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

Philippe Louapre, Karen Muller, Soline Bettencourt-Amarante, Denis Thiery, Jérôme Moreau

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

2 **reproductive outputs**

3

4 Philippe LOUÂPRE [a*], Karen MULLER [a], Soline BETTENCOURT-AMARANTE [a], Denis

5 THIERY [b, c], Jérôme MOREAU [a]

6

7 [a] UMR CNRS 6282 Biogeosciences, Université Bourgogne Franche-Comté, 6 Boulevard

8 Gabriel, 21000 Dijon, France

9 [b] INRA UMR 1065 Santé et Agroecologie du Vignoble, Institut des Science de la Vigne et du

10 Vin, Villenave d'Ornon Cedex, France

11 [c] Université de Bordeaux, INRA UMR 1065, Save, Bordeaux Sciences Agro, Villenave

12 d'Ornon Cedex, France

13

14 *Correspondence: P. LOUÂPRE, UMR CNRS 6282 Biogeosciences, Université Bourgogne

15 Franche-Comté, 6 Boulevard Gabriel, 21000 Dijon, France

16 E-mail address: philippe.louapre@u-bourgogne.fr

17

ABSTRACT

Males evolved plastic strategies to respond to male-male competition and exhibit adaptive

traits and behaviors maximizing their access to the females and limiting sperm competition.

Mating behaviors allow males to express quick responses to current sexual audience, i.e the

number of nearby conspecifics prone to mate. In contrast, physiological responses are

frequently delayed because they are constrained by the time and resources having to be

mobilized to produce and export sperm and associated products. This is especially critical in

species for which males produce spermatophores. Here we investigated in what extend moth

males (the tortricid moth *Lobesia botrana*) producing spermatophores exhibit plastic

behavioral and physiological responses to different sexual audiences before and during

mating and the consequences for their reproductive output. We found that males adjusted their

mating behaviors and spermatophore size to a potentially elevated risk of sperm competition

perceived before mating. In addition, males responded to the closed presence of females

during mating by reducing their mating duration. Surprisingly, the various behavioral and

physiological responses we highlighted here were not fully reflected in their reproductive

performance as we did not reveal any effect on fecundity and fertility of their mate. The

selective pressure exerted on males experiencing male-male competition could thus be

sufficient to trigger adjustment in male mating behaviors but constrains physiological

responses according to the perception of competition.

KEY WORDS

Male-male competition, Sperm competition, *Lobesia botrana*, sexual conflict, sexual

selection, mating behavior

INTRODUCTION

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

Because males' response to sexual audience is essential for fitness, plastic male mating 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 though 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.

MATERIAL AND METHODS

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. Dissected females were frozen at -25 °C for 10 min in a freezer prior to decapitation.

Field sampling

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

General design

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

Competition experiments

Experiment 1: Male-male competition prior to mating

The experiment started at dusk. One 2-day-old virgin male was placed into a plastic box (15 x 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 side. 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

Statistical analysis

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

RESULTS

Experient 1: male-male competition prior to mating

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

Experiment 2: male-male competition during mating

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

DISCUSSION

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

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

AUTHOR CONTRIBUTION STATEMENT

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

COMPILANCE WITH ETHICAL STANDARDS

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

REFERENCES

Allen, L.E., Barry, K.L., Holwell, G.I. & Herberstein, M.E. (2011) Perceived risk of sperm competition affects juvenile development and ejaculate expenditure in male praying mantids. *Animal Behaviour*, **82**, 1201–1206.

Bonduriansky, R. (2001) The evolution of male mate choice in insects: A synthesis of ideas and evidence. *Biological Reviews of the Cambridge Philosophical Society*, **76**, 305–339.

Bretman, A., Fricke, C. & Chapman, T. (2009) Plastic responses of male *Drosophila melanogaster* to the level of spermcompetition increase male reproductive fitness. *Proceedings of the Royal Society B: Biological Sciences*, **276**, 1705–1711.

