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1 **Sexual audience affects male's reproduction investment without consequences on**  
2 **reproductive outputs**

3

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17

18 **ABSTRACT**

19 Males evolved plastic strategies to respond to male-male competition and exhibit adaptive  
20 traits and behaviors maximizing their access to the females and limiting sperm competition.  
21 Mating behaviors allow males to express quick responses to current sexual audience, i.e the  
22 number of nearby conspecifics prone to mate. In contrast, physiological responses are  
23 frequently delayed because they are constrained by the time and resources having to be  
24 mobilized to produce and export sperm and associated products. This is especially critical in  
25 species for which males produce spermatophores. Here we investigated in what extend moth  
26 males (the tortricid moth *Lobesia botrana*) producing spermatophores exhibit plastic  
27 behavioral and physiological responses to different sexual audiences before and during  
28 mating and the consequences for their reproductive output. We found that males adjusted their  
29 mating behaviors and spermatophore size to a potentially elevated risk of sperm competition  
30 perceived before mating. In addition, males responded to the closed presence of females  
31 during mating by reducing their mating duration. Surprisingly, the various behavioral and  
32 physiological responses we highlighted here were not fully reflected in their reproductive  
33 performance as we did not reveal any effect on fecundity and fertility of their mate. The  
34 selective pressure exerted on males experiencing male-male competition could thus be  
35 sufficient to trigger adjustment in male mating behaviors but constrains physiological  
36 responses according to the perception of competition.

37

38 **KEY WORDS**

39 Male-male competition, Sperm competition, *Lobesia botrana*, sexual conflict, sexual  
40 selection, mating behavior

## 41 INTRODUCTION

42 Accessing females, producing and transferring sperm to fertilize the eggs are costly for males  
43 (Dewsbury, 1982; Nakatsuru & Kramer, 1982; Scharf *et al.*, 2013). Males thus employ  
44 strategies to optimize their mating frequency and fertilization success (Parker, 1978;  
45 Bonduriansky, 2001; Wedell *et al.*, 2002; Louâpre *et al.*, 2015). In polygamous mating systems,  
46 one of the main constraints males face with to increase their paternity is their ability to overpass  
47 the access of females by competitors (or rivals) (Emlen & Oring, 1977). The presence of rivals  
48 is indeed a key determinant of the sexual environment leading to sometimes strong male-male  
49 competition to access females, and intense sperm competition within the female reproductive  
50 tract (Simmons, 2002). Risk models predict an increase of male reproductive investment for  
51 accessing the females and fertilizing the eggs when competing with rivals (Parker *et al.*, 1997;  
52 Engqvist & Reinhold, 2005; Parker & Ball, 2005). In this regard, males express plastic response  
53 to male-male competition by perceiving direct cues, i.e the presence of potentially rival males  
54 (Bretman *et al.*, 2009), and indirect cues, those arising as a consequence of females mating by  
55 other males (Friberg, 2006). Therefore, males generally express adaptive reproductive  
56 strategies when mating, with a higher investment during and/or after mating (Wedell *et al.*,  
57 2002; Bretman *et al.*, 2011a). For instance, they ejaculate more sperm (Gage & Baker, 1991;  
58 Gage & Barnard, 1996; Wedell & Cook, 1999; Thomas & Simmons, 2007; Bretman *et al.*,  
59 2011a; Garbaczewska *et al.*, 2013), transfer more viable sperm (Magris 2021) and seminal fluid  
60 proteins (Wigby *et al.*, 2009) to the females. Such plastic response to male-male competition  
61 also involves modifications of the mating behaviors such as a more intense harassment of  
62 females (Sih & Krupa, 1995) and an extended mating duration under strong competition  
63 (Friberg, 2006; Bretman *et al.*, 2009).

64 Because males' response to sexual audience is essential for fitness, plastic male mating  
65 strategies should be adjusted to the level of male-male competition they perceive at the

66 appropriate timing (Parker *et al.*, 1997; Engqvist & Reinhold, 2005). In stable or predictably  
67 fluctuant sexual environment, many male species are known to anticipate future reproductive  
68 competition during development through physiological, neural and genomic mechanisms  
69 (Bretman *et al.*, 2016; Kasumovic & Brooks, 2011). When the operational local sex ratio is  
70 dynamic in time and space at small scales, as is frequently the case, efficient reproductive  
71 behaviors of males should be more responsive to the sexual environment experiencing when  
72 mating than to cues perceived during the development or before mating (Kasumovic *et al.*,  
73 2008; Punzalan *et al.*, 2010; Bretman *et al.*, 2016). Indeed, mating behaviors allow males to  
74 express quick responses to current male-male competition (Bretman *et al.*, 2010, 2011b). In  
75 contrast, to express a physiological response to male-male competition at the time of mating is  
76 challenging for males because it may be constrained by the time and resources having to be  
77 mobilized to produce and export sperm and associated products (Dewsbury, 1982; Wedell *et*  
78 *al.*, 2002). This sometime explains delayed development of juvenile males exposed to a higher  
79 risk of sperm competition allowing higher investment in testing development (Allen *et al.*,  
80 2011), and time lags between the perception of competition by mature males and the expression  
81 of the male responses (Rouse & Bretman, 2016).

