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1 **Direct benefits from choosing a virgin male in the European**
2 **grapevine moth (*Lobesia botrana*)**

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21 In Lepidoptera, male investment in the ejaculate usually declines over consecutive matings, a
22 depletion that could have profound consequences for female reproductive output. Since
23 successive matings can affect the ability of males to provide phenotypic benefits, strong
24 selection for females to discriminate among males based on the male mating experience may
25 exist. The aim of our study was to determine if monandrous females of the European
26 grapevine moth (*Lobesia botrana*) are able to discriminate among males of different qualities
27 based on their mating experience in order to maximise direct benefits (by receiving large
28 spermatophores from virgin males) and minimise mating costs (by avoiding low-quality non-
29 virgin males). Non-virgin males produced spermatophores five times smaller than those of
30 virgin males; consequently, mating with non-virgin males significantly reduced female
31 fecundity (by ~25%) and increased their motivation to remate. In a mate-preference
32 experiment, we found that females were more likely to mate with virgin males and more
33 frequently rejected non-virgin mates. Moreover, non-virgin males required more time to
34 achieve mating than virgin males. Our results suggest that females are able to discriminate
35 among males based on male mating experience, and demonstrate female preference for virgin
36 males, thereby maximising direct benefits associated with receiving large spermatophores.

37

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

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

55 In most insect species, one of the direct benefits that might drive female preference is access
56 to nutritive resources, such as nuptial gifts offered by males before or during mating (Lewis &
57 South, 2012; Vahed, 1998). These nuptial gifts include food items or male accessory gland
58 secretions, and generally have a direct positive effect on female fitness through beneficial
59 effects on a variety of fitness-related traits, including fecundity, fertility, and longevity (South
60 & Lewis, 2011). However, in some species, the nuptial gifts apparently provide no
61 significantly nutritional fitness benefits to females (for example, in the bushcricket,
62 *Leptophyes laticauda*, Vahed & Gilbert, 1997, or in the decorated cricket, *Gryllodes*
63 *sigillatus*, Will & Sakaluk, 1994). In Lepidoptera, nuptial gifts consist of a spermatophore
64 containing sperm and accessory gland products rich in nutrients such as proteins (Marshall,
65 1982). Several studies using radiolabelled substances (e.g., amino acids, zinc, phosphorus,
66 sodium) support the prediction that females allocate male-derived nutrients from
67 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)

118 and the European corn borer, *Ostrinia nubilalis* (Milonas et al., 2011). In some of these,
119 species, females prefer to mate with experienced males based on indirect benefits related to
120 genetically superior partners or inherited male attractiveness (Iyengar, 2009; Krupke et al.,
121 2008). Moreover, male mating history can have positive direct effects on female fitness
122 through the reduction of male manipulations, especially in species in which male ejaculates
123 contain manipulative substances that are harmful to females (Edvardsson et al., 2008). The
124 lack of overall consensus could reflect the fact that mating patterns greatly depend on the
125 species under consideration (with or without nuptial gifts), feeding habits (capital vs. income
126 breeders) mating system (monandrous vs. polyandrous females), and the effect of male
127 mating experience on female reproductive output. To our knowledge, there has never been a
128 conclusive study that explicitly tested predictions about female preferences driven by
129 potential direct benefits in a capital breeding monandrous species in which nuptial gifts
130 represent a crucial advantage for female reproductive output.

131 The European grapevine moth, *Lobesia botrana* (Lepidoptera: Tortricidae) (Denis and
132 Schiffermüller), a very important pest of grapes worldwide, is an ideal candidate for assessing
133 female preferences based on male mating experience. This moth is a capital breeder that
134 acquires most of the resources necessary for its adult life during larval stages in grapes. Adult
135 females can also obtain resources during the adult stage, not by feeding but by receiving
136 spermatophore-derived nutrients from males during mating (Muller et al., 2015; Torres-Vila
137 et al., 1997). This species is considered monandrous, with about 70% of wild females mating
138 only once during their lifespan (Torres-Vila et al., 1997). The remnant polyandry (involving
139 less than 30% of the population) is an heritable trait ($h^2 = 0.40 \pm 0.12$, Torres-Vila et al.,
140 2002) and is strongly associated with physiological factors, such as larval food nutrition
141 (Torres-Vila et al., 2004) and the size of the spermatophore received by females (Torres-Vila
142 et al., 1997). Thus, all the ecological conditions (a capital breeding monandrous species in

143 which there is a strong effect of spermatophore on female fitness) are present in this species to
144 detect female preference related to male mating experience.

