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

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

The time required to produce, mature, and transfer viable sperm to female is amplified in taxa where males produce spermatophores, such as in Lepidoptera and Orthoptera (Mann, 2012). Spermatophore contains sperm and accessory gland secretions that could be reinvested into female reproduction (Vahed, 1998). It can also play an important role in interindividual sperm competition by increasing the length of the refractory period since it was shown that a bigger spermatophore increases the female latency to remate (McNamara *et al.*, 2009). Given its content, spermatophore is thus a key determinant of the female reproductive output, and therefore, of the male fitness. When compete for mating, males producing a bigger spermatophore may overpass sperm competition within the female reproductive tract and constrain the propensity of the mated female to remate. We can predict a strong positive relationship between the level of male-male competition in Lepidoptera and the size of the spermatophore transferred to the female, as it was shown for example in Orthoptera (Simmons *et al.*, 1993; Gage & Barnard, 1996). However, to exhibit such a plastic reproductive strategy sounds difficult for males producing spermatophores as their production is time and energy consuming (Muller *et al.*, 2016). We thus expect that the investment into the spermatophore is a delayed response of males to the reproductive competition, such plastic response would not be likely to be express at the time of mating.

Here, we used the European grapevine moth *Lobesia botrana* as a model system to investigate plastic responses to the risk of male-male competition and in what extend behavioral and physiological responses vary according to the sexual audience perceived before and during mating. In this species, polyandry is a heritable trait ($h^2 = 0.40 \pm 0.12$) (Torres-Vila *et al.*, 2002) and is strongly associated with physiological factors, such as larval food nutrition (Torres-Vila *et al.*, 2004, Thiéry *et al.*, 2014a,b) and the size of the spermatophore received by females (Torres-Vila *et al.*, 1997). To assess the effect of reproductive competition, we measured the volume of spermatophore, the duration of mating and the latency before mating of partners experiencing different sexual audiences. We tested whether male can plastically adjust these traits to the presence of one or three rival males added to the mating arena 24 hours before mating or during mating. We also added one or three supplementary females in mating area to test if the plastic response of males to the socio-sexual environment, is specifically determined 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 (moderate competition treatment), (iii) or three 2-day-old virgin males (high competition treatment) during 24 h. At dusk of the next day, each male of each treatment was placed into a new plastic box (mating chamber hereafter) with one 2-day-old virgin female originating from the stock population. The male and female sexual activities were then observed continuously during the following 4 hours. Mating was considered successful if the pair formation lasted more than one minute, which is the threshold over which genital coupling is completed. Once mating finished, both females engaged in pair were collected.

Experiment 2: Male-male competition during mating

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

Behavioral and life history traits measurements

Mating behaviors

For the first experiment (male-male competition prior to mating), we recorded the mating latency (time elapsed from the session's start until genital coupling) and the mating duration (time between the pair formation and separation) of each pair. For the second treatment (male-male competition during mating), we measured the mating duration.

The spermatophore volume

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

The female reproductive output

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

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 influenced by the male mass engaged in mating (Table 2), as larger spermatophores were transferred by heavier males (Pearson's r = 0.32, *t* = 3.75, *df* = 122, P < 0.001). Regarding the reproductive output of the mated female, neither the total number of eggs laid (fecundity) nor the proportion of hatching eggs (fertility) were influenced by the number of conspecifics exposed to the focal male during mating, whatever their sex (Table 2). As for the first competition experiment, fecundity was the only trait positively correlated with the mass of the 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 prolonged mating duration may generate at least two benefits for the male: to have a privileged access to the females by outperforming the mating ability of the other males, and to decrease the propensity of the mated females to remate subsequently. Supplementary mating by the female indeed drastically alters the reproductive outcome of the male by decreasing its likelihood of paternity (Jennions & Petrie, 2000). Faced with this significant risk emerging when multiple males look for females, extended mating duration is viewed as a “mate guarding strategy” which significantly decreases sperm competition intensity in the female genital tractus (Carazo *et al.*, 2007; Mazzi *et al.*, 2009). Combined with a bigger spermatophore transfer in this case, this strategy also led to an extended mating refractoriness in the females, as it was showed for example, in the fruitfly *Drosophila montana* (Mazzi *et al.*, 2009), the almond moth *Cadra cautella* (McNamara *et al.*, 2009) and the apple moth *Epiphyas postvittana* (Foster & Ayers, 1996). Similarly, in *L.botrana*, the size of the spermatophore transferred to the female affects its motivation to remate: the bigger the spermatophore, the longer the refractory period after mating (Torres-Vila *et al.*, 1997; Muller *et al.*, 2016). In our study system, the extended mating duration likely initiated by the male seems beneficial for him as it may prevent females from remating immediately after the pair separation, ensuring the transferred sperm to fertilize the eggs (Gilchrist & Partridge, 2000). Remaining in pair for more than 1 hour is sufficient to reduce the probability that a female remates on the same day, mating occurring *in natura* between 2-3 hrs at dusk (Louâpre and Moreau, personal observation). Moreover, sperm generally reaches the spermatheca between 2 and 5 hours after mating in several butterfly and moth species (Seth *et al.*, 2002; Marcotte *et al.*, 2005). The plastic behavior expressed by males experiencing male-male competition prior to mating could be particularly efficient in *L. botrana*, but it is usually observed in species with short mating or external spermatophore transfer (Simmons, 2002). To express specific behaviors when perceived sexual audience before mating may thus evolved in species with internal spermatophore transfer as a “spermatophore guarding strategy” (Carazo *et al.*, 2007).

