



## Interaction between females and males grapevine moth *Lobesia botrana* modifies further mating preference

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### ABSTRACT

During reproduction, females may boost their fitness by being selective based on direct material benefits provided by the males, such as nuptial gifts. In Lepidoptera, male provides a spermatophore containing nutrients. However, virgin males produce a bigger spermatophore, containing spermatozoa and nutrients, allowing higher female fertility. Lepidoptera females that could detect the sexual status of males may thus prefer a male without previous mating experience (i.e. a virgin male). This mate selection could be achieved by the use of chemical indices, such as sexual pheromones and cuticular compounds, known to be possibly exchanged during reproduction, and which can be indicators of a previous mating experience and known to be possibly sources of information exchanged. In this study, we experimentally presented *Lobesia botrana* virgin males with females in order for them to be exposed to females' natural sexual pheromones or cuticular compounds. 12 or 48 h after the exposure of males to either females' sexual pheromones or cuticular compounds, these males were confronted to naïve females, which have a choice between them or a virgin non-exposed males. We highlighted that, despite producing a spermatophore of similar volume, all exposed virgin males were less likely to mate with females 12 h after exposure, while after 48 h of exposure this is only the case for virgin males exposed to sexual pheromones. *L. botrana* females may thus discriminate male sexual experience based on chemical cues (either from cues transferred directly from females to males, or from changes in the cuticular or pheromone males' profile) indicating past mating experiences. Mating duration was longer for males exposed to sexual pheromones after 12 h only, and for males exposed to cuticular compounds after 48 h only. Pheromones signal might be more persistent over time and seems to more easily gather information for males. The physiological reasoning behind this result still needs to be investigated.

### 1. Introduction

Mate preference, whereby the selection of a suitable, see optimal, reproductive partner based on perceived benefits, is a key process within the realm of sexual selection (Andersson, 1994). Classical sexual selection theory predicts that males should maximize their reproductive success by mating with several females, whereas females boost their fitness by being selective and choosing the highest quality mate (Andersson and Simmons, 2006; Kokko et al., 2003). Indeed, reproduction is known to be energetically and physiologically costly, especially for females (Navarro, 2020; Stearns, 1989; Webb et al., 2019), and females' choices may have consequences on population dynamic (Cally

et al., 2019).

Females may choose mates based on secondary sexual characteristics that are indicative of indirect benefits including good genes or superior health (Andersson and Simmons, 2006; Kokko et al., 2003), or based on direct material benefits, such as parental care from the male (Alonso, 2011; Cally et al., 2019; Møller and Thornhill, 1998). In some species, males might also offer even more direct benefits, such as nuptial gifts (Lewis and South, 2012; Vahed, 1998). They are offered during mating and have a very strong positive impact on female fitness, both in terms of fertility and/or longevity (Bergström and Wiklund, 2002; Lewis and South, 2012; Vahed, 1998). These gifts are particularly important in capitals breeders where reproduction is a nutrient-limited process for

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both males and females, and is largely related to their energy reserves obtained as larvae (Boggs, 1997; Davis et al., 2016). For example, in Lepidoptera, male provides a spermatophore, containing spermatozoa as well as nutrients such as proteins that allow the female to boost her egg production (Rooney and Lewis, 2002; Wedell and Karlsson, 2003). Indeed, a larger spermatophore allows higher female fertility (South and Lewis, 2011; Torres-Vila and Jennions, 2005). Females may thus find a mate able to produce the biggest spermatophore, in order to maximize their fitness and produced numerous offspring of good quality. However, quantity and quality of spermatophore can greatly vary between males (He and Tsubaki, 1992; Lehmann and Lehmann, 2009; Muller et al., 2015; Torres-Vila et al., 1995).

Production of spermatophores in males can represent an energetic cost (Dewsbury, 1982; Oberhauser, 1989; Scharf et al., 2013; Wedell and Cook, 1999), so that size, quality and number of spermatophores can be influenced by many variables, including males competition (He and Tsubaki, 1992), age (Lehmann and Lehmann, 2009; Torres-Vila et al., 1995), males' size and weight (Torres-Vila et al., 1995; Wedell, 1993) or food as a larva (Muller et al., 2015; Torres-Vila et al., 1995). More importantly, if, in Lepidoptera, male strategy to maximize fitness is generally to acquire as many mates as possible (Thornhill and Alcock, 2013), mating order and the time that elapses between consecutive mating can also influence the spermatophore production (Torres-Vila et al., 1995), the spermatophore size decreasing with the number of male matings (Hughes et al., 2000; Lauwers and Van Dyck, 2006). Indeed, virgin males have been shown to produce a bigger spermatophore (Muller et al., 2016a; Torres-Vila and Jennions, 2005). As examples, in *Pararge aegeria* and *Lobesia botrana*, it has been shown that virgin males produce a spermatophore 337 % larger (Lauwers and Van Dyck, 2006) or with a volume 250 % higher (Muller et al., 2016a) respectively, compared to non-virgin males of the same age. This is noteworthy given the fact that increase spermatophore size has been shown to increase female fecundity by 30 % in *Lobesia botrana* (Muller et al., 2016a). As a consequence, and as it is widely known that females may adjust their reproductive investment regarding males traits (Uller et al., 2005), it is widely expected that Lepidoptera females that could detect the sexual status of males would prefer, and thus choose for, a mate with the least possible copulations, i.e. a virgin male if possible.