Bretman, A., Fricke, C., Hetherington, P., Stone, R. & Chapman, T. (2010) Exposure to rivals and plastic responses to sperm competition in *Drosophila melanogaster*. *Behavioral Ecology*, **21**, 317–321.

Bretman, A., Fricke, C., Westmancoat, J.D. & Chapman, T. (2016) Effect of competitive cues on reproductive morphology and behavioral plasticity in male fruitflies. *Behavioral Ecology*, **27**, 452–461.

Bretman, A., Gage, M.J.G. & Chapman, T. (2011a) Quick-change artists: Male plastic behavioural responses to rivals. *Trends in Ecology and Evolution*, **26**, 467–473.

Bretman, A., Westmancoat, J.D. & Chapman, T. (2013) Male control of mating duration following exposure to rivals in fruitflies. *Journal of Insect Physiology*, **59**, 824–827.

Bretman, A., Westmancoat, J.D., Gage, M.J.G. & Chapman, T. (2011b) Males use multiple, redundant cues to detect mating rivals. *Current Biology*, **21**, 617–622.

Carazo, P., Font, E. & Alfthan, B. (2007) Chemosensory assessment of sperm competition levels and the evolution of internal spermatophore guarding. *Proceedings of the Royal Society B: Biological Sciences*, **274**, 261–267.

Cook, P.A. & Gage, M.J.G. (1995) Effects of risks of sperm competition on the numbers of

426   eupyrene and apyrene sperm ejaculated by the moth *Plodia interpunctella* (Lepidoptera:

427   Pyralidae). *Behavioral Ecology and Sociobiology*, **36**, 261–268.

428   Dewsbury, D.A. (1982) Ejaculate cost and male choice. *American Naturalist*, **119**, 601–610.

429   Dore, A.A., Rostant, W.G., Bretman, A. & Chapman, T. (2021) Plastic male mating behavior

430   evolves in response to the competitive environment. *Evolution*, **75**, 101–115.

431   Eichhorn, K.W. & Lorenz, D.H. (1977) Phenological development stages of the grape vine.

432   *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes*, **29**, 119–120.

433   Emlen, S.T. & Oring, L.W. (1977) Ecology, sexual selection, and the evolution of mating

434   systems. *Science*.

435   Engqvist, L. & Reinhold, K. (2005) Pitfalls in experiments testing predictions from sperm

436   competition theory. *Wiley Online Library*, **18**, 116–123.

437   Foster, S.P. & Ayers, R.H. (1996) Multiple mating and its effects in the lightbrown apple

438   moth, *Epiphyas postvittana* (walker). *Journal of Insect Physiology*, **42**, 657–667.

439   Friberg, U. (2006) Male perception of female mating status: its effect on copulation duration,

440   sperm defence and female fitness. *Animal Behaviour*, **72**, 1259–1268.

441   Gage, A.R. & Barnard, C.J. (1996) Male crickets increase sperm number in relation to

442   competition and female size. *Behavioral Ecology and Sociobiology*, **38**, 349–353.

443   Gage, M.J.G. & Baker, R.R. (1991) Ejaculate size varies with socio-sexual situation in an

444   insect. *Ecological Entomology*, **16**, 331–337.

445   Gage, M.J.G. & Cook, P.A. (1994) Sperm size or numbers? Effects of nutritional stress upon

446   eupyrene and apyrene sperm production strategies in the moth *Plodia interpunctella*

447   (Lepidoptera: Pyralidea). *Functional Ecology*, 594–599.

448   Garbaczewska, M., Billeter, J.C. & Levine, J.D. (2013) *Drosophila melanogaster* males

449   increase the number of sperm in their ejaculate when perceiving rival males. *Journal of*

450   *Insect Physiology*, **59**, 306–310.