82         The time required to produce, mature, and transfer viable sperm to female is amplified  
83 in taxa where males produce spermatophores, such as in Lepidoptera and Orthoptera (Mann,  
84 2012). Spermatophore contains sperm and accessory gland secretions that could be reinvested  
85 into female reproduction (Vahed, 1998). It can also play an important role in interindividual  
86 sperm competition by increasing the length of the refractory period since it was shown that a  
87 bigger spermatophore increases the female latency to remate (McNamara *et al.*, 2009). Given  
88 its content, spermatophore is thus a key determinant of the female reproductive output, and  
89 therefore, of the male fitness. When compete for mating, males producing a bigger  
90 spermatophore may overpass sperm competition within the female reproductive tract and

91 constrain the propensity of the mated female to remate. We can predict a strong positive  
92 relationship between the level of male-male competition in Lepidoptera and the size of the  
93 spermatophore transferred to the female, as it was shown for example in Orthoptera (Simmons  
94 *et al.*, 1993; Gage & Barnard, 1996). However, to exhibit such a plastic reproductive strategy  
95 sounds difficult for males producing spermatophores as their production is time and energy  
96 consuming (Muller *et al.*, 2016). We thus expect that the investment into the spermatophore is  
97 a delayed response of males to the reproductive competition, such plastic response would not  
98 be likely to be express at the time of mating.

99         Here, we used the European grapevine moth *Lobesia botrana* as a model system to  
100 investigate plastic responses to the risk of male-male competition and in what extend behavioral  
101 and physiological responses vary according to the sexual audience perceived before and during  
102 mating. In this species, polyandry is a heritable trait ( $h^2 = 0.40 \pm 0.12$ ) (Torres-Vila *et al.*, 2002)  
103 and is strongly associated with physiological factors, such as larval food nutrition (Torres-Vila  
104 *et al.*, 2004, Thiéry *et al.*, 2014a,b) and the size of the spermatophore received by females  
105 (Torres-Vila *et al.*, 1997). To assess the effect of reproductive competition, we measured the  
106 volume of spermatophore, the duration of mating and the latency before mating of partners  
107 experiencing different sexual audiences. We tested whether male can plastically adjust these  
108 traits to the presence of one or three rival males added to the mating arena 24 hours before  
109 mating or during mating. We also added one or three supplementary females in mating area to  
110 test if the plastic response of males to the socio-sexual environment, is specifically determined  
111 by the presence of rival males, not by the presence of conspecific females.

## 112 MATERIAL AND METHODS

113

### 114 Ethical Note

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

121

### 122 Field sampling

123 Larvae of *L. botrana* were collected on June 2015 (corresponding to the first larvae generation  
124 of the year) at the end of their larval cycle (fifth instar) on floral clusters (grape phenology 17  
125 – 25) (Eichhorn & Lorenz, 1977) in one vineyard planted with a single cultivar (*Vitis vinifera*  
126 cv Grenache, Senas plot, Roquemartine, France). Classically in this pest species, most larvae  
127 accomplish their whole development on a single grape stock or even a single bunch. Larvae  
128 completed their life cycle in the laboratory in small polyethylene boxes (60 x 40 cm, height 21  
129 cm) and fed *ad libitum* on bunches of the same cultivar sampled in the same place, at  $22 \pm 1$   
130 °C,  $60 \pm 10$  % RH at natural photoperiod. Larvae were checked daily until pupation, and pupae  
131 were gently removed from the grape clusters. Pupae were weighed to the nearest 0.01 mg  
132 (Precisa 262 SMA-FR microbalance) and placed individually in glass tubes (70 x 9 mm  
133 diameter) stoppered with cotton plugs, and then stored at 22 °C under natural photoperiod.  
134 Pupae were checked every morning, and newly emerged adults sexed.

135 **General design**

136 We performed two experiments for testing the ability of males to perceive and respond to the  
137 level of male-male competition prior (experiment 1) or during (experiment 2) mating. In the  
138 first experiment, virgin males were kept either alone, or by batch of two or four during 24h,  
139 before individually exposed to a virgin female. This experiment allowed to test for the ability  
140 of males to respond to the male's density before encountering a female. In the second  
141 experiment, a virgin female was proposed to a virgin male without prior male-male competition.  
142 Once the copulation started, either one or three supplementary virgin males were added to the  
143 mating chamber. This experiment allowed to test for a plastic response of the male engaged in  
144 copulation depending on the sexual audience. In this experiment, we also exposed some of the  
145 males to either one or three virgin females to assess the specific response of the male depending  
146 on the sex of the audience. For all matings occurring during the two experiments, half of the  
147 mated females were used to evaluate the male reproductive performance (i.e. spermatophore  
148 volume transferred to the female). The other half of the females allowed to evaluate the  
149 consequences of the male donation on the reproductive output of females (i.e. laying latency,  
150 fecundity, fertility). For all the experiments, only males from the field sampling were used.  
151 Females came from a laboratory breeding to minimize variance due to a female effect on the  
152 male behaviors (see Muller *et al.*, 2015 for a detailed procedure of the female rearing and  
153 selection procedure). For the two competition experiments, the sample sizes for every modality  
154 are given in the corresponding figures.