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

Besides plastic mating behaviors expressed by males responding to the sexual context before and during mating, they also exhibit plastic physiological response. They transferred a bigger spermatophore when exposed to conspecifics, this effect was observed within the two competition experiments when males were exposed to competitors 24 hours before mating, or during mating. We thus highlight the ability of *L. botrana* males to express a physiological response quickly depending on the perceived sexual context. Surprisingly, such plastic response was not followed by an increase in fecundity or fertility by the mated female. In a previous study on *L. botrana*, it was showed that a bigger spermatophore (for which a significant part of variance was attributed to the host plant consumed by the males at larval stages) induced higher fecundity (Muller *et al.*, 2015). The present result appears to contrast with previous observations where higher investment in the male's ejaculate – in this case, after exposure to rival males – translates into an increase of its reproductive success, as it was also the case for example in *D. melanogaster* (Bretman *et al.*, 2009) and the beetle, *Tenebrio molitor* (Gage & Baker, 1991). This intuitively response may be explained by the ability of males to plastically adjust the number of sperm inseminated when exposed to rival males before or during mating. Regarding our results, two plausible explanations of these diverging results may emerge from the reproduction mode of moths. First, males produce internal spermatophores containing sperm and various secretions produced by accessory glands (Gillott, 2003; Ramm, 2020). Non-sperm components (e.g. parasperm, water, proteins, macromolecules) modulate mating behaviors and reproductive output of the females and play key roles in male-male competition (Perry *et al.*, 2013). Here, the lack of the expected fitness gain for *L. botrana* males faced with rival males suggests that the bigger spermatophores transferred by the male result from higher investment in non-sperm components rather than on sperm allocation. It is nonetheless possible that males *L. botrana* adjust the number of sperm in their spermatophore but to a less extend way. In this hypothesis, distinction must be done regarding the sperm type to be produced: moths are known to produce a fertile eupyrene form and a non-fertile apyrene form (Gage & Cook, 1994). Apyren sperm lacks genetic material and play various roles such as, nutrient provisioning, sperm facilitation and competition (Silberglied *et al.*, 1984; Swallow & Wilkinson, 2002). We can thus hypothesize that males *L. botrana* exposed to rivals also express a physiological plastic response to the sexual audience by modifying their investment in the two sperm forms without any consequences on the female fecundity and fertility (a potential consequence on the mating behaviors of females, especially their propensity for remating is not excluded). This hypothesis seems unlikely for at least two reasons. The first reason reflects the lack of evidence for such a plastic response in moths. In moth species, such as *Plodia interpunctella*, the presence of a rival male closed to the focal male for 24 hours has no effect upon the number of both sperms – eurpyren nor apyren forms – within the spermatophore (Cook & Gage, 1995). The second reason is based on the adequate timing required for adjustment of sperm quality and quantity. While anticipatory plasticity is now coming to be recognized in ejaculate adjustment in response to male-male competition, adjustment of spermatogenesis is time consuming (Magris, 2021). Hypothetic mechanisms behind adjustment of sperm quality and quantity mostly involve changes occurring during sperm maturation, which require at least several days (Magris, 2021). Consequently, during the course of our experiments lasting from 24 hours prior mating to the moment of mating, rapid changes in spermatophore composition in response to the sexual audience are more likely to be mediated by adjustment of non-sperm component rather than by sperm modification. Further investigation is required to test for the specific effect of the plastic response highlighted here on the female's propensity to remate and paternity when several mattings occur.