451 Gilchrist, A.S. & Partridge, L. (2000) Why It Is Difficult to Model Sperm Displacement in
452   *Drosophila Melanogaster*: The Relation Between Sperm Transfer and Copulation
453   Duration. *Evolution*, **54**, 534–542.

454 Gillott, C. (2003) Male Accessory Gland Secretions: Modulators of Female Reproductive
455   Physiology and Behavior. *Annual Review of Entomology*, **48**, 163–184.

456 Jarrige, A., Kassis, A., Schmoll, T. & Goubault, M. (2016) Recently mated males of a lek-
457   mating insect intensify precopulatory mate guarding under male competition. *Animal*
458   *Behaviour*, **117**, 21–34.

459 Jennions, M.D. & Petrie, M. (2000) Why do females mate multiply? A review of the genetic
460   benefits. *Biological Reviews*, **75**, 21–64.

461 Kasumovic, M.M. & Brooks, R.C. (2011) It's All Who You Know: The Evolution Of
462   Socially Cued Anticipatory Plasticity As A Mating Strategy. *The Quarterly Review of*
463   *Biology*, **86**, 181–197.

464 Kasumovic, M.M., Bruce, M.J., Andrade, M.C.B. & Herberstein, M.E. (2008) Spatial and
465   Temporal Demographic Variation Drives Within-Season Fluctuations in Sexual
466   Selection. *Evolution*, **62**, 2316–2325.

467 Lessells, C.M. & Boag, P.T. (1987) Unrepeatable repeatabilities: a common mistake. *The*
468   *Auk*, **104**, 116–121.

469 Liu, J., Zhang, Y., Zheng, X.L., He, X.Z. & Wang, Q. (2020) Combined cues of male
470   competition influence spermatozoal investment in a moth. *Functional Ecology*, **34**,
471   1223–1234.

472 Louâpre, P., Fauvergue, X., Baaren, J. Van & Martel, V. (2015) The male mate search: An
473   optimal foraging issue? *Current Opinion in Insect Science*, **9**.

474 Magris, M. (2021) Strategic adjustment of ejaculate quality in response to variation of the
475   socio-sexual environment. *Behavioral Ecology and Sociobiology*, **75**.

476   Mann, T. (2012) *Spermatophores: Development, Structure, Biochemical Attributes and Role*

477   *in the Transfer of Spermatozoa*. Springer Science & Business Media.

478   Marcotte, M., Delisle, J. & McNeil, J.N. (2005) Impact of male mating history on the

479   temporal sperm dynamics of *Choristoneura rosaceana* and *C. fumiferana* females.

480   *Journal of Insect Physiology*, **51**, 537–544.

481   Mazzi, D., Kesäniemi, J., Hoikkala, A. & Klappert, K. (2009) Sexual conflict over the

482   duration of copulation in *Drosophila montana*: Why is longer better? *BMC*

483   *Evolutionary Biology*, **9**.

484   McNamara, K.B., Elgar, M.A. & Jones, T.M. (2009) Large spermatophores reduce female

485   receptivity and increase male paternity success in the almond moth, *Cadra cautella*.

486   *Animal Behaviour*, **77**, 931–936.

487   Muller, K., Thiéry, D., Moret, Y. & Moreau, J. (2015) Male larval nutrition affects adult

488   reproductive success in wild European grapevine moth (*Lobesia botrana*). *Behavioral*

489   *Ecology and Sociobiology*, **69**, 39–47.

490   Muller, K., Thiéry, D., Motreuil, S. & Moreau, J. (2016) What makes a good mate? Factors

491   influencing male and female reproductive success in a polyphagous moth. *Animal*

492   *Behaviour*, **120**, 31–39.

493   Nakatsuru, K. & Kramer, D.L. (1982) Is sperm cheap? Limited male fertility and female

494   choice in the lemon tetra (pisces, characidae). *Science*, **216**, 753–755.