155

156 **Competition experiments**

157 *Experiment 1: Male-male competition prior to mating*

158 The experiment started at dusk. One 2-day-old virgin male was placed into a plastic box (15 x  
159 10 x 8 cm) either (i) alone (no competition treatment), (ii) with one 2-day-old virgin males



160 (moderate competition treatment), (iii) or three 2-day-old virgin males (high competition  
161 treatment) during 24 h. At dusk of the next day, each male of each treatment was placed into a  
162 new plastic box (mating chamber hereafter) with one 2-day-old virgin female originating from  
163 the stock population. The male and female sexual activities were then observed continuously  
164 during the following 4 hours. Mating was considered successful if the pair formation lasted  
165 more than one minute, which is the threshold over which genital coupling is completed. Once  
166 mating finished, both females engaged in pair were collected.

167

### 168 *Experiment 2: Male-male competition during mating*

169 The experiment started at dusk. One 2-day-old virgin male was placed into a mating plastic  
170 chamber (15 x 10 x 8 cm) with one 2-day-old virgin standardized female originating from the  
171 stock population for 4 hours. During this session, male and female sexual activities were  
172 continuously observed to detect the beginning of mating. Once mating occurred and lasted more  
173 than one minute, either no male, one (moderate competition treatment) or three (high  
174 competition treatment) rival field males of the same age were immediately added in the mating  
175 chamber using a small hole previously drilled in the lid (1 cm diameter). To control if mating  
176 males can distinguish the sex of the audience in the mating chamber, some of the males were  
177 exposed to one or three additional 2-day-old virgin standardized female (instead of males)  
178 originating from the stock population. Once mating finished, the females engaged in pair were  
179 collected.

180

## 181 **Behavioral and life history traits measurements**

### 182 *Mating behaviors*

183 For the first experiment (male-male competition prior to mating), we recorded the mating  
184 latency (time elapsed from the session's start until genital coupling) and the mating duration

185 (time between the pair formation and separation) of each pair. For the second treatment (male-  
186 male competition during mating), we measured the mating duration.

187

### 188 *The spermatophore volume*

189 Immediately after the end of mating, half of the mated females in all modalities of the two  
190 experiments were frozen at -25 °C for ten minutes and then were dissected on a glass slide. The  
191 bursa copulatrix containing the male spermatophore was removed to measure its size.  
192 Estimating spermatophore size by extrapolating its volume is a well-established method used  
193 in several studies on moths (Royer & McNeil, 1993; Foster & Ayers, 1996) and in previous  
194 works on *L. botrana* (Torres-Vila *et al.*, 1999; Muller *et al.*, 2016). We measured its length *l*,  
195 width *w* and thickness *t* under a stereomicroscope (NIKON SMZ1500) with a magnification of  
196 20X. The volume of the spermatophore was estimated as an ellipsoid balloon as in Torres Vila  
197 *et al.* (1999) ( $V = \pi/6 (l \times w \times t)$ ) after preliminary measures to check that this process is  
198 repeatable ( $n = 47$ ; *repeatability coefficient* = 0.863) (Lessells & Boag, 1987).

199

### 200 *The female reproductive output*

201 After one successful mating and natural separation of the pair, half of the females were  
202 individualized in glass tubes (70 x 9 mm diameter) stoppered with moistened cotton plugs, and  
203 then stored at 22 °C under natural photoperiod. These females were allowed to oviposit freely  
204 on the surface of the glass tub until their death. Female survival was checked daily. After the  
205 female's death, the eggs were incubated under the same conditions as moth maintenance for  
206 seven days. We recorded the achieved fecundity (mean number of eggs laid per female), and  
207 the female fertility (proportion of hatched eggs).

208

209

210 **Statistical analysis**

211 All the measured traits (mating behaviors, spermatophore volume and female reproductive  
212 output) were studied with linear models after applying square root or log transformations if  
213 necessary (mating latency and mating duration) or with GLM-quasi poisson errors (fertility).  
214 For the first experiment (male-male competition prior to mating), the number of conspecific  
215 males exposed to the focal male prior to mating, as well as the mass of the male and the female  
216 pupae engaged in mating were included in the model as independent factor and covariates. For  
217 the second experiment (male-male competition during mating), models incorporated the  
218 number of conspecifics exposed to the focal male during mating, their sex, and their interaction  
219 as factors. The mass of the males and the females engaged in mating were also included in the  
220 model as covariates. All these data were studied with analyses of covariance (ANCOVAs).  
221 Analysis that revealed significant effects were followed by post-hoc paired comparisons. All  
222 statistical analyses were carried out using R 4.0.5 software.