This mate selection could be achieved by the use of chemical indices. First, in insects, sexual pheromones are known to play a crucial role in sexual communication (Hansson, 1995; Harari and Steinitz, 2013; Krieger and Breer, 1999; Thiéry et al., 2023). They facilitate mate attraction, recognition, and mating behaviour display (Cardé and Millar, 2004; Symonds and Elgar, 2008; Wyatt, 2003). The structure of sexual pheromones varies widely among insect taxa, encompassing diverse chemical classes such as hydrocarbons, alcohols, aldehydes, and esters (Wyatt, 2003). Chemoreceptor organs, particularly antennae, play a key role in detecting these pheromones, initiating specific behavioural responses in the recipient insect, including orientation, courtship rituals, and mating behaviour (Cardé and Haynes, 2004). These sexual pheromones can enable individuals to recognize the quality of potential partners, including their sexual status and fertility signals (Blomquist et al., 2020; Conte and Hefetz, 2008; Harari et al., 2011).

Additionally to these volatile pheromones (Blomquist et al., 2020; Bordereau and Pasteels, 2011), tactile sensory stimuli may be involved by both sexes during courtship (Zweerus et al., 2021). Insects exhibit a diverse array of chemical compounds on their external layers (cuticular compounds), including hydrocarbons, lipids, amino acid derivatives, and other chemical classes, contributing to various aspects of communication and ecological interactions (Ozaki and Wada-Katsumata, 2010). Cuticular hydrocarbons (CHCs), long-chain hydrocarbons found on the insect cuticle, play a pivotal role in species and mate recognition (Blomquist and Bagnères, 2010; Howard and Blomquist, 2005). Female insects, in particular, release specific blends of CHCs that signal species identity, relatedness, and reproductive status (Blomquist and Bagnères, 2010; Sprenger and Menzel, 2020). Additionally,

cuticular lipids, including fatty acids and wax esters, contribute to the structural integrity of the cuticle and may participate in chemical signaling (Batalha et al., 2020; Ginzler and Blomquist, 2016). Lastly, glycosides and proteins on the cuticle may play roles in mate recognition and signaling of reproductive status (Dettner, 2015; Hackman, 1965). The diverse functions and structures of these cuticular compounds underscore their importance in the intricate communication systems and adaptive strategies of insects.

As insects females have been shown to prefer virgin males compared to non-virgin males (Harris and Moore, 2005; Muller et al., 2016a; Torres-Vila and Jennions, 2005), it could be expected that such differentiation could be made using these chemical cues. Importantly, some studies have demonstrated that some cuticular compounds (Everaerts et al., 2010; Polerstock et al., 2002) and sexual pheromones (Scott et al., 1988) can be produced by males or females and be exchanged during reproduction, thus being acquired by the opposite sex during mating, by mechanical transfer and direct contact (Everaerts et al., 2010; Scott et al., 1988). These transfer have been shown to have various behavioural consequences (Scott et al., 1988; Yew et al., 2008). Additionally, and perhaps more importantly, as cuticular compounds are very plastic and related to environmental changes (Botella-Cruz et al., 2021; Otte et al., 2018; Sprenger and Menzel, 2020), it is now recognized that contacts with another individual (chemical, visual and physical information) may contribute to a change in an individual's cuticular compounds (Blomquist et al., 2020; Drijfhout et al., 2009). But while the potential effects of these transfers or change in chemical profile has been discussed as an indicator of a previous mating experience for the other sex (Scott et al., 1988), the overtime persistence of these signals remained poorly understood.

In this study, we experimentally exposed *L. botrana* virgin males to females' natural sexual pheromones or cuticular scent compounds. Females were then exposed for 4 h, after 12 h or 48 h the treatment was applied to the male, to a choice between virgin non-exposed males, or virgin males exposed to either sexual pheromones or cuticular compounds. We expected that male's treatment will not influence male spermatophore size, as all tested males were still virgin males. *Lobesia botrana* females were already proved to choose when possible for virgin males (Muller et al., 2016a). Since sexual pheromones or cuticular compounds originating from females might biased information about the male's sexual status (Chenoweth and Blows, 2005; Ferveur, 2005; Gemeno and Schal, 2004), we hypothesize that a female should mate preferentially with virgin odourless male. This choice is likely to last even after 48 h, as cuticular scents are thought to be persistent (Bordereau and Pasteels, 2011; Hemptinne et al., 2001).

## 2. Material and methods

### 2.1. Ethical note

All experiments complied with International laws on animal experimentation. All individuals were reared in reasonable population density under controlled laboratory conditions and fed regularly to maintain a healthy population (see below for rearing methods). Moths were handled carefully, and the abiotic conditions (temperature, humidity and photoperiod) they experienced corresponded to the natural conditions in their native habitat (see studies under natural conditions (Iltis et al., 2020; Rank et al., 2020)). Before dissection, females were frozen at  $-25^{\circ}\text{C}$  for 10 min in a freezer prior to decapitation.