145 Here, we experimentally evaluated the influence of male mating experience on male
146 spermatophore size, female reproductive output, and motivation to remate in *L. botrana*. We
147 first investigated the existence of direct benefits to females from mating with virgin males in
148 terms of fecundity, fertility, and longevity. Secondly, we quantified the motivation to remate
149 in females mated with virgin or non-virgin males. Finally, we assessed if whether *L. botrana*
150 females preferred to mate with virgin males rather than with recently mated males, with the
151 assumption that female should optimise mate choice to obtain male-derived nutrients. We also
152 monitored the premating behaviour of virgin and non-virgin males to characterise behavioural
153 differences of virgin and non-virgin males that might explain female mating preference. We
154 predicted that female would be able to discriminate between a non-virgin and a virgin male
155 and, given the choice, would preferentially mate with the virgin.

156

157 **MATERIAL & METHODS**

158 *Moth rearing and general procedures*

159 All adults used were obtained from an inbred strain of *L. botrana* (INRA, UMR Save
160 Bordeaux). The stock colony was maintained without diapause on a semi-artificial diet (1000
161 mL water, 15 g agar, 84.63 g maize flour, 41.25 g wheat germ, 45.48 g yeast, 6.03 g ascorbic
162 acid, 3.35 g Wesson salts, 0.32 mL Scala, 5 mL ethanol [95%], 2.65 g benzoic acid, 2.76 g
163 Nipagin), as described previously (Thiéry & Moreau, 2005), and maintained at $22 \pm 1^\circ\text{C}$, $60 \pm$
164 10% relative humidity, under a 16:8 hour light:dark photoperiod. Males and females were
165 placed in a large cage and bands of waxed paper (15×2 cm) were hung for oviposition
166 support. Once the paper had received a sufficient number of eggs, it was placed in a plastic
167 box containing the semi-artificial larval diet. The larvae were maintained at a density of 100

168 individuals per 300 ml of diet. Larvae were checked daily until pupation, and then were
169 weighed to the nearest 0.01 mg on a Precisa 262 SMA-FR microbalance and placed
170 individually in glass tubes (70 × 9 mm diameter) stoppered with cotton plugs. Pupae were
171 checked every morning, and newly emerging virgin adults were used for subsequent
172 experiments under the same conditions as rearing.

173

174 *Ethical Note*

175 All experiments complied with French laws on animal experimentation. All individuals were
176 reared under controlled laboratory conditions and fed regularly to maintain a healthy
177 population (see below for rearing methods). Moths were treated carefully, and the abiotic
178 conditions (temperature, humidity and photoperiod) they experienced corresponded to the
179 natural conditions in their native habitat. Females submitted to dissection were chilled in a
180 freezer prior to decapitation.

181

182 *Experiment 1: No choice mating trials*

183 *General procedure*

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

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

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

204 *Experiment 1a: Effect of mating experience on spermatophore volume*

205 Immediately after the end of mating with a virgin (n = 76) or a non-virgin (n = 118) male,
206 females were frozen at -25 °C for ten minutes and then dissected on a glass slide. The bursa
207 copulatrix containing the male spermatophore was removed in order to estimate its size.