223 **RESULTS**

224 *Experiment 1: male-male competition prior to mating*

225 The number of conspecific males exposed to the focal male 24h prior to mating influenced  
226 both the mating latency and the mating duration (Table 1): the higher the number of  
227 conspecific males, the shorter the mating latency of the focal male (Figure 1A) and the longer  
228 the mating duration (Figure 1B). The number of conspecific males exposed to the focal male  
229 24h prior to mating also influenced the spermatophore volume transfer by the focal male to  
230 the female (Table 1): the spermatophore volume increased when the focal male was exposed  
231 to conspecific males prior to mating (Figure 1C). It was also influenced by the mass of the  
232 male (Table 1), as the spermatophore volume was positively correlated with the mass of the  
233 male engaged in mating (Pearson's  $r = 0.28$ ,  $t = 2.63$ ,  $df = 78$ ,  $P = 0.01$ ). After mating, neither  
234 the total number of eggs laid by the females (fecundity), nor the proportion of hatching eggs  
235 (fertility) were influenced by the number of conspecific males exposed to the focal male 24h  
236 prior to mating (Table 1). Fecundity was the only trait positively correlated with the mass of  
237 the females (Pearson's  $r = 0.58$ ,  $t = 5.96$ ,  $df = 71$ ,  $P < 0.001$ ).

238

239 *Experiment 2: male-male competition during mating*

240 The mating duration was influenced by the number of conspecifics exposed to the focal male  
241 during mating, this effect depended on the sex of the audience (Table 2): the mating duration  
242 shortened only when the focal males was exposed to females (either one or three) during  
243 mating (Figure 2A). Both the number of conspecifics exposed to the focal male and the sex of  
244 the audience influenced the spermatophore volume (Table 2): the focal male transferred a  
245 larger spermatophore when mating in the presence of one conspecific in comparison with no  
246 audience (Figure 2B). This effect was sex specific as larger spermatophores were transferred  
247 only in the case of a male sex audience (Figure 2C). To note, the spermatophore volume was

248 influenced by the male mass engaged in mating (Table 2), as larger spermatophores were  
249 transferred by heavier males (Pearson's  $r = 0.32$ ,  $t = 3.75$ ,  $df = 122$ ,  $P < 0.001$ ). Regarding the  
250 reproductive output of the mated female, neither the total number of eggs laid (fecundity) nor  
251 the proportion of hatching eggs (fertility) were influenced by the number of conspecifics  
252 exposed to the focal male during mating, whatever their sex (Table 2). As for the first  
253 competition experiment, fecundity was the only trait positively correlated with the mass of the  
254 females (Pearson's  $r = 0.39$ ,  $t = 4.41$ ,  $df = 109$ ,  $P < 0.001$ ).

255 **DISCUSSION**

256 The aim of our study was to quantify the ability of Lepidopteran males producing  
257 spermatophores to respond and adjust to male-male competition. Sexual audience consisted in  
258 three different densities of potential rivals exposed to focal males 24h before mating or during  
259 mating. Our results showed that the sexual audience strongly influenced the mating behaviors  
260 expressed by the males and the volume of the spermatophore they transferred to the females,  
261 but the effects differed according to the time at which the sexual audience is perceived. The  
262 mating latency decreased while the mating duration increased when the males were exposed  
263 to a larger number of competitors 24 hours before mating. During mating, the effect of the  
264 sexual audience depended on the sex of the audience as the mating duration decreased and the  
265 spermatophore size increased when the focal males were exposed to females. Despite these  
266 clear effects of the sexual audience on the male mating behaviors, we did not find any  
267 consequences on the reproductive output of the females mated by males experiencing  
268 different sexual audiences. Our results showed that males adjust their mating behaviors to a  
269 potentially elevated risk of sperm competition, but the various behavioral and physiological  
270 responses are not fully reflected in their reproductive performance.

271 Males exposed to different sexual audiences before and during mating expressed  
272 plastic sexual behavior and physiological traits, consistent with risk model predictions (Parker  
273 *et al.*, 1997; Engqvist & Reinhold, 2005). They showed higher motivation to access the  
274 available females and invest more during mating through longer mating duration and bigger  
275 spermatophore transfer when having exposed to potential rival males before mating. Previous  
276 studies in various species have reported extended mating duration when males were exposed  
277 to competitors prior to mating (Bretman *et al.*, 2009, 2013; Price *et al.*, 2012). Mating  
278 duration is known as a plastic trait highly responsive to the socio-sexual context during which  
279 it is expressed (Bretman *et al.*, 2011a, 2013). A reduced mating latency associated with a