### 2.2. Insect model, stock populations and rearing protocols

*Lobesia botrana* is one of the major pests of grapes (*Vitis vinifera*) (Gilligan et al., 2011; Rank et al., 2020), which is widely distributed, occurring in almost all European vineyards (Benelli et al., 2023). Females are polyandrous, but in natural situation this polyandry is often masked by low rates of mating due to the probability of encountering

(Gabel and Thiéry, 1994).

The strain of *L. botrana* used for this study originated from individuals collected in a French Sauternes vineyard (cultivar Semillon) in 1997, to which wild adults are periodically added (UMR Save, INRA-Nouvelle Aquitaine). This rearing line is maintained with a substantial number of caged adults (several thousand a week) to avoid genetic drift. Considerable variation is found in the larval and adult behaviours and in larval immune parameters, ensuring genetic variability (Vogelweith et al., 2011).

The stock colony is maintained without diapause on a semi-artificial diet (as described in (Thiéry and Moreau, 2005)), with the following composition: 150 ml water, 3 g agar, 9 g maize flour, 11 g wheat germ, 9 g yeast, 0.9 g ascorbic acid, 0.3 g benzoic acid, 0.3 ml maize oil, 0.3 g nipagin and 0.2 g iprodione, at  $24 \pm 1$  °C,  $60 \pm 10$  % RH with a photoperiod of 15:8h light/dark and 1 h of dusk. The first 15 photophase hours were at 1000 lx luminosity, and the last hour (dusk) was at 25 lx. Males and females were placed in a large cage and bands of waxed paper (15\*2 cm) were hung for oviposition support. Once the paper had received a sufficient number of eggs, it was placed in a plastic box containing the semiartificial larval diet. The larvae were maintained at a density of 100 individuals per 300 ml of diet.

We collected final instar larvae from our colony stocks daily and placed them in small piece of cardboard. Larvae were checked daily until pupation, upon which pupae were gently extracted from cardboard. Pupae were weighed to the nearest 0.1 mg (Precisa 262 SMA-FR microbalance) and placed individually in glass tubes (70\*9 mm diameter), stoppered with cotton plugs, at 22 °C under natural photoperiod. Pupae were checked every morning, and newly emerged adults sexed. Newly emerging virgin adults were used for subsequent experiments, conducted during April and May 2016, under the same conditions as rearing.

### 2.3. Males exposed to females' cuticular compounds

Two days old males were exposed to cuticular compounds (a probable mix of cuticular hydrocarbons, cuticular lipids, glycosides and proteins). During the day, just before dusk, a randomly selected male was placed in a coupling tube (100\*15 mm) along with a virgin female which did not call, to allow the passage of cuticular compounds between the two individuals by contact. Indeed, cuticular compounds on females can be transmitted directly by contact (Blomquist and Bagnères, 2010; Howard and Blomquist, 2005; Lockey, 1988). Since individuals were not active during the day, the contacts between male and female were established by gently stirring the tube for 5 min and ensuring that at least 10 contacts were obtained between both sexes. This also prevent individuals from copulating. Two days old control males in this experiment were also placed, just before dusk, in a coupling tube, but without the presence of a virgin female. The tube was gently stirring for 5 min as for exposed males. Males were then returned to their respective tubes until the beginning of the experiment. Control and exposed males were always the same age.

### 2.4. Males exposed to females' sexual pheromone

At dusk, two days-old pheromone-emitting females were randomly selected and placed individually in mating tubes (100 x 15 mm) closed for 30 min. Every 3 min, the call behaviour of the female was noted. Only females calling more than half the time were used to expose males. Indeed, when female moths adopt the calling posture, they emit pheromones that serve as signals to attract potential mates (Cardé and Haynes, 2004; Stepien et al., 2020). Female was then removed from the mating tube and one randomly selected two days-old male was immediately placed inside for 5 min, in order to be impregnated by sexual pheromones previously deposited in the tube by the female. Two days old control males in this experiment were also placed for 5 min in a coupling tube, but which was not impregnated by sexual pheromones.

Males were then returned to their respective tubes until the beginning of the experiment. Control and exposed males were always the same age.

### 2.5. Behavioural experiment

To explore whether females preferred to mate with virgin males over virgin exposed males (cuticular compounds or sexual pheromones), we performed a choice trial test 12 h or 48 h after the exposure, using different males and females for each experiments, under the same conditions, to see if the chemical cues of the cuticular compounds and the sexual pheromones persist over time. In selecting the time intervals of 12 h and 48 h for our study, we aimed to capture both the immediate and more sustained effects of sexual pheromones and cuticular compounds on mate choice behaviours in insects. The initial 12-hour period allows for the assessment of immediate responses to freshly emitted chemical cues, which may reflect rapid behavioural adjustments in mate recognition and selection. Additionally, by extending our observation to 48 h, we aimed to encompass both short-term behavioural responses and potential changes in chemical cues dynamics over time (Wyatt, 2003). For these tests, two males (one 2-day-old-virgin and one 2-day-old-exposed [to cuticular compounds or sexual pheromones] virgin [for the test after 12 h] or one 4-day-old-virgin and one 4-day-old- exposed [to cuticular compounds or sexual pheromones] virgin [for the test after 48 h]) were simultaneously presented to one 2-day-old virgin female in a glass mating tube (100\*15 mm diameter) and observed until the end of copulation, as already described in (Muller et al., 2016a). The onset time (time elapsed from the start of the session until genital coupling) and the duration of mating (time during which the pair was observed) were noted. Pairs were observed for 4 h, corresponding to the period during which females called males by releasing their sexual pheromone (K. Muller, personal observations), and pairs that failed to mate within this period were excluded from the experiment. For cuticular compounds, 43 trials were performed at 12 h [4 failed trials] and 22 trials at 48 h [no failed trials], while for sexual pheromones 47 trials were performed at 12 h [8 failed trials] and 39 trials at 48 h [8 failed trials].