208 Estimating spermatophore size by extrapolating its volume is the method classically used in
209 moths, including *L. botrana* (Milonas et al., 2011; Muller et al., 2015; Torres-Vila et al.,
210 1999). To assess spermatophore size, we measured its dimensions (length [l], width [w], and
211 thickness [t]) under a stereomicroscope (NIKON SMZ1500) with a magnification of 20X. The
212 volume of the spermatophore was estimated as an ellipsoid balloon [$V = \pi/6 (l \times w \times t)$], as
213 described previously (Torres-Vila et al., 1999).

214 *Experiment 1b: Effect of mating experience on both female oviposition and recalling*
215 *dynamics and on female reproductive output*

216 After the end of copulation with virgin (n = 34) or non-virgin (n = 36) males, males were
217 removed and females were maintained in their mating tube until death to assess reproductive
218 output and motivation to remate. These females were allowed to oviposit freely on the inside
219 surface of the glass tub until their death. Every day, newly laid eggs were counted daily
220 through the tube. Female survival was also checked daily; after death, the eggs were
221 incubated for 7 days under the same conditions as used for moth maintenance. With this
222 procedure, we recorded (i) time spent ovipositing (number of days a female oviposited), (ii)
223 daily fecundity (number of eggs laid per day), (iii) achieved fecundity (total number of eggs
224 laid during a female's lifespan), (iv) female fertility (proportion of hatched eggs), and (v)
225 longevity.

226 To assess motivation to remate, each day until their death we simultaneously monitored the
227 behaviour of females that had previously copulated with either a virgin or non-virgin male. In
228 this species, a female that is ready to remate signals her readiness by releasing sex pheromone
229 at dusk, an action that represents a fitness cost to her (Harari et al., 2011). The female does
230 this by assuming a calling position with wings raised and pheromone gland exposed. To
231 obtain an index of female motivation, we recorded the occurrence of this calling behaviour
232 every 3 minutes during a 1-hour period at dusk (each day, females that did not recall at all
233 were excluded from the analysis) – the longer the recalling period, the higher the motivation
234 to mate (Torres-Vila et al., 2002). With this procedure, we also obtained the refractory period
235 of females, corresponding to the number of days elapsed between the mating and the first day
236 we observed the recalling behaviour. The female motivation to mate was measured by (i) the
237 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 $\times 100$. 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 $\times 100$. 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

263 from a male during courtship, the male could not mate with her), suggesting that mating is to
264 a large degree under female control. Preliminary tests suggested that *L. botrana* females
265 rejected their mate several times before copulation (Muller, unpublished results); thus, we
266 scored these female rejection behaviours against virgin and non-virgin males.

267

268 *Statistical analysis*

269 All statistical tests were performed using R Software version 3.2.0 (R Core Team, 2015).
270 Each model includes all the main effects, deleting non-significant interactions. For each
271 analysis, we first tested the full model with the main effects and their interactions and then,
272 we deleted the interactions when they were not significant. However, all main effects were
273 always included in the model, following the recommendations of Forstmeier & Schielzeth
274 (2011). To assess the mating success of males (percentage of successful mating) according to
275 male mating experience (virgin vs. non-virgin) in no-choice mating trials, we used Pearson's
276 χ^2 tests. The effects of mating experience on spermatophore volume and mating duration were
277 analysed with ANCOVAs, with the pupal mass of males and females as covariates.
278 We used generalised linear modeling to estimate the sources of variation in fecundity.
279 Because lifetime fecundity was best approximated by an over-dispersed Poisson distribution,
280 we fitted the model with a negative binomial error structure (Bolker et al., 2009). We also
281 improved the model by specifying that the data were zero inflated. This analysis was
282 performed using the glmmADMB library (Skaug et al., 2013), including time, male mating
283 experience and their interactions as fixed effects, male and female mass as covariates, and
284 female identity as a random factor. A generalised linear model (GLM) using a quasi-binomial
285 error structure and a logit link function was used to analyse the proportion of eggs hatched by
286 females mated with virgin and non-virgin males. Finally, a Cox regression analysis was