280 prolonged mating duration may generate at least two benefits for the male: to have a  
281 privileged access to the females by outperforming the mating ability of the other males, and to  
282 decrease the propensity of the mated females to remate subsequently. Supplementary mating  
283 by the female indeed drastically alters the reproductive outcome of the male by decreasing its  
284 likelihood of paternity (Jennions & Petrie, 2000). Faced with this significant risk emerging  
285 when multiple males look for females, extended mating duration is viewed as a “mate  
286 guarding strategy” which significantly decreases sperm competition intensity in the female  
287 genital tractus (Carazo *et al.*, 2007; Mazzi *et al.*, 2009). Combined with a bigger  
288 spermatophore transfer in this case, this strategy also led to an extended mating refractoriness  
289 in the females, as it was showed for example, in the fruitfly *Drosophila montana* (Mazzi *et*  
290 *al.*, 2009), the almond moth *Cadra cautella* (McNamara *et al.*, 2009) and the apple moth  
291 *Epiphyas postvittana* (Foster & Ayers, 1996). Similarly, in *L.botrana*, the size of the  
292 spermatophore transferred to the female affects its motivation to remate: the bigger the  
293 spermatophore, the longer the refractory period after mating (Torres-Vila *et al.*, 1997; Muller  
294 *et al.*, 2016). In our study system, the extended mating duration likely initiated by the male  
295 seems beneficial for him as it may prevent females from remating immediately after the pair  
296 separation, ensuring the transferred sperm to fertilize the eggs (Gilchrist & Partridge, 2000).  
297 Remaining in pair for more than 1 hour is sufficient to reduce the probability that a female  
298 remates on the same day, mating occurring *in natura* between 2-3 hrs at dusk (Louâpre and  
299 Moreau, personal observation). Moreover, sperm generally reaches the spermatheca between  
300 2 and 5 hours after mating in several butterfly and moth species (Seth *et al.*, 2002; Marcotte *et*  
301 *al.*, 2005). The plastic behavior expressed by males experiencing male-male competition prior  
302 to mating could be particularly efficient in *L. botrana*, but it is usually observed in species  
303 with short mating or external spermatophore transfer (Simmons, 2002). To express specific

304 behaviors when perceived sexual audience before mating may thus evolved in species with  
305 internal spermatophore transfer as a “spermatophore guarding strategy” (Carazo *et al.*, 2007).

306 Plasticity in the male reproductive investment and mating behaviors is now known to  
307 evolve rapidly in populations depending on the sexual audience males are faced with (Dore *et*  
308 *al.*, 2021). Here, such plasticity in mating behaviors is only expressed when males perceived  
309 potential rival males 24 hours before mating. We did not reveal any effect of the close  
310 presence of rival males on the mating duration when they were perceived by the focal male  
311 during mating. Our results thus showed that the sexual audience is a key information  
312 perceived and retained by the insect males for at least 24 hours, and responsible for the  
313 expression of subsequent plastic mating behaviors, even when potential rivals disappear at the  
314 moment of mating. Rouse *et al.* (2018) demonstrated that plastic response of *Drosophila*  
315 *melanogaster* males to sperm competition is based on their ability to assess sexual audience  
316 when exposed to rival males through olfactory learning and memory. Long-lasting memory of  
317 sperm competition risk experienced by males is suspected to be involved to assess the socio-  
318 sexual context through various cues (acoustic, chemical, visual, tactile), as it was showed in  
319 *D. melanogaster* (Bretman *et al.*, 2011b) and the seed beetle *Callosobruchus maculatus* (Liu  
320 *et al.*, 2020). Such a cognitive ability is likely to guide *L. botrana* males for the expression of  
321 their plastic mating behaviors: the presence and/or the number of rival males may be  
322 information retained by the nervous system causing later arousal of the male’s motivation  
323 faced with females. However, males are able to perceive the sexual audience during mating if  
324 composed of females, as in our experiments, the mating duration decreased when  
325 supplementary females were in closed proximity of the pair. Such a sex-specific effect,  
326 presumably caused by detecting further opportunity of mating by the male, reinforces the  
327 hypothesis that multiple cues are perceived by males engaged in mating to assess the quality  
328 and the density of the sexual audience.



329 Besides plastic mating behaviors expressed by males responding to the sexual context  
330 before and during mating, they also exhibit plastic physiological response. They transferred a  
331 bigger spermatophore when exposed to conspecifics, this effect was observed within the two  
332 competition experiments when males were exposed to competitors 24 hours before mating, or  
333 during mating. We thus highlight the ability of *L. botrana* males to express a physiological  
334 response quickly depending on the perceived sexual context. Surprisingly, such plastic  
335 response was not followed by an increase in fecundity or fertility by the mated female. In a  
336 previous study on *L. botrana*, it was showed that a bigger spermatophore (for which a  
337 significant part of variance was attributed to the host plant consumed by the males at larval  
338 stages) induced higher fecundity (Muller *et al.*, 2015). The present result appears to contrast  
339 with previous observations where higher investment in the male's ejaculate – in this case,  
340 after exposure to rival males – translates into an increase of its reproductive success, as it was  
341 also the case for example in *D. melanogaster* (Bretman *et al.*, 2009) and the beetle, *Tenebrio*  
342 *molitor* (Gage & Baker, 1991). This intuitively response may be explained by the ability of  
343 males to plastically adjust the number of sperm inseminated when exposed to rival males  
344 before or during mating. Regarding our results, two plausible explanations of these diverging  
345 results may emerge from the reproduction mode of moths. First, males produce internal  
346 spermatophores containing sperm and various secretions produced by accessory glands  
347 (Gillott, 2003; Ramm, 2020). Non-sperm components (e.g. parasperm, water, proteins,  
348 macromolecules) modulate mating behaviors and reproductive output of the females and play  
349 key roles in male-male competition (Perry *et al.*, 2013). Here, the lack of the expected fitness  
350 gain for *L. botrana* males faced with rival males suggests that the bigger spermatophores  
351 transferred by the male result from higher investment in non-sperm components rather than  
352 on sperm allocation. It is nonetheless possible that males *L. botrana* adjust the number of  
353 sperm in their spermatophore but to a less extend way. In this hypothesis, distinction must be