495   Parker, D.J. & Vahed, K. (2010) The intensity of pre-and post-copulatory mate guarding in

496   relation to spermatophore transfer in the cricket *Gryllus bimaculatus*. *Journal of*

497   *Ethology*, **28**, 245–249.

498   Parker, G.A. (1978) Evolution of Competitive Mate Searching. *Annual Review of*

499   *Entomology*, **23**, 173–196.

500   Parker, G.A. & Ball, M.A. (2005) Sperm competition, mating rate and the evolution of testis

501   and ejaculate sizes: A population model. *Biology Letters*, **1**, 235–238.

502   Parker, G.A., Ball, M.A., Stockley, P. & Gage, M.J.G. (1997) Sperm competition games: A

503   prospective analysis of risk assessment. *Proceedings of the Royal Society B: Biological*

504   *Sciences*, **264**, 1793–1802.

505   Perry, J.C., Sirot, L. & Wigby, S. (2013) The seminal symphony: How to compose an

506   ejaculate. *Trends in Ecology and Evolution*, **28**, 414–422.

507   Price, T.A.R., Lizé, A., Marcello, M. & Bretman, A. (2012) Experience of mating rivals

508   causes males to modulate sperm transfer in the fly *Drosophila pseudoobscura*. *Journal*

509   *of Insect Physiology*, **58**, 1669–1675.

510   Punzalan, D., Rodd, F.H. & Rowe, L. (2010) Temporally Variable Multivariate Sexual

511   Selection on Sexually Dimorphic Traits in a Wild Insect Population. *The American*

512   *Naturalist*, **175**, 401–414.

513   Ramm, S.A. (2020) Seminal fluid and accessory male investment in sperm competition:

514   Seminal fluid and sperm competition. *Philosophical Transactions of the Royal Society*

515   *B: Biological Sciences*, **375**.

516   Rouse, J. & Bretman, A. (2016) Exposure time to rivals and sensory cues affect how quickly

517   males respond to changes in sperm competition threat. *Animal Behaviour*, **122**, 1–8.

518   Rouse, J., Watkinson, K. & Bretman, A. (2018) Flexible memory controls sperm competition

519   responses in male *Drosophila melanogaster*. *Proceedings of the Royal Society B:*

520   *Biological Sciences*, **285**.

521   Royer, L. & McNeil, J.N. (1993) Male investment in the European corn borer, *Ostrinia*

522   *nubilalis* (Lepidoptera: Pyralidae): impact on female longevity and reproductive

523   performance. *Functional Ecology*, 209–215.

524   Scharf, I., Peter, F. & Martin, O.Y. (2013) Reproductive Trade-Offs and Direct Costs for

525   Males in Arthropods. *Evolutionary Biology*, **40**, 169–184.

526 Seth, R.K., Kaur, J.J., Rao, D.K. & Reynolds, S.E. (2002) Sperm transfer during mating,

527   movement of sperm in the female reproductive tract, and sperm precedence in the

528   common cutworm *Spodoptera litura*. *Physiological Entomology*, **27**, 1–14.

529 Sih, A. & Krupa, J.J. (1995) Interacting effects of predation risk and male and female density

530   on male/female conflicts and mating dynamics of stream water striders. *Behavioral*

531   *Ecology*, **6**, 316–325.

532 Silberglied, R.E., Shepherd, J.G. & Dickinson, J.L. (1984) Eunuchs: the role of apyrene

533   sperm in Lepidoptera? *The American Naturalist*, **123**, 255–265.

534 Simmons, L.W. (2002) *Sperm Competition and Its Evolutionary Consequences in the Insects*.

535   *Sperm Competition and Its Evolutionary Consequences in the Insects*. Princeton

536   University Press, Princeton.

537 Simmons, L.W., Craig, M., Llorens, T., Schinzig, M. & Hosken, D. (1993) Bushcricket

538   spermatophores vary in accord with sperm competition and parental investment theory.