287 applied to assess the influence of male mating experience, female pupal mass, and fecundity
288 on female longevity.
289 Because percent calling could not be normalised, a nonparametric factorial repeated measures
290 analysis of longitudinal data was performed using the package nparLD in R (Noguchi et al.,
291 2012) to determine how time, male mating experience, and their interaction affected the
292 intensity of female motivation to remate. Repeated measures were analysed using the F1-LD-
293 F1 model of the nparLD function to calculate an ANOVA-type statistic. Due to
294 heteroscedasticity, female motivation to remate (percent of time spent in recalling position)
295 and male activity (percent of time spent in movement) were arcsin square root-transformed
296 prior to analysis. The effect of male mating experience on male and female precopulatory
297 behaviours were analysed with ANCOVAs, with the pupal mass of males and females as
298 covariates, respectively. Because data on male mating attempts were counted and were over-
299 dispersed, a GLM with a negative binomial distribution (NBGLM) was used to assess the
300 effect of male mating experience on mating attempts.

301

302 **RESULTS**

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

304 In no-choice mating trials, no effect of male mating experience (virgin vs. non-virgin) was
305 detected on mating success, with approximately 85% of males successfully copulating
306 ($\chi^2_1 = 0.001$, $P = 0.973$; Table 1). The onset time of mating did not vary with male mating
307 experience ($W = 0.3$, $P = 0.586$; Table 1) or male mass ($W = 1.4$, $P = 0.237$). Mating duration
308 was affected by male mating experience; copulation with a non-virgin male lasted 33% longer
309 than that with a virgin male ($F_{1,192} = 128.1$, $P < 0.0001$, corrected for male mass; Table 1).
310 Spermatophores transferred by virgin males were on average more than five times larger than
311 those transferred by non-virgin males (Table 1). In addition, heavier males produced larger

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

315

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

318 Females that mated with virgin males had higher achieved fecundity (corresponding to the
319 total number of eggs laid during a female's lifespan) than those mated with males that had
320 previously mated ($F_{1,67} = 10.03$, $P = 0.002$, corrected for male and female mass; Table 1). As
321 shown in Figure 1, daily fecundity, corresponding to the number of eggs laid by females per
322 day, greatly depended on time elapsed since mating ($F = 191.56$, $df = 14$, $P < 0.0001$), male
323 mating experience ($F = 8.78$, $df = 1$, $P = 0.003$) and their interaction ($F = 49.94$, $df = 14$, $P <$
324 0.0001), and was positively correlated with female mass ($F = 11.52$, $df = 1$, $p = 0.0007$). The
325 number of eggs laid was the highest 2 days after mating and decreased daily until the death of
326 the female, regardless of the experience of males (Fig. 1). However, females mated with
327 virgin males laid more eggs at the end of their life than females mated with non-virgin males
328 (Figs 1 and 2). This increase in eggs laid by females mated with virgin males at the end of
329 their life was confirmed by dividing oviposition period into two sequences: eggs laid in the
330 first half of the oviposition period versus eggs laid in the second half of the oviposition period
331 for a given female (Fig. 2). Females mated with virgin males laid more eggs than females
332 mated with non-virgin males in the second half of the oviposition sequence, whereas there
333 was no difference during the first half of the oviposition sequence (first sequence: $F_{1,67} = 2.0$,
334 $P = 0.163$; second sequence: $F_{1,67} = 23.7$, $P < 0.0001$; corrected for male and female mass, Fig.
335 2). In addition, females mated with virgin males spent more time ovipositing ($F_{1,67} = 14.41$, P
336 $= 0.0003$, corrected for male and female mass; Table 1). Male mating experience affected

337 neither female fertility (corresponding to the proportion of hatched eggs) ($F_{1,67} = 9.67$, $P =$
338 0.447 , corrected for male and female mass) nor female longevity (Cox regression, $W = 2.46$, P
339 $= 0.29$, Table 2). However, we found a trade-off between fecundity and longevity: females
340 with higher fecundity had diminished longevity regardless of the experience of their mate
341 (Cox regression: $W = 8.91$, $P = 0.012$).