354 done regarding the sperm type to be produced: moths are known to produce a fertile eupyrene  
355 form and a non-fertile apyrene form (Gage & Cook, 1994). Apyren sperm lacks genetic  
356 material and play various roles such as, nutrient provisioning, sperm facilitation and  
357 competition (Silberglied *et al.*, 1984; Swallow & Wilkinson, 2002). We can thus hypothesize  
358 that males *L. botrana* exposed to rivals also express a physiological plastic response to the  
359 sexual audience by modifying their investment in the two sperm forms without any  
360 consequences on the female fecundity and fertility (a potential consequence on the mating  
361 behaviors of females, especially their propensity for remating is not excluded). This  
362 hypothesis seems unlikely for at least two reasons. The first reason reflects the lack of  
363 evidence for such a plastic response in moths. In moth species, such as *Plodia interpunctella*,  
364 the presence of a rival male closed to the focal male for 24 hours has no effect upon the  
365 number of both sperms – eurpyren nor apyren forms – within the spermatophore (Cook &  
366 Gage, 1995). The second reason is based on the adequate timing required for adjustment of  
367 sperm quality and quantity. While anticipatory plasticity is now coming to be recognized in  
368 ejaculate adjustment in response to male-male competition, adjustment of spermatogenesis is  
369 time consuming (Magris, 2021). Hypothetic mechanisms behind adjustment of sperm quality  
370 and quantity mostly involve changes occurring during sperm maturation, which require at  
371 least several days (Magris, 2021). Consequently, during the course of our experiments lasting  
372 from 24 hours prior mating to the moment of mating, rapid changes in spermatophore  
373 composition in response to the sexual audience are more likely to be mediated by adjustment  
374 of non-sperm component rather than by sperm modification. Further investigation is required  
375 to test for the specific effect of the plastic response highlighted here on the female's  
376 propensity to remate and paternity when several matings occur.

377         To conclude, this study clearly shows that males are highly sensitive to the sexual  
378 audience before and during mating and can adapt their mating behavior and physiological

379 response quickly. These plastic responses are expected to evolve in situations where the  
380 probability to find a mate is stochastic in a short temporal window, that is the case of *L.*  
381 *botrana* in the field when encountering a female is rare and when the first mating induce a  
382 strong inhibition of female mating, a situation also frequently encountered in other  
383 lepidopteran species (Parker & Vahed, 2010; Jarrige *et al.*, 2016). The selective pressure  
384 exerted on males experiencing male-male competition could be sufficient to trigger  
385 adjustment in male mating behavior according to the perception of competition and to select  
386 sensitive mechanisms allowing to perceive competition.

387 **AUTHOR CONTRIBUTION STATEMENT**

388 Conceptualization and methodology (KM, JM, DT), Investigation (KM, SBA), Formal analysis  
389 (PL), Writing (PL, JM), Review and editing (PL, JM).

390

391 **COMPILANCE WITH ETHICAL STANDARDS**

392 Conflict of interest - The authors declare they have no conflict of interest. Ethical Approval -  
393 All applicable institutional and/or national guidelines for the care and use of animals were  
394 followed.

395

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573

574 **FIGURE LEGENDS**

575

576 **Figure 1.** Effects of the number of conspecific males (0, 1 or 3) on A-mating latency (time  
577 elapsed from the session's start until genital coupling), B-mating duration (time between the  
578 pair formation and separation), C-spermatophore volume, in the case where the focal males  
579 have been exposed to them 24h prior to mating. Represented values correspond to means  $\pm$   
580 95% confidence intervals. Asterisks highlight significant differences (\*\*\*P < 0.001, \*\*P <  
581 0.01, \*P < 0.05, n.s. non-significant) and numbers refer to sample sizes.

582

583 **Figure 2.** Effects of the number of conspecifics (0, 1 or 3) and their sex (males are  
584 represented by triangles, females by points, squares represent the combined effect of the two  
585 sexes) on A-mating duration (time elapsed from the session's start until genital coupling),  
586 B/C-spermatophore volume, in the case where the focal males are exposed to a sexual  
587 audience when mating. Represented values correspond to means  $\pm$  95% confidence intervals.  
588 Letters and asterisks highlight significant differences \*P < 0.05, n.s. non-significant) and  
589 numbers refer to sample sizes.

590

591 **TABLES**

592 **Table 1.** Effects of the number of conspecific males (0, 1 or 3) and the mass of the two partners engaged in mating on the reproductive traits and  
 593 behaviors when focal males were exposed to the conspecifics 24h prior to mating (experiment 1). Bold font indicates statistical significance.