To control for the effect of male mass on female choice, males with equal pupal masses were selected (Linear Models, using the lme4 package (Bates et al., 2015), pheromones experiment after 12 h: Estimate = 0.145, SE = 0.219,  $t_{1,76} = 0.664$ , p-value = 0.509; pheromones experiment after 48 h: Estimate = 0.080, SE = 0.246,  $t_{1,60} = 0.325$ , p-value = 0.746; cuticular compounds experiment after 12 h: Estimate = 0.041, SE = 0.170,  $t_{1,76} = 0.238$ , p-value = 0.813; cuticular compounds experiment after 48 h: Estimate = 0.048, SE = 0.274,  $t_{1,42} = 0.174$ , p-value = 0.863) and were marked similarly by removing the scales on one of their wings. Marking did not influence female choice (trials for cuticular compounds and sexual pheromones taken together: after 12 h:  $X^2 = 0.628$ , df = 1, p-value = 0.214; after 48 h:  $X^2 = 0.075$ , df = 1, p-value = 0.608).

Immediately after the end of mating, females were frozen and then dissected on a glass slide. The bursa copulatrix containing the male spermatophore was removed in order to estimate its size. Estimating spermatophore size by extrapolating its volume is the method classically used in moths, including *L. botrana* (Milonas et al., 2011; Muller et al., 2015; Torres-Vila et al., 1999). To assess spermatophore size, we measured its dimensions (length [l], width [w] and thickness [t]) under a stereomicroscope (NIKON SMZ1500) with a magnification of 20. The volume of the spermatophore was estimated as an ellipsoid balloon [ $V = 1/4\pi/6 (l w t)$ ], as previously described (Torres-Vila et al., 1999). Spermatophore volume were measured after 12 h but not after 48 h, as only females behaviour was of interest during this phase in our experimental design.

### 2.6. Statistical analysis

All statistical analysis were performed using R 3.6.3 (R Core Team, 2020) and Rstudio v1.1.419. We computed Linear Models (LMs) using

the *lme4* package (Bates et al., 2015). For all test computed, models accuracy was tested using the *check\_model* function from the *performance* package (Lüdtke et al., 2020), and using a shapiro test (*shapiro.test* function, implemented in the R environment (R Core Team, 2020)). When models did not fit a normal distribution, the response variable was  $\log_{10} + 1$  transformed. Significance level was set at 0.05, and comparisons were considered marginally significant at a 0.10 level, which still can be interpreted, due to our relatively small sample size (Jennions and Møller, 2003). Effects size were also given for all tests using the Cohen's d indicator in the *effsize* package (Torchiano, 2020), a measure of effect size that quantifies the difference between two group means in standard deviation units, and allow to interpret the magnitude of the difference between groups (e.g. values of 0.2, 0.5 and 0.8 represent respectively a small, medium, and large effect size).

For both tests performed after 12 h and after 48 h, in each independent trial (females choice between males non exposed or males exposed to cuticular compounds, and females choice between males non exposed or males exposed to sexual pheromones), proportion of mating with exposed or non-exposed males were compared using the *prop.test* function in stats (implemented in the R environment (R Core Team, 2020)). To assess the effect of males' treatment during trials between males non-exposed and exposed to cuticular compounds, we used LMs with males treatment as an explanatory variable, and either latency to mating ( $\log_{10} + 1$  transformed), mating duration ( $\log_{10} + 1$  transformed) and spermatophore volume (only for the test performed after

12 h) as dependant variables.