342 A higher proportion of females were motivated to remate during their lifetime after mating
343 with a non-virgin male ($n = 32/36$, 88.9%) compared to females mated with a virgin male
344 ($n = 22/34$, 64.7%) ($\chi^2_1 = 4.51$, $P = 0.034$). The mean length of the refractory period was
345 longer in females mated with virgin males (4.0 ± 0.3 days) than for those mated with non-
346 virgin males (2.0 ± 0.3 days) (Cox regression: $W = 12.42$, $P = 0.0004$). Females mated with
347 non-virgin males resumed calling longer (5.7 ± 0.6 days) than females mated with virgin
348 males (3.5 ± 0.4 days) ($F_{1,52} = 7.54$, $P = 0.008$, corrected for female mass). In addition,
349 females mated with non-virgin males were more motivated to remate than females mated with
350 virgin males and their motivation increased over time (time effect: $F = 3.29$, $df = 4.92$, $P =$
351 0.006 ; male mating experience effect: $F = 48.4$, $df = 1$, $P < 0.0001$; Fig. 3).

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

354 When females could choose between two males, they were more likely to mate with a virgin
355 than a non-virgin male (respectively 68.18% of virgin vs. 31.82% of non-virgin males mated
356 with females, $\chi^2_1 = 10.23$, $P = 0.001$; Table 2). Non-virgin males required more time to
357 successfully mate than virgin males (Cox regression, $W = 5.31$, $P = 0.021$; Table 2). As in no-
358 choice tests, mating duration was affected by male mating experience and was 50% longer for
359 non-virgin than for virgin males ($F_{1,42} = 14.43$, $P = 0.0004$, corrected for male mass; Table 2).
360 Female motivation to mate was comparable among all females regardless of the mating status
361 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 ($\chi^2_1 = 0.83$, $P = 0.361$) or non-virgin ($\chi^2_1 = 0.07$, $P = 0.789$). 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 (Bezzarides 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

437 recalled at dusk and were motivated to remate, whereas only 64.7% of those mated with
438 virgin males did. Moreover, females mated with non-virgin males recalled males sooner (2
439 days) than females mated with non-virgin males (4 days), a finding consistent with the
440 literature on Lepidoptera species (Marcotte et al., 2005; Torres-Vila et al., 1997; Wedell &
441 Cook, 1999). Moreover, the length of the refractory period in moths can be affected by
442 several factors, including spermatophore size (McNamara et al., 2009) and the quality and
443 quantity of viable sperm and secretions of the male accessory gland transferred to the female
444 during mating (Simmons, 2001; Wedell, 2005). In our experiment, it is possible that larger
445 spermatophores produced by virgin males activate stretch receptors in the female reproductive
446 tract (Sugarawa, 1979), inducing a long refractory period. In addition to this proximate
447 mechanism for post-mating non-receptivity, the presence of sperm in the spermatheca may be
448 required to elicit pheromonostasis in *L. botrana*, similar to other Lepidoptera species in which
449 females only become unreceptive if their spermatheca is full of sperm (Giebultowicz et al.,
450 1991; Karube & Kobayashi, 1999). However, female receptivity in butterflies is not only
451 affected by the presence of sperm; some males transfer anti-aphrodisiacs at mating that
452 temporarily reduce female attractiveness (Andersson et al., 2003). Further investigations on
453 the composition of the male spermatophore (sperm and anti-aphrodisiac substances) in *L.*
454 *botrana* are needed to better understand the effect of male mating experience on female
455 motivation to remate.

456 Because of the beneficial effect of mating with a virgin male, one might expect that virgin
457 females would avoid mating with non-virgin males and would prefer virgin males so as to
458 acquire more direct benefits (Wedell et al., 2002). Consistent with these predictions, our study
459 revealed that virgin males were twice as likely to mate with females compared to non-virgin
460 males in choice trials. Some studies have shown that the level of activity of males may play a
461 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.