	Number of conspecific males		Mass of the male		Mass of the female	
	<i>Test value</i>	<i>P</i>	<i>Test value</i>	<i>P</i>	<i>Test value</i>	<i>P</i>
Mating latency <sup>a</sup>	<b><math>F_{2,145} = 14.03</math></b>	<b>&lt;0.001</b>	$F_{1,145} = 0.01$	0.94	$F_{1,145} = 0.1$	0.76
Mating duration <sup>a</sup>	<b><math>F_{2,148} = 6.28</math></b>	<b>0.002</b>	$F_{1,148} = 2.79$	0.1	$F_{1,148} = 0.1$	0.28
Spermatophore volume <sup>a</sup>	<b><math>F_{2,75} = 5.59</math></b>	<b>0.005</b>	<b><math>F_{1,75} = 23.21</math></b>	<b>&lt;0.001</b>	$F_{1,75} = 2.83$	0.1
Fecundity <sup>a</sup>	$F_{2,68} = 2.32$	0.11	$F_{1,68} = 0.69$	0.41	<b><math>F_{1,68} = 35.16</math></b>	<b>&lt;0.001</b>
Fertility <sup>b</sup>	$\chi^2_2 = 0.003$	0.34	$\chi^2_1 = 0$	0.76	$\chi^2_1 = 0$	0.89

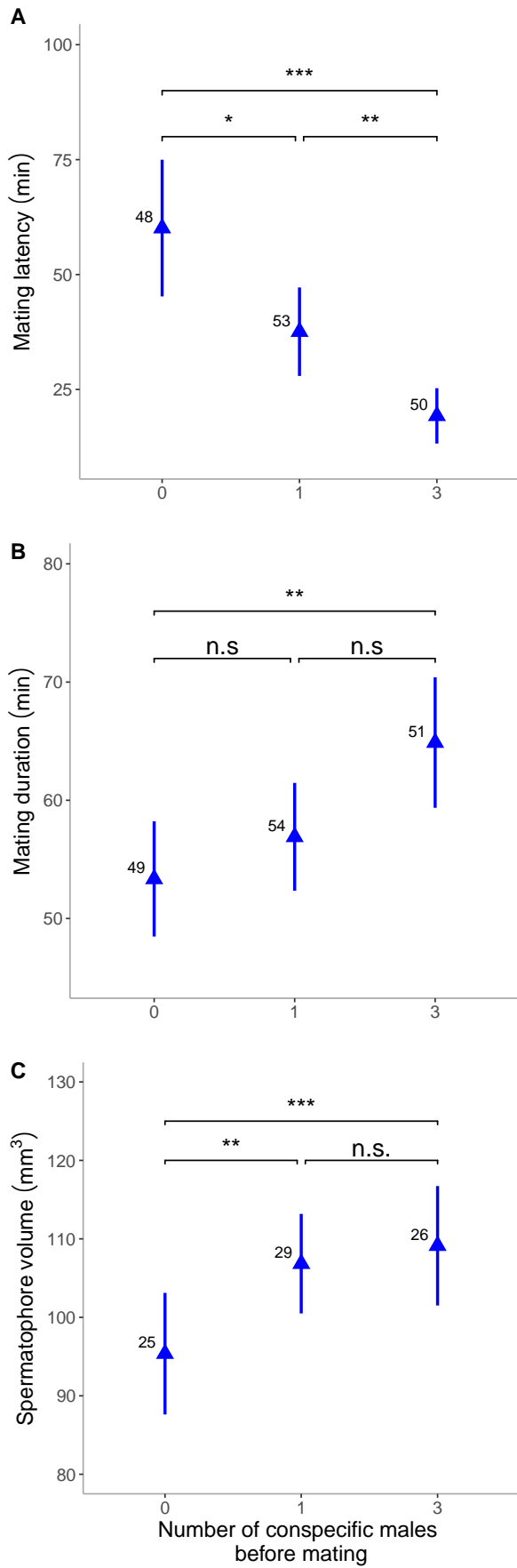
<sup>a</sup> ANCOVA, <sup>b</sup> GLM with quasi-poisson errors

595 **Table 2.** Effects of the number of conspecifics (0, 1 or 3), the sex of the audience added in mating box (male or female), their interaction, and the  
596 mass of the two partners engaged in mating on the reproductive traits and behaviors when focal males were exposed to the conspecifics during  
597 mating (experiment 2). Bold font indicates statistical significance.