### 3. Results

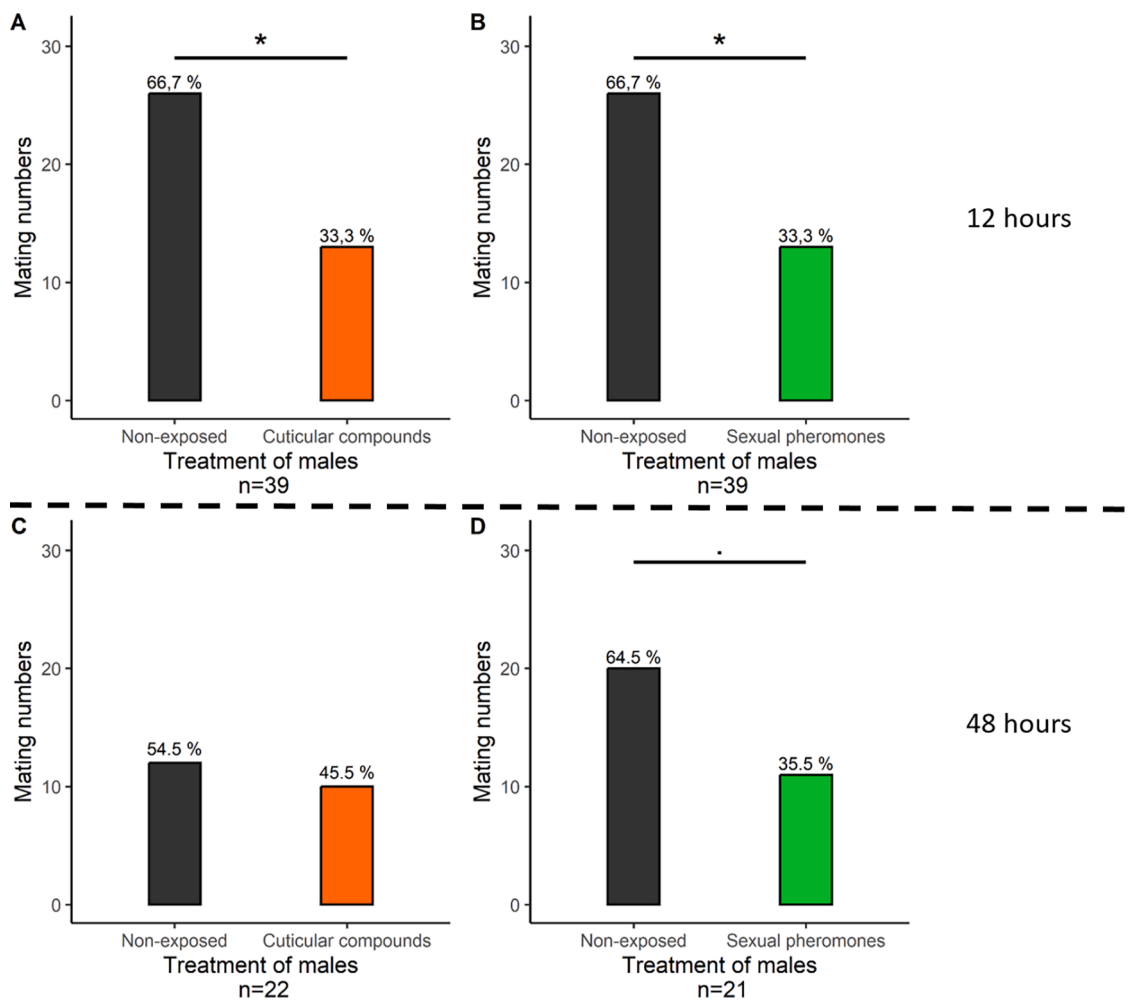
#### 3.1. Mating number

After 12 h, when confronted with a non-exposed male and a male exposed to cuticular compounds, females were more likely to mate with a non-exposed male (66.7 % versus 33.3 %,  $X^2 = 3.692$ ,  $df = 1$ ,  $p$ -value = 0.027, Fig. 1a). When confronted to a non-exposed male and a male exposed to sexual pheromones, females were more likely to mate with a non-exposed male (66.7 % versus 33.3 %,  $X^2 = 3.692$ ,  $df = 1$ ,  $p$ -value = 0.027, Fig. 1b).

48 h after treatment, females did not discriminate between a non-exposed and an exposed to cuticular compounds male (54.5 % of non-exposed versus 45.5 % of exposed males mated with females,  $X^2 = 0.045$ ,  $df = 1$ ,  $p$ -value = 0.416, Fig. 1c), but were marginally more likely to mate with a non-exposed compared to an exposed to pheromones male (64.5 % of non-exposed versus 35.5 % of exposed males mated with females,  $X^2 = 2.065$ ,  $df = 1$ ,  $p$ -value = 0.075, Fig. 1d)

#### 3.2. Latency to mating

After 12 h, latency to mating was equivalent whether females mated with non-exposed males (7.12 min  $\pm$  1.43 SE) or males exposed to



**Fig. 1.** Mating numbers (and proportion) for non-exposed males compared to males exposed to cuticular compounds (AC) and non-exposed males compared to males exposed to sexual pheromones (BD), after 12 h exposure (upper line) or 48 h exposure (lower line). Number of successful trials are given for each group. “\*” represent significant differences at  $\alpha = 0.05$ , “·” Represent significant differences at  $\alpha = 0.1$ .

cuticular compounds (16.08 min  $\pm$  9.43 SE, Estimate = 0.071, SE = 0.143,  $t_{1,37}$  = 0.500, p-value = 0.620, Cohen's d = 0.442), and for males non-exposed (4.38 min  $\pm$  0.97 SE) or males exposed to sexual pheromones (3.62 min  $\pm$  1.04 SE, Estimate = 0.075, SE = 0.115,  $t_{1,37}$  = 0.352, p-value = 0.518, Cohen's d = 0.168).