486 Such “perfumed” non-virgin males could be recognised and avoided by virgin females in
487 order to minimise the mating costs associated with sperm-depleted males.

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

503

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696

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

700

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

705

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

710 Table 1. Summary results from no-choice trials showing mating success, onset time, duration of mating and spermatophore volume (Experiment
711 1), and reproductive traits and longevity of *L. botrana* females (Experiment 2) according to the mating experience of males (virgin vs. non-
712 virgin). Values are expressed as a percentage (mating success) or as means \pm SEM (all other parameters). Values in each column denoted by
713 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
Virgin	84.89	4.6 \pm 0.3	62.8 \pm 1.3 (a)	133.3 \pm 2.8 (a)	103.2 \pm 7.9 (a)	7.1 \pm 0.4 (a)	85.8 \pm 2.6	11.5 \pm 0.4
Non-virgin	87.36	5.0 \pm 0.6	83.7 \pm 1.2 (b)	26.7 \pm 1.1 (b)	77.9 \pm 7.7 (b)	5.3 \pm 0.3 (b)	87.9 \pm 2.9	11.7 \pm 0.5

714 ¹Pearson χ^2
715 ²Cox regression
716 ³ANCOVA
717 ⁴GLMMADMB
718 ⁵GLM with quasi-binomial error
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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 \pm 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
Virgin	30/44 (a)	182.6 \pm 22.6 (a)	60.9 \pm 2.1 (a)	27.2 \pm 3.6	72.4 \pm 17.1	52.0 \pm 4.2	4.4 \pm 0.5 (a)
Non-virgin	14/44 (b)	309.0 \pm 57.1 (b)	91.9 \pm 9.7 (b)	32.9 \pm 5.3	90.6 \pm 17.1	47.5 \pm 3.6	7.5 \pm 0.9 (b)