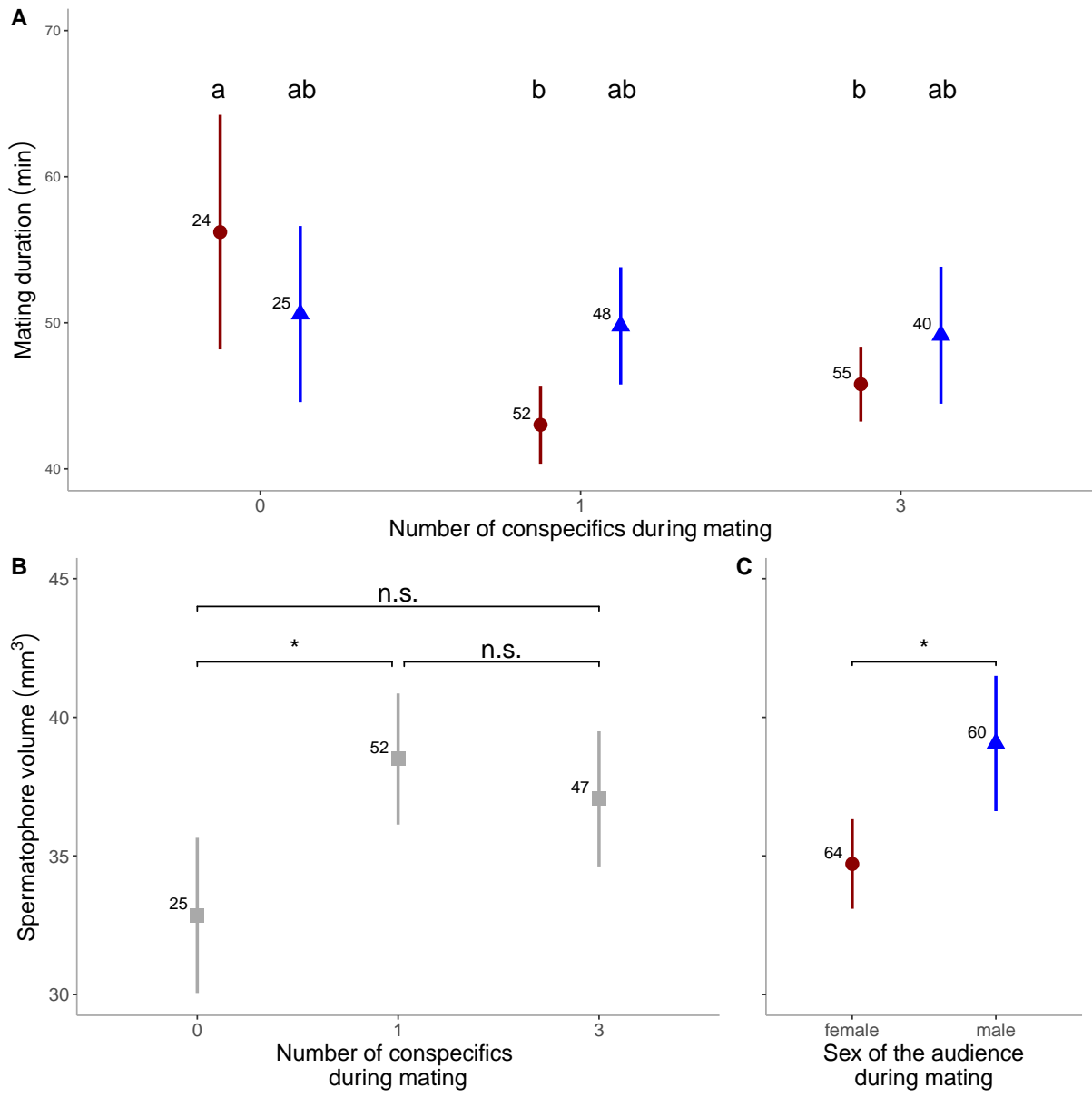
	Number of conspecifics		Sex of the conspecifics		Interaction Number:Sex		Mass of the male		Mass of the female	
	<i>Test value</i>	<i>P</i>	<i>Test value</i>	<i>P</i>	<i>Test value</i>	<i>P</i>	<i>Test value</i>	<i>P</i>	<i>Test value</i>	<i>P</i>
Mating duration	<b><math>F_{2,236} = 5.06</math></b>	<b>0.007</b>	$F_{1,236} = 3.08$	0.08	<b><math>F_{2,236} = 3.33</math></b>	<b>0.037</b>	$F_{1,236} = 0.15$	0.69	$F_{1,236} = 1.87$	0.17
Spermatophore volume	<b><math>F_{2,116} = 3.84</math></b>	<b>0.02</b>	<b><math>F_{1,116} = 6.69</math></b>	<b>0.01</b>	$F_{2,116} = 1.77$	0.17	<b><math>F_{1,116} = 20.4</math></b>	<b>&gt;0.001</b>	$F_{1,116} = 0.94$	0.33
Fecundity	$F_{2,103} = 1.53$	0.22	$F_{1,103} = 2.30$	0.13	$F_{2,103} = 0.39$	0.68	$F_{1,103} = 0.06$	0.81	<b><math>F_{1,103} = 15.11</math></b>	<b>&gt;0.001</b>
Fertility	$\chi_2^2 = 0.008$	0.39	$\chi_1^2 = 0.005$	0.29	$\chi_2^2 = 0$	0.93	$\chi_1^2 = 0.003$	0.39	$\chi_1^2 = 0$	0.69

<sup>a</sup> ANCOVA, <sup>b</sup> GLM with quasi-poisson errors

598



601 **Figure 2.**



602