After 48 h, latency to mating was equivalent whether females mated with non-exposed males (5.75 min  $\pm$  1.77 SE) or males exposed to cuticular compounds (11.70 min  $\pm$  5.03 SE, Estimate = 0.129, SE = 0.214,  $t_{1,20}$  = 0.600, p-value = 0.555, Cohen's d = 0.513), and for males non-exposed (7.15 min  $\pm$  2.16 SE) or males exposed to sexual pheromones (8.27 min  $\pm$  3.43 SE, Estimate = 0.061, SE = 0.175,  $t_{1,29}$  = 0.349, p-value = 0.730, Cohen's d = 0.109).

### 3.3. Mating duration

After 12 h, mating duration was equivalent for non-exposed males compared to those exposed to cuticular compounds (Estimate = 0.025, SE = 0.033,  $t_{1,37}$  = 0.768, p-value = 0.448, Cohen's d = 0.245, Fig. 2a), while mating duration was marginally longer for males exposed to sexual pheromones compared to non-exposed males (Estimate = 9.038, SE = 4.859,  $t_{1,37}$  = 1.860, p-value = 0.071, Cohen's d = 0.632, Fig. 2b).

Comparatively, after 48 h, mating duration was longer for males exposed to cuticular compounds compared to non-exposed males (Estimate = 0.096, SE = 0.040,  $t_{1,20}$  = 2.385, p-value = 0.027, Cohen's d = 0.963, Fig. 2c), while mating duration was equivalent for non-exposed males compared to those exposed to sexual pheromones (Estimate =

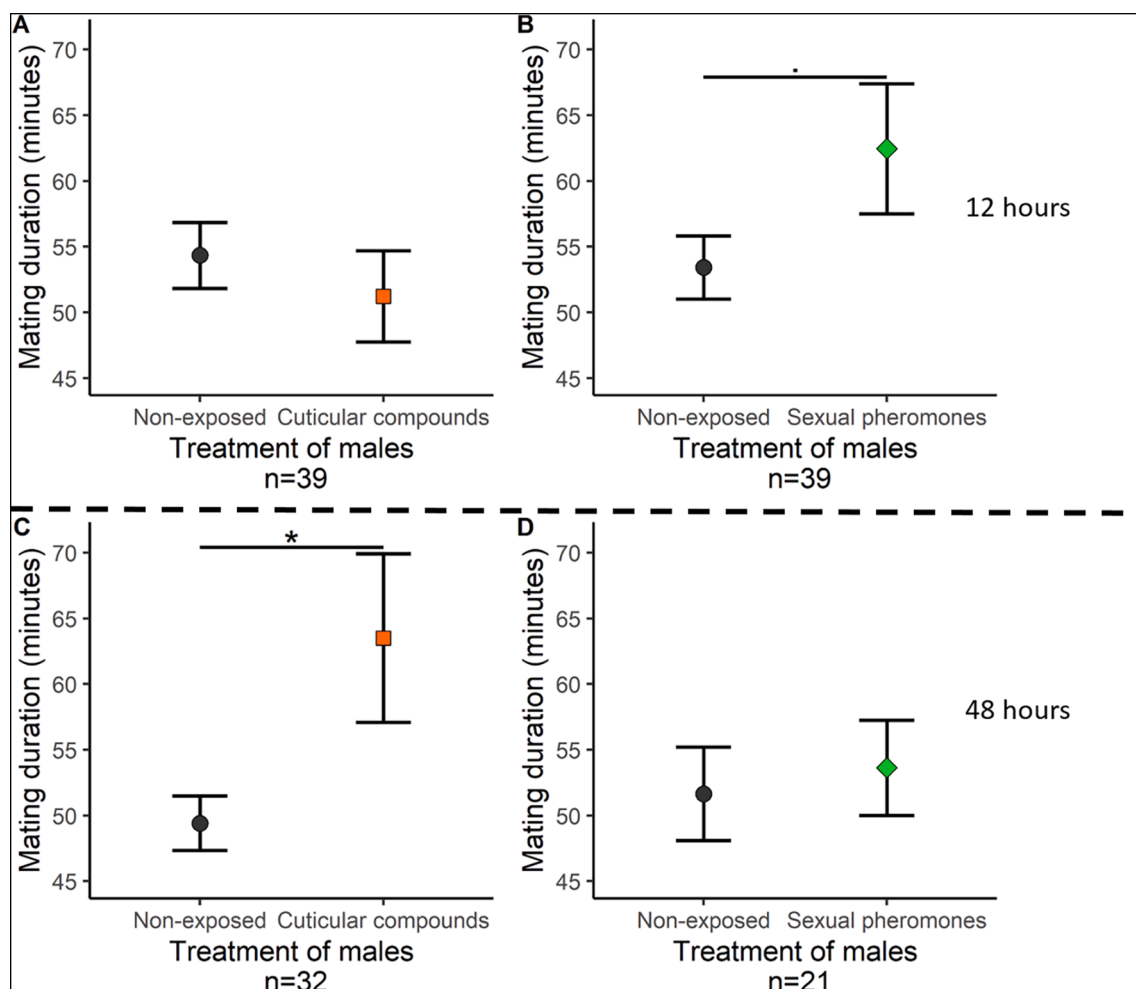
1.986, SE = 5.523,  $t_{1,29}$  = 0.360, p-value = 0.722, Cohen's d = 0.135, Fig. 2d).