To conclude, this study clearly shows that males are highly sensitive to the sexual audience before and during mating and can adapt their mating behavior and physiological response quickly. These plastic responses are expected to evolve in situations where the probability to find a mate is stochastic in a short temporal window, that is the case of *L. botrana* in the field when encountering a female is rare and when the first mating induce a strong inhibition of female mating, a situation also frequently encountered in other lepidopteran species (Parker & Vahed, 2010; Jarrige *et al.*, 2016). The selective pressure exerted on males experiencing male-male competition could be sufficient to trigger adjustment in male mating behavior according to the perception of competition and to select 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.

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FIGRE LEGENDS

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

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

TABLES

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 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]	**$F_{2,145} = 14.03$**	**<0.001**	$F_{1,145} = 0.01$	0.94	$F_{1,145} = 0.1$	0.76
Mating duration[a]	**$F_{2,148} = 6.28$**	**0.002**	$F_{1,148} = 2.79$	0.1	$F_{1,148} = 0.1$	0.28
Spermatophore volume[a]	**$F_{2,75} = 5.59$**	**0.005**	**$F_{1,75} = 23.21$**	**<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	**$F_{1,68} = 35.16$**	**<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

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 mass of the two partners engaged in mating on the reproductive traits and behaviors when focal males were exposed to the conspecifics during 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	**$F_{2,236}$ = 5.06**	**0.007**	$F_{1,236}$ = 3.08	0.08	**$F_{2,236}$= 3.33**	**0.037**	$F_{1,236}$ = 0.15	0.69	$F_{1,236}$ = 1.87	0.17
Spermatophore volume	**$F_{2,116}$ = 3.84**	**0.02**	**$F_{1,116}$ = 6.69**	**0.01**	$F_{2,116}$ = 1.77	0.17	**$F_{1,116}$ = 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	**$F_{1,103}$ = 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

Figure 1.

