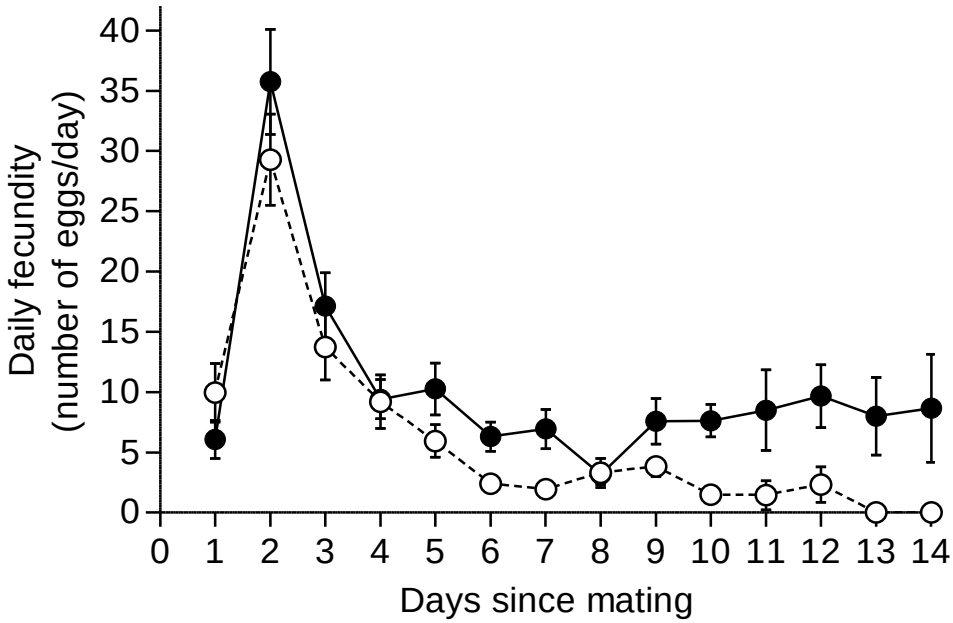

Figure 1

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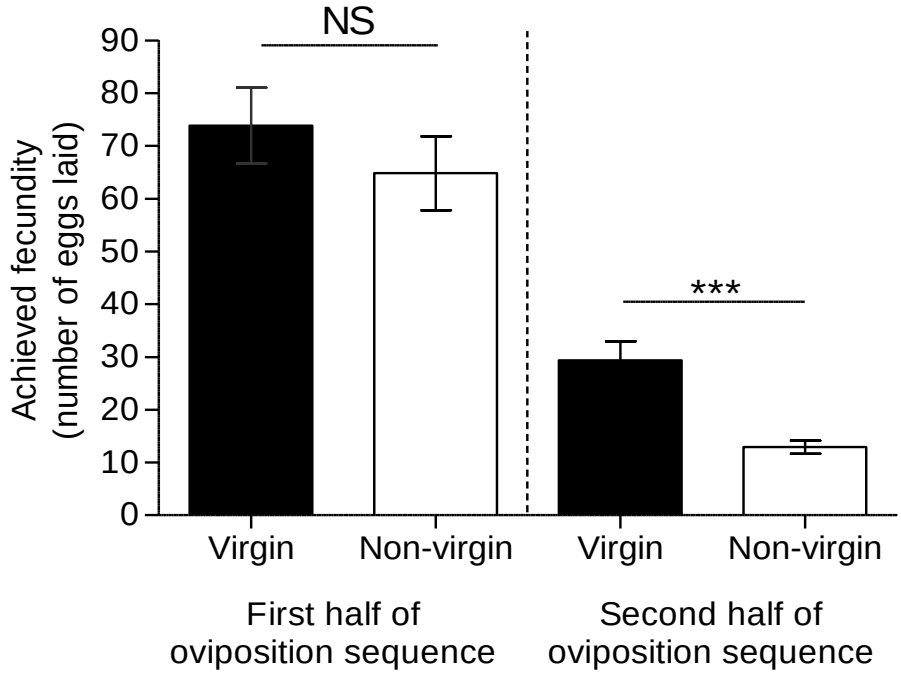

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745 Figure 2

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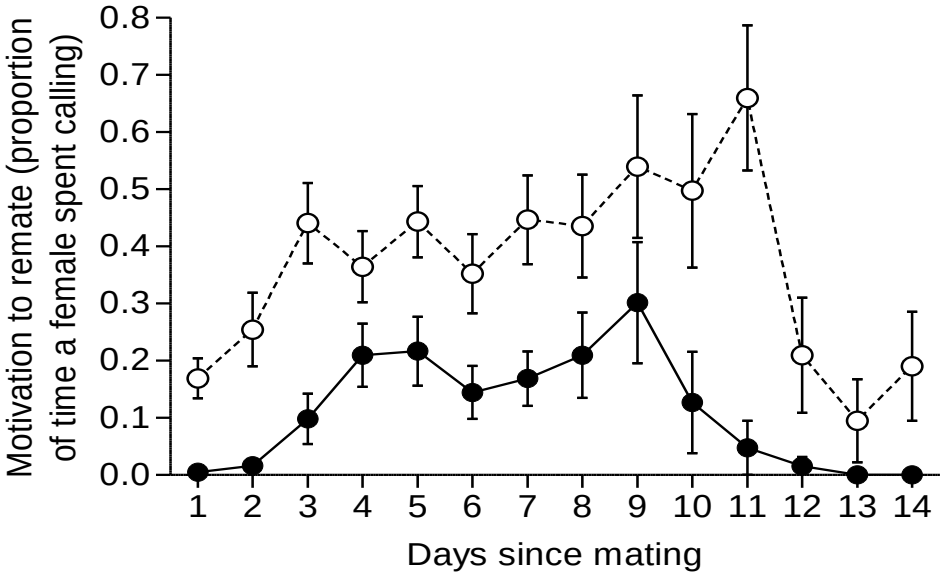

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751 Figure 3

752 Muller et al., 2015