### 3.4. Size of the spermatophore transferred to females

After 12 h, spermatophore volume transferred to females was equivalent to non-exposed males compared in either male exposed to cuticular compounds (Estimate = 0.019, SE = 0.038,  $t_{1,37}$  = 0.485, p-value = 0.631, Cohen's d = 0.092) or sexual pheromones (Estimate = 0.006, SE = 0.031,  $t_{1,37}$  = 0.182, p-value = 0.857, Cohen's d = 0.026).

## 4. Discussion

Lepidoptera females have previously been emphasized to preferentially choose virgin males rather than non-virgin males as a function of spermatophore size (Muller et al., 2016a, 2016b; Torres-Vila and Jennions, 2005). Our study allows to emphasize that, despite producing a spermatophore of similar volume, virgin males that are exposed to sexual pheromones or cuticular compounds were less likely to mate with females 12 h after exposure compared to non-exposed ones. After 48 h, only virgin males exposed to sexual pheromones were less likely to mate with females. Importantly, male exposure to sexual pheromones or cuticular compounds also increase the duration of mating, but at differing timings. Indeed, males exposed to sexual pheromones had longer mating duration after 12 h only, while males exposed to cuticular compounds had longer mating duration after 48 h only.



**Fig. 2.** Mating duration (mean  $\pm$  SE) for non-exposed males compared to males exposed to cuticular compounds (AC) and non-exposed males compared to males exposed to sexual pheromones (BD), after 12 h exposure (upper line) or 48 h exposure (lower line). Number of successful trials are given for each group. "\*" represent significant differences at  $\alpha$  = 0.05, "." Represent significant differences at  $\alpha$  = 0.1.

#### 4.1. Size of the spermatophore transferred to females

In Lepidoptera, to boost females fertility (South and Lewis, 2011) male provides a spermatophore, containing spermatozoa as well as nutrients such as proteins that allow the female to boost her egg production (Rooney and Lewis, 2002; Wedell and Karlsson, 2003). However, it has been emphasized that mating order and the time that elapses between consecutive mating can also influence the spermatophore production (Torres-Vila et al., 1995), the spermatophore size and quality decreasing with the number of male matings (Hughes et al., 2000; Lauwers and Van Dyck, 2006; Muller et al., 2016a; Torres-Vila and Jennions, 2005). In this study, we demonstrated that contact with females' sexual pheromones or cuticular compounds did not influence males investment in spermatophore size transferred to females. This strengthens that future female choice might be influenced by males' odor only. However, we did not study spermatophore content in nutrient, which has already been shown to vary with environmental variables in this species (Delisle and Bouchard, 1995; Muller et al., 2015). Given the lack of effect on spermatophore size, and as it is the main reason why females prefer to mate with virgin males (Muller et al., 2016a), we could expect no effects on males' reproductive investment and thus direct benefits to females fitness. However, our results show that females may choose their mates according to their previous encounter with females, despite the absence of mating.

#### 4.2. Directional choices

*L. botrana* females are known to be able to discriminate virgin males from non-virgin one when confronted to a choice (Muller et al., 2016a), which could be due to a possible chemical recognition signal between virgin and non-virgin males as sexual pheromones or cuticular compounds substances produced by females can be acquired by males during copulation and advertise their previous mating experience (Everaerts et al., 2010; Harris and Moore, 2005; Polerstock et al., 2002; Scott et al., 1988). Accordingly, after 12 h exposure, we showed that virgin males that are exposed to sexual pheromones or cuticular compounds were less likely to mate with females, while transferring a spermatophore of similar size to females. The greater mating probability of non-exposed males could therefore be related to an active choice of the female, based on a chemical recognition of the status of their mate (Everaerts et al., 2010; Harris and Moore, 2005; Polerstock et al., 2002; Scott et al., 1988), which strengthens that females might be more motivated to mate with virgin males, as already highlighted in other studies (Muller et al., 2016a; Torres-Vila and Jennions, 2005). This could be due to a reduce spermatophore size in mated males (Muller et al., 2016a; Torres-Vila and Jennions, 2005), or to a reduced risk of pathogen or sexually transmitted disease (STD) transmission (Able, 1996; Knell and Webberley, 2004; Kokko et al., 2002). Virgin males, having no prior mating experience, are less likely to carry infections that could be detrimental to females or their offspring. Such behavior minimizes the risk of acquiring infections that could compromise reproductive success or the health of future generations.

After 48 h exposure, we showed that virgin males that are exposed to sexual pheromones were still less likely to mate with females, while males exposed to cuticular compounds were as likely to mate with females compared to non-exposed males. This result might indicate that sexual pheromones would be a chemical cue of recognition more durable over time for females, as compared to cuticular compounds which effect decrease over time (Scott et al., 1988). Additionally, sexual pheromones can be emitted long range (Blomquist et al., 2020; Bordenau and Pasteels, 2011), unlike cuticular compounds which have low volatility and are principally detected by contact (Drijfhout et al., 2009), which might explain lower discrimination on this chemical. Previous studies have shown that sexual pheromones can still convey information for at least one hour in females (Sivinski et al., 1994), and up to 24 h for males (Glass et al., 1970). Our study seems to demonstrate that sexual

pheromones emitted by females can emit on males up to 48 h, which might still convey information for females.