729 ¹Pearson χ^2

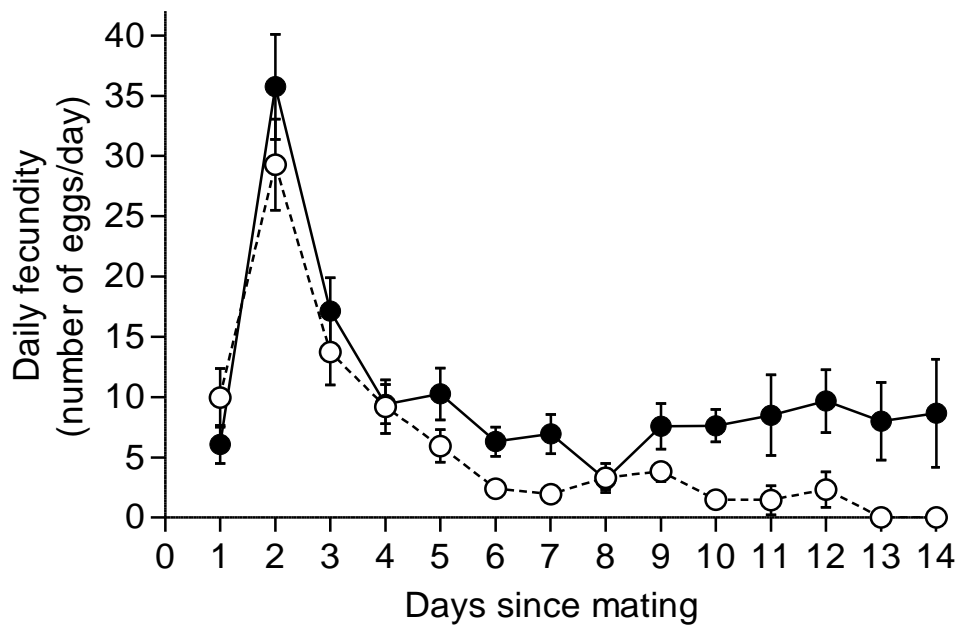
730 ²Cox regression

731 ³ANCOVA

732 ⁴NBGLM

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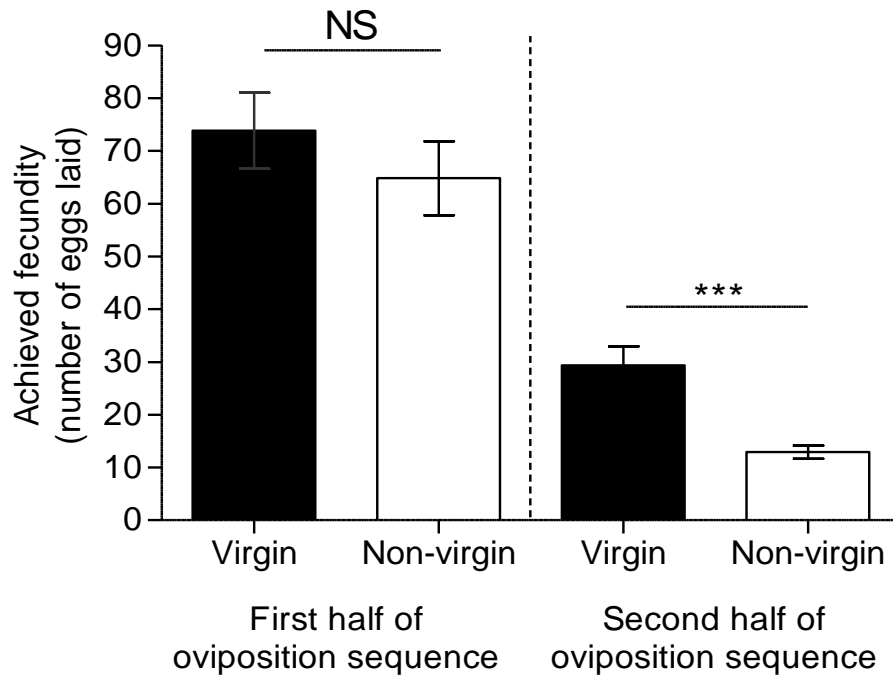
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Figure 1

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Muller et al., 2015



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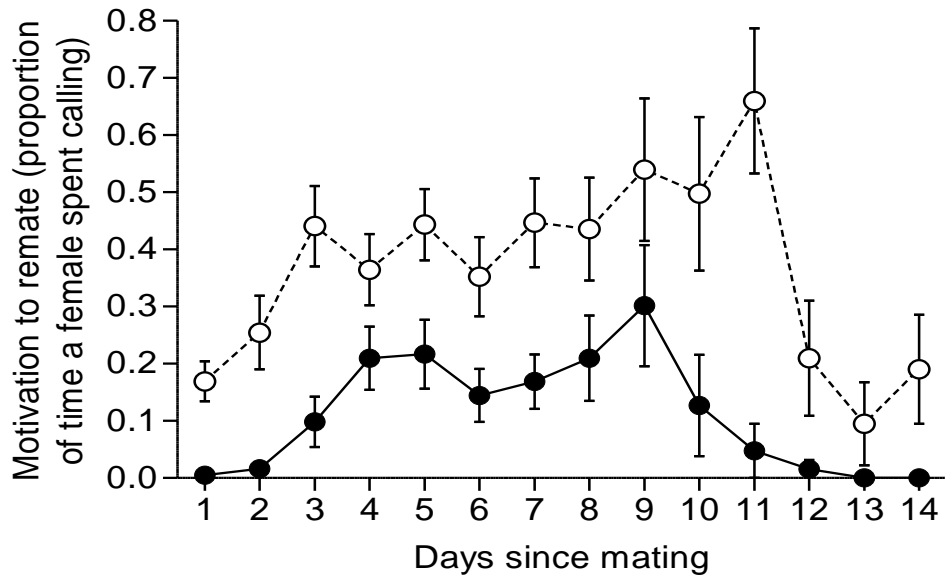
Figure 2

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Muller et al., 2015

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Figure 3

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Muller et al., 2015