539   *Proceedings of the Royal Society B: Biological Sciences*, **251**, 183–186.

540 Swallow, J.G. & Wilkinson, G.S. (2002) The long and short of sperm polymorphisms in

541   insects. *Biological Reviews of the Cambridge Philosophical Society*, **77**, 153–182.

542 Thiéry, D., Monceau, K., Moreau, J. (2014a) Different emergence phenology of European

543   grapevine moth (*Lobesia botrana*, Lepidoptera: Tortricidae) on six varieties of grapes.

544   *Bulletin of Entomological Research*, **104**, 277-287.

545 Thiéry D., Monceau K., Moreau J. (2014b) Larval intraspecific competition for food in the

546   European grapevine moth *Lobesia botrana*. *Bulletin of Entomological Research*, **104**,

547   517-524.

548 Thomas, M.L. & Simmons, L.W. (2007) Male crickets adjust the viability of their sperm in

549   response to female mating status. *The American Naturalist*, **170**, 190–195.

550 Torres-Vila, L.M., Rodríguez-Molina, M.C. & Jennions, M.D. (2004) Polyandry and

551   fecundity in the Lepidoptera: can methodological and conceptual approaches bias

552   outcomes? *Behavioral Ecology and Sociobiology*, **55**, 315–324.

553   Torres-Vila, L.M., Rodriguez-Molina, M.C., Roehrich, R. & Stockel, J. (1999) Vine

554   phenological stage during larval feeding affects male and female reproductive output of

555   *Lobesia botrana* (Lepidoptera: Tortricidae). *Bulletin of Entomological Research*, **89**,

556   549–556.

557   Torres-Vila, L.M., Rodríguez-Molina, M.C. & Stockel, J. (2002) Delayed mating reduces

558   reproductive output of female European grapevine moth, *Lobesia botrana* (Lepidoptera:

559   Tortricidae). *Bulletin of Entomological Research*, **92**, 241.

560   Torres-Vila, L.M., Stockel, J. & Rodríguez-Molina, M.C. (1997) Physiological factors

561   regulating polyandry in *Lobesia botrana* (Lepidoptera: Tortricidae). *Physiological*

562   *Entomology*, **22**, 387–393.

563   Vahed, K. (1998) The function of nuptial feeding in insects: a review of empirical studies.

564   *Biological Reviews*, **73**, 43–78.

565   Wedell, N. & Cook, P.A. (1999) Butterflies tailor their ejaculate in response to sperm

566   competition risk and intensity. *Proceedings of the Royal Society B: Biological Sciences*,

567   **266**, 1033.

568   Wedell, N., Gage, M.J.G. & Parker, G.A. (2002) Sperm competition, male prudence, and

569   sperm-limited females. *Trends in Ecology and Evolution*, **17(7)**, 313–320.

570   Wigby, S., Sirot, L.K., Linklater, J.R., Buehner, N., Calboli, F.C.F., Bretman, A., *et al.* (2009)

571   Seminal Fluid Protein Allocation and Male Reproductive Success. *Current Biology*, **19**,

572   751–757.

573

574 **FIGRE 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

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.