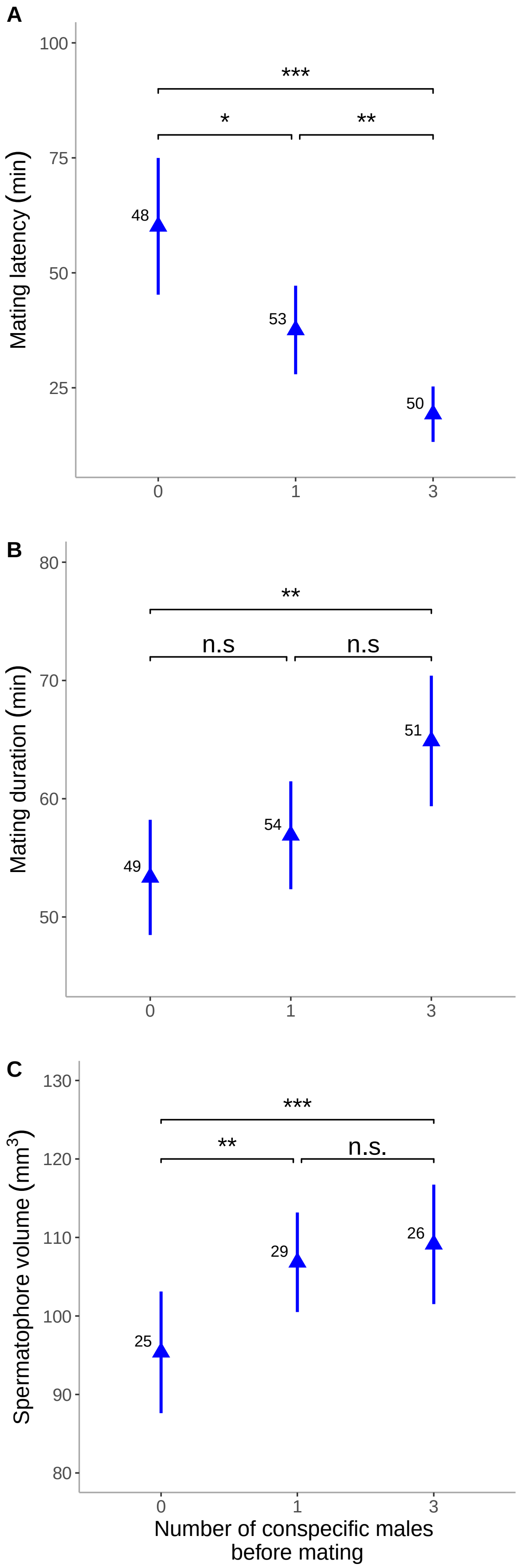
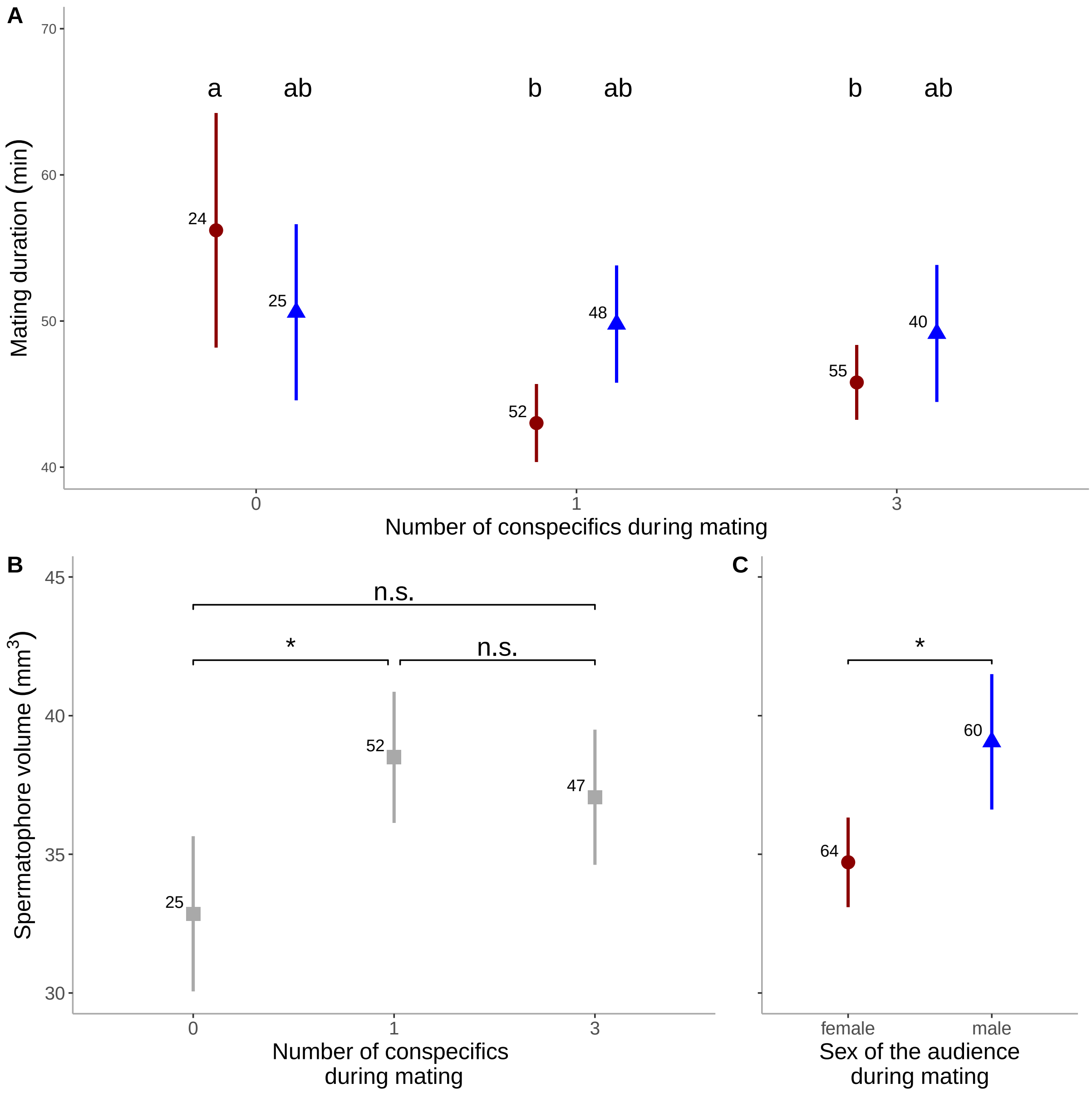

A
Mating latency (min)
100
75
50
25
***
*
**
48
53
50
0
1
3
B
Mating duration (min)
80
70
60
50
**
n.s
n.s
51
54
49
0
1
3
C
Spermatophore volume (mm^3)
130
120
110
100
90
80
***
**
n.s.
29
26
25
0
1
3
Number of conspecific males before mating

Figure 2.


A
70
60
50
40
Mating duration (min)
a
ab
b
ab
b
ab
24
25
52
48
55
40
0
1
3
Number of conspecifics during mating
B
45
40
35
30
Spermatophore volume (mm³)
n.s.
*
n.s.
52
47
25
0
1
3
Number of conspecifics during mating
C
*
60
64
female
male
Sex of the audience during mating