#### 4.3. Latency to mating

Mating latencies were similar between non-exposed virgin males and virgin males exposed to cuticular compounds or sexual pheromones, either after 12 h or after 48 h exposure. Mating latency is a result of female choice, in order to maximize their fitness by finding a more favorable male, or potentially investing more in their offspring, to assure a better development success (Katlav et al., 2023; Koons et al., 2008). Indeed, females may choose to mate depending on the availability of energy resources (Koons et al., 2008) or males quality (Aluja et al., 2009; Torres-Vila and Jennions, 2005). In this study, females were exposed to sufficiently good conditions to ensure reproduction, but also to two potential mates, ensuring reproductive success. As a result, it is not surprising that mating latency did not vary. However, this may indicate that mating selection occur across a relatively long distance range, sufficient for the female to a priori detect for the status of a male, for it to be virgin or non-virgin.

#### 4.4. Mating duration

Importantly, for males that were exposed to sexual pheromones, mating duration increase after 12 h exposure. Increase mating duration can be a direct response of males to the presence of competing males (Bretman et al., 2013b, 2013a; Jarrige et al., 2016; Lane et al., 2015). In our study, virgin males were all confronted to one rival, so that it is unlikely to explain the differences we found. However, those that were exposed had access to one females, which there were not able to mate with, which might have simulated an indirect competition. As a result, this increased mating duration might be linked to the need for males to monopolize female (Parker, 1974), or to produce a higher quality spermatophore. To test such hypothesis, spermatophore quality in such context need to be assessed. Still, the fact that mating duration increased after 12 h exposure only for males exposed to females sexual pheromones, but females discriminate for males exposed to sexual pheromones and cuticular compounds, might indicate that, at least after 12 h, sexual pheromones might convey more information to males than cuticular compounds.

However, importantly, after 48 h, mating duration increased only in males exposed to cuticular scents. Females cuticular compounds might interact with those of the male, due to the general complexity of insect cuticular profiles (Blomquist et al., 2020; Drijfhout et al., 2009), thus expressing a new kind of signal, which might not be detected by the female (explaining similar probability of mating). This kind of interaction between signals need to be tested, but might explain a subsequent behavioural change, as in insects males behaviour can be changed if they have been previously exposed to females (Krupp et al., 2008). These point highlight one other possible explanation of our results. Indeed, an alternative interpretation suggests that female responses may not be exclusively attributable to the fact that males are exposed to female cuticular compounds or pheromone. Instead, it is plausible that a broader array of social cues, encompassing chemical, visual, and physical stimuli, could have influenced males' cuticular profiles during the contacts between male and female (Chenoweth et al., 2010; Gershman et al., 2014; Gershman and Rundle, 2017; Kent et al., 2008; Krupp et al., 2008). In fact, the female presence might have induce a change in males' cuticular compounds or behaviour, which may have been detected by females (Blomquist et al., 2020; Drijfhout et al., 2009). This is even more plausible that the effects of cuticular compounds deposited on individuals have been shown to decrease over time (Scott et al., 1988), and this may highlight that change in cuticular compounds profile might be predominant after a certain amount of time. This alternative perspective underscores the complex interplay of environmental factors in insect chemical communication mechanisms. To test for such effects, future

studies should investigate females' selection when males are previously given information about the presence of a female without there being any physical contact.

Our results might thus indicate both that differing chemicals signal can be used according to timing of encounter, but also that males and females can use different cues to assess reproductive status. Studies combining behavioural and chemical analyses might be required to a better understanding of these findings (Butterworth et al., 2018). However, these experiments were carried out in the laboratory and the results may differ under natural conditions. Indeed, in the wild, males that have just mated are impregnated by both sexual pheromones and cuticular compounds, partners being contiguous throughout the duration of mating. It is possible that the female uses the two joint signals to evaluate the partner's status, these signals also being strengthened by volatile emissions from host plants (von Arx et al., 2012).

## 5. Conclusion

Our study tends to demonstrate that *L. botrana* females may discriminate males sexual experience based on chemical cues, but also that they are able to behaviourally distinguish between these males according to signals indicating virginity, even though no direct benefits can be inferred by reproducing to these males. If both cuticular compounds and sexual pheromones allow female to know the reproductive status of the male, different signals seem to be used for short terms (12 h) and longer terms (48 h) responses. Pheromones signal might also seem more persistent over time and seems to more easily gather information for males. The physiological reasoning behind this result still needs to be investigated, by studying persistence of these signals, their chemical structure, and the precise compounds involved.

## 6. Data archiving statement

The data that supports the findings of this study will be made available by the corresponding author upon request.

## 7. Funding statement

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## CRedit authorship contribution statement

**Léa Lorrain-Soligon:** Writing – review & editing, Writing – original draft, Visualization, Formal analysis. **Karen Muller:** Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. **Camille Delaby:** Writing – review & editing, Methodology, Investigation. **Denis Thiéry:** Writing – review & editing, Visualization, Validation, Methodology, Investigation. **Jérôme Moreau:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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