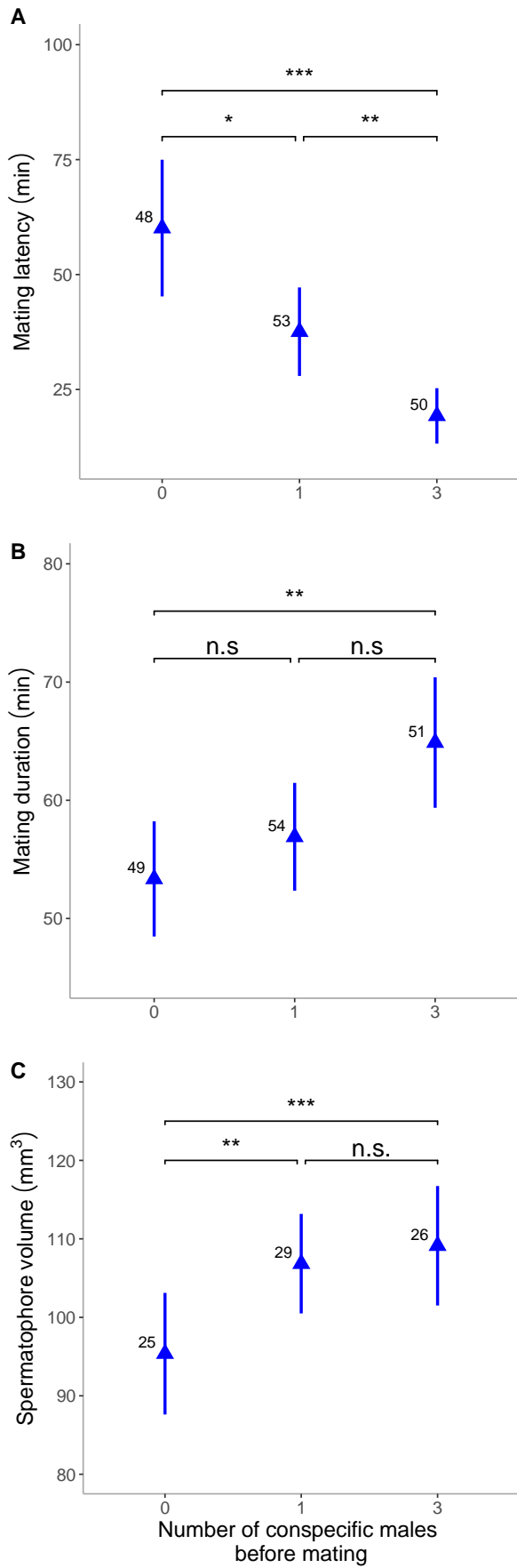|	Number of conspecific males		Mass of the male		Mass of the female	
	Test value	*P*	*Test value*	*P*	*Test value*	*P*
Mating latency[a]	$\boldsymbol{F_{2,145} = 14.03}$	**<0.001**	$F_{1,145} = 0.01$	0.94	$F_{1,145} = 0.1$	0.76
Mating duration[a]	$\boldsymbol{F_{2,148} = 6.28}$	**0.002**	$F_{1,148} =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

[a] ANCOVA, [b] GLM with quasi-poisson errors

594

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

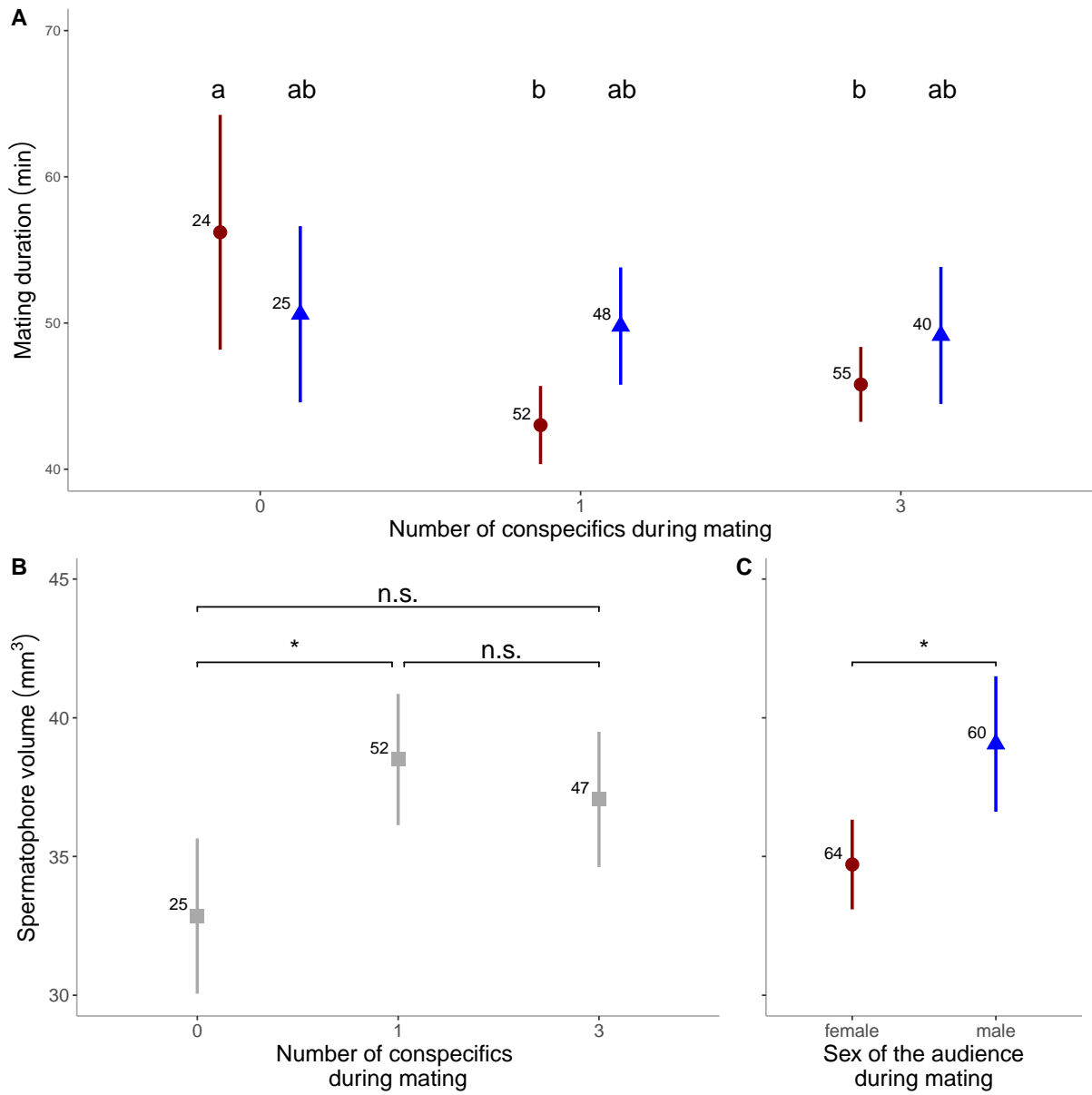597 mating (experiment 2). Bold font indicates statistical significance.

	Number of conspecifics		Sex of the conspecifics		Interaction Number:Sex		Mass of the male		Mass of the female	
	Test value	*P*	*Test value*	*P*	*Test value*	*P*	*Test value*	*P*	*Test value*	*P*
Mating duration	$\boldsymbol{F_{2,236} = 5.06}$	**0.007**	$F_{1,236} = 3.08$	0.08	$\boldsymbol{F_{2,236} = 3.33}$	**0.037**	$F_{1,236} = 0.15$	0.69	$F_{1,236} = 1.87$	0.17
Spermatophore volume	$\boldsymbol{F_{2,116} = 3.84}$	**0.02**	$\boldsymbol{F_{1,116} = 6.69}$	**0.01**	$F_{2,116} = 1.77$	0.17	$\boldsymbol{F_{1,116} = 20.4}$	**>0.001**	$F_{1,116} = 0.94$	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	$\boldsymbol{F_{1,103} = 15.11}$	**>0.001**
Fertility	$\chi^2_2 = 0.008$	0.39	$\chi^2_1 = 0.005$	0.29	$\chi^2_2 = 0$	0.93	$\chi^2_1 = 0.003$	0.39	$\chi^2_1 = 0$	0.69

[a] ANCOVA, [b] GLM with quasi-poisson errors

598

Figure 1.

601   **Figure 